UNIVERSITY OF CALIFORNIA
RIVERSIDE

Molecular Biological and Genetic Characterization of Synthetic Elicitor Activity in *Arabidopsis thaliana*

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Plant Biology

by

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December 2015

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Acknowledgements

I would like to express the deepest appreciation to my advisor and mentor Dr. Thomas Eulgem. Over the last five years, his continuous enthusiasm and scientific curiosity pushed me farther and gave me inspirations in research. Without his guidance this dissertation would not have been possible. I would also like to thank to my committee members, Dr. Isgouhi Kaloshian and Dr. Sean Cutler for their invaluable critiques and suggestions throughout my project. I would like to express my gratitude to all of the past and present members of the Eulgem lab. I have always felt well in the lab while working with them and enjoyed a lot through the years. I need to acknowledge the help I had from my undergraduate volunteers, without their help I could never have gotten all of this work completed. I would like to send a special thanks to my husband Harun. He helped me a lot to survive and stay positive in all of the stresses of life. Also, I would like to thank to our families little member; Irem for her patience, while waiting at home for her mom through long nights. Big thanks to all of my family members and friends. Without their supports, it was not possible to come to this point. Especially I would like to thank to my mom and dad for their love, support and guidance on my entire life. Finally, I am also grateful to the many people in the UCR community that have helped, encouraged, and pushed me over the years. I would particularly like to thank my funding agency Turkish Republic Ministry of National Education for supporting my PhD program. This work was supported by NSF-EAGER-IOS-1313814 grant to TE.

Dedication

to my Family

ABSTRACT OF THE DISSERTATION

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University of California, Riverside, December 2015
Dr. Thomas A. Eulgem, Chairperson

Providing food for human beings is one of the major challenges for the future. Plant diseases cause massive losses in crop production all over the world. Pesticides have been used as a major strategy of crop disease control, however conventional pesticides typically rely on toxic activity leading to environmental problems. Synthetic elicitors can protect plants from diseases by activating host immune responses. The identification and characterization of synthetic elicitors can result in valuable tools for the dissection of the plant defense network as well as leads for the development of environmentally-safe pesticide alternatives.

Synthetic elicitors have been classified and the vast majority of known them belong to the large group of functional SA analogs. Additionally

imprimatins, sulfonamides, adipic acid derivatives and jasmonic acid analogs were found to have synthetic elicitor activity.

By high-throughput screening we identified over 100 synthetic elicitors. By using model pathosystems Arabidopsis/*Hpa*, I functionally characterized two novel synthetic elicitors, BHTC and DPMP and their effects on plant defense pathways. BHTC can induce disease resistance quickly and transiently, has a distinct mode-of- action from already characterized synthetic elicitors. BHTC can enhance root elongation on Arabidopsis when applied at low doses, while induces defense reactions at high doses. This phenomenon is known as hormesis. Transcriptional patterns associated with BHTC-mediated hormesis were different from those associated BHTC-mediated defense. Furthermore, the WRKY70 transcription factor is required for both BHTC-mediated immunity and hormetic root elongation and links plant defense signaling to hormetic developmental responses.

DPMP is one of the strongest synthetic elicitors that were identified by our screening. It also exhibited hormesis effect at low doses and induces disease resistance at high doses. Its activity is fully dependent on NPR1 and partially dependent on WRKY70. Interestingly, two separate moieties of DPMP can independently induce immune responses. While their direct targets in plant defense are still yet to be defined, it is clear that they are powerful tools to dissect plant defense networks as well as develop novel pesticide alternatives.

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General Introduction

The Plant Immune System

Plants are subjected as a host to various types of potential pathogens and exposed to thousands of infectious diseases caused by microbial organisms. Most of the time, plants can recognize the attacking microbes and resist to diseases due to an efficient immune system consisting of multiple layers of defense mechanisms (Baker 1997; Jones and Dangl 2006; Chisholm et al. 2006). Highly conserved microbe associated molecular patterns (MAMPs) such as bacterial flagellin and microbial cell wall components are recognized by plant pattern recognition receptors (PRRs). PRRs are either surface-localized receptor-like kinases (RLKs) or receptor-like proteins (RLPs) and activate pattern-triggered immunity (PTI), a broad specificity immune mechanism (Gómez-Gómez and Boller 2002; Zipfel et al. 2004; Segonzac and Zipfel 2011; Macho and Zipfel 2014).

In *Arabidopsis thaliana* (Arabidopsis) FLAGELLIN SENSING2 (FLS2) is a well-studied PRRs. It binds to bacterial peptide flg22 that are derived from most conserved domain of eubacterial flagellin (Zipfel et al. 2006; Chinchilla et al. 2007). Upon recognition of flg22, FLS2 is activated and leads to recruitment of BAK1 as an co-receptor for flg22 (Lozano-Duran and Zipfel 2015; Zipfel 2014; Chinchilla et al. 2007). In addition to BAK1, also other related SERK (SOMATIC EMBRYOGENESIS RECEPTOR KINASE) proteins might act as co-receptor for

other ligands (Zipfel 2008, 2014; Roux et al. 2011). This first level of cellular recognition of flagellin or other MAMPs activates downstream signal transduction processes, which includes rapid ion fluxes across the plasma membrane, production of reactive oxygen species (ROS) and mitogen-activated protein kinases (MAPKs) activation (Macho and Zipfel 2014; Zipfel 2008; Zhang and Klessig 2001). Studies on early defense gene expression controlled by MAPKs in response to FLS2 mediated flg22 recognition in an Arabidopsis protoplast system revealed that a MAPK cascade containing MEKK1, MKK4/MKK5 and MPK3/MPK6 controls the WRKY22 and WRKY29 transcription factors (Asai et al. 2002). These and other signaling processes result in massive transcriptional reprogramming (Thilmony et al. 2006; Eulgem et al. 2004).

Typically, PTI is sufficient to mediate resistance against a broad range of pathogens and it is often referred to as non-host resistance (Jones and Dangl 2006). To counteract PTI, pathogens have evolved strategies to escape from recognition or suppress defined host defense signaling steps. They have evolved virulence effector molecules that are secreted into host tissues to suppress PTI-associated processes resulting in compatible plant/microbe interactions (aka effector-triggered susceptibility (ETS)) (Chisholm et al. 2006; Zipfel 2008). For example, the bacterial pathogen *Pseudomonas syringae* can inject the virulence effector AvrPtoB into plant cells, where it functions as an E3 ligase targeting FLS2 for degradation through the 26S proteasome (Göhre and Robatzek 2008). However, suppression of PTI by virulence effectors is typically incomplete and

plants exhibit weakened disease resistance, which is known as basal defense. Basal defense still can limit the growth of a pathogen but is insufficient in preventing diseases (Abramovitch and Martin 2004; Nimchuk et al. 2003). To counter this virulence strategy, plants have evolved resistance proteins (R proteins) and can recognize pathogen effector molecules. In this case the recognized virulence effector is termed an avirulence (Avr) protein. The mode of Avr protein recognition by R proteins can be direct or indirectly. In any case R-mediated Avr recognition results in a strong immune response termed effector-triggered immunity (ETI). ETI has been historically described as gene-for-gene resistance or race-specific resistance and leads to incompatible plant/microbe interactions. As a result of ETI the pathogen is avirulent and the plant is resistant (Jones and Dangl 2006; Chisholm et al. 2006; Flor 1971). For example, in tomato the activity of the bacterial AvrPtoB virulence effector is detected by the R protein Prf resulting in ETI (Ntoukakis et al. 2014).

R proteins are a second class of plant immune receptors (besides PRRs) and commonly contain a central nucleotide-binding (NB) sites and C-terminal leucine rich repeats (LRRs) domains. They can be grouped into two main classes according to their N-terminal domains. The CC-NB-LRR class contains a putative heptad leucine- zipper or coiled-coil (CC) motif. The TIR-NB-LRR contains a similar structure to the Drosophila Toll and human interleukin-1 transmembrane receptors (TIR). The TIR and CC portions are involved activation of downstream defense signaling rather than pathogen recognition, but can also mediate effector

recognition (van der Biezen et al. 2002; Rafiqi et al. 2009; Bonardi and Dangl 2012).

Mode of virulence effector recognition by R proteins can be explained by various distinct mechanistic models. The receptor-ligand model proposes a direct interaction between the R protein and effector protein. However, this model seems only to apply to a small number of R/effector interactions and virulence effectors seem in most cases to be recognized by R proteins in an indirect manner (Dangl and Jones, 2001). Such indirect recognition can be described either by the guard hypothesis or its derivative the decoy model (Ntoukakis et al. 2014; Rafiqi et al. 2009). The guard hypothesis postulates that the respective plant R protein (guard) monitors an accessory host protein (guardee), which is a direct target of the virulence effector (Van der Biezen and Jones 1998). Modification of the effector target by the effector (e.g. by degradation or chemical modification) results in activation of the R protein and initiates defense signaling. A critical element of “guard mechanisms” is that the respective effector target is an authentic component of the plant immune system and that its interference with effectors suppresses PTI. The decoy model, however, postulates the existence of “effector target mimics” which are host proteins that do not serve any function in plant immunity, except for interacting with virulence effectors and mediating their R-dependent recognition (van der Hoorn and Kamoun 2008). Thus loss-of-function mutations of authentic effector targets should affect the level of susceptibility of a given host to a virulent pathogen, while such mutations of a

decoy should not have any effect on the outcome of a compatible host pathogen interaction. Effector recognition processes conforming both the guard hypothesis as well as the decoy model have been described (van der Hoorn and Kamoun 2008).

ETI is a stronger and faster immune response than PTI and efficiently protects plants from avirulent pathogens. It is often accompanied by the hypersensitive reaction (HR) that is a form of programmed plant cell death at infection sites (Jones and Dangl 2006; Nimchuk et al. 2003). To counteract ETI some pathogens gained the ability to suppress this immune response using additional effectors. This dynamic co-evolution between plants and microbes is described by the so called “zig-zag” model that postulates that the history of a given plant/microbe interaction is mainly shaped by the evolution of new virulence effectors on the pathogen side and matching *R* gene varieties on the plant side (Jones and Dangl 2006; Dodds and Rathjen 2010; Bent and Mackey 2007). Microbes have a substantial advantage in this “evolutionary arms race”, as their generation times are shorter and the evolution of their effectors can be very fast. Still plants are able to keep up with pathogen evolution and *R* genes are the fastest evolving plant genes.

Numerous studies have shown that ETI, basal defense and PTI use a common set of signaling components including multiple regulatory proteins, reactive oxygen intermediates (ROIs) as well as the phytohormones salicylic acid

(SA), ethylene (ET) and jasmonic acid (JA). Levels of ROI, SA, ET, or JA often increase in plant tissues after pathogen infections (Nimchuk et al. 2003; Glazebrook 2005; Spanu 2012).

While PTI, basal defense and ETI are transient local responses, plant can also induce long-lasting systemic immunity (systemic acquired resistance (SAR)), which protect uninfected tissues against subsequent pathogen attack (Cui et al. 2015). SAR provides a memory and broad-spectrum resistance against pathogenic bacteria, oomycetes, fungi, virus and this immune memory can last for weeks to months or even whole growing seasons (Fu and Dong 2013). Induction of SAR requires production and accumulation of the endogenous signaling molecule SA. SA induces defense gene expression mediated by the master regulator protein NPR1 (Nonexpressor of pathogenesis related (PR) genes 1). The resulting accumulation of numerous PR proteins and antimicrobial metabolites in uninfected tissues provides long-lasting protection against a wide variety of different secondary infections (Spoel and Dong 2012; Durrant and Dong 2004).

The natural plant defense hormone SA (2-hydroxybenzoic acid) functions as an endogenous signal to activate certain immune responses and to establish disease resistance against various pathogens with biotrophic or hemibiotrophic lifestyles (Glazebrook 2005; Vlot et al. 2009). SA is a phenolic compound and in plants SA can be synthesized from primary metabolite chorismate by the

shikimate pathway (Pieterse et al. 2012; An and Mou 2014). The production of SA and its levels are normally tightly regulated and SA biosynthesis is activated upon perception of MAMPs or effector proteins during PTI and ETI respectively (Pieterse et al. 2012; Wildermuth 2006). In Arabidopsis, isochorismate synthase 1 (ICS1/SID2) mediates the production of the majority of defense-associated SA (Wildermuth et al., 2001). On the subsequent steps, during PTI and TIR-NBS-LRR-type R protein triggered ETI, ENHANCED DISEASE SUSCEPTIBILITY1 (EDS1) and PHYTOALEXIN DEFICIENT4 (PAD4) act in the onset of SA biosynthesis (Wiermer et al. 2005). When ETI is induced by CC-NBS-LRR-type R proteins, NON-RACE-SPECIFIC DISEASE RESISTANCE1 (NDR1) is taking a role on SA production (Pieterse et al. 2012). The defense-related roles of SA have been analyzed in transgenic *nahG* plants that express a bacterial SA hydroxylase which suppress endogenous SA levels and display enhanced susceptibility to a variety of pathogens (Delaney et al. 1994; Gaffney et al. 1993; Friedrich et al. 1995). In Arabidopsis various mutants with defects in SA biosynthesis and perception are used as well (Nimchuk et al. 2003).(Tsuda et al. 2009; Glazebrook et al. 2003).

Elevated SA levels can induce a set of downstream defense responses and lead to defense reactions during both local and systemic resistance. Transduction of SA signaling requires NPR1, which is also required for *PR* gene expression, local defense and SAR (Cao et al. 1994; Fu and Dong 2013). NPR1 is transcriptional co-regulator and of central importance for the activation of a

large set of defense genes in response to SA-related signals (Maleck et al., 2000). NPR1-dependent defense genes include a set of *PR* genes which encode small secreted or vacuole-targeted proteins with antimicrobial activity (Spoel and Dong 2012; Dong 2004; Fu and Dong 2013).

SA-induced cellular redox state changes activate NPR1 by reducing inactive NPR1 oligomers to active monomers , which are translocated into the nucleus where they interact with transcription factors and defense regulate gene expression (Tada et al. 2008; Mou et al. 2003). It was shown that NPR1 physically interacts with members of the TGA subfamily of bZIP transcription factors (TGA-bZIPs) and activate SA-responsive genes (Fu and Dong 2013; Dong 2004; Pieterse et al. 2009; Despres et al. 2003; Despres et al. 2000). In addition the putative SA receptor proteins NPR3 and NPR4 were recently reported to control the nuclear NPR1 concentration in a SA-level dependent manner (Fu et al. 2012; Fu and Dong 2013). The family of WRKY transcription factors has also been shown to contribute to transcriptional reprogramming during plant immune responses (Eulgem et al. 2000; Eulgem 2006; Eulgem and Somssich 2007). For example, it was shown that WRKY70 is required for multiple layers of plant defense responses to various microbes as well as cross talk between separate signal transduction pathways (Atamian et al. 2012; Hu et al. 2012; Knoth et al. 2007; Ulker et al. 2007; Li et al. 2006). WRKY70 is a member of the *LURP* (late/sustained up-regulation in response to *Hpa)* gene set, that show a highly coordinated transcriptional response during ETI and some

members of which are important for plant immunity against Hpa (Eulgem et al., 2004; Knoth et al., 2007; Knot & Eulgem, 2008). Another member of this cluster, *CaBP22* represents the average expression behavior of this gene set (Eulgem et al., 2004; Knoth et al., 2009). A chimeric reporter gene consisting of a pathogen-responsive CaBP22 promoter fragment fused to the bacterial GUS gene was used for a high throughput synthetic elicitor screen (Knoth et al. 2009; Knoth and Eulgem 2014). This screen resulted in the identification of 114 different candidate synthetic elicitors that activated the *CaBP22*$^{-333}$*::GUS* reporter gene in transgenic Arabidopsis seedlings.

In addition to SA, the stress hormones Jasmonic acid (JA) and ethylene (ET) are also control various defense responses. Comparative studies on the roles of SA, JA and ET in pathogen defense indicated that SA typically mediates immunity against biotrophic/hemibiotrophic pathogens, while JA and ET are active against necrotophic pathogens as well as insects (McDowell and Dangl 2000; Glazebrook 2005; Tsuda et al. 2009). Pathways controlled by each of these signaling molecules cause activation of numerous defense-related genes. They interact with each other in a complex manner (Glazebrook et al. 2003).

Jasmonic acid and its methylester, methyl-jasmonate (MeJA), are important members of the family of jasmonates and are broadly present in the plant kingdom. Upon pathogen or insect attack, their biosynthesis via the octadecanoid biosynthesis pathways is induced (Howe 2010). In addition to

defense responses against nectrotrophic pathogens, they are also known to control stress responses against herbivores and wounding, and perform various important roles in plant development (He et al. 2002; Balbi and Devoto 2008; Zhang and Turner 2008; Oh et al. 2013; Santino et al. 2013). Upon its biosynthesis, JA can either metabolized to MeJA or conjugated to L-isoleucine leading to jasmonoyl-isoleucine (JA-Ile), an active form of JA (Svoboda and Boland 2010; Pieterse et al. 2012). Along with Jasmonate ZIM-domain (JAZ)-type transcriptional repressors, the F-box protein Coronatine Insensitive1 (COI1) functions as JA-Ile receptors. Upon recruitment of JAZ proteins into COI1-containing SKP1-Cullin-F-box (SCF^{COI1}) complexes, they are targeted to proteasome-mediated breakdown. As a result, the expression of a large number of JA-responsive genes is de-repressed and defense responses are activated (Browse 2009; Pieterse et al. 2012; Monte et al. 2014) (Browse, 2009; Pieterse et al., 2012; Monte et al., 2014).

In addition to crosstalk between the canonical defense hormones SA, JA and ET, the balance of other phytohormones such as abscisic acid (ABA), auxin, gibberellic acid (GA), cytokinin (CK), brassinosteroid (BR) can strongly affect the outcome of plant-pathogen interactions (Pieterse et al. 2012; Robert-Seilaniantz et al. 2011; Fu and Dong 2013).

Pathosystems

Oomycetes are a clade of eukaryotic microorganisms containing various agriculturally important plant pathogens such as *Phytophthora infestans*, *P. ramorum* and *P. sojae* (Goritschnig et al. 2012). *Hyaloperonospora arabidopsidis* (*Hpa*, formerly known as *Peronospora parasitica*) *is* a natural pathogen of Arabidopsis. Asexual propagation of Hpa on its host Arabidopsis plants is initiated by asexual conidiospores that are in nature dispersed by rain or wind. If they land on the surface of the host's leaf, the conidiophores germinate and for a network of hyphae in the intracellular space of Arabidopsis mesophyll or other tissues. Eventually they form sporangiophores which emerge through the stomata and expand into tree-shaped structures that carry the asexual conidiospores. In addition to formation of asexual conidiospores, also sexual oospores can be produced in the leaf/cotyledon tissues approximately a week after infection with a conidiospores (Coates and Beynon 2010; Slusarenko and Schlaich 2003). *Hpa* is obligate biotrophic, and thus requires its host to remain alive in order to complete its life cycle. It causes a disease in its host called downy mildew (Coates and Beynon 2010).

Interactions between *Hpa* and Arabidopsis have been established as a model for race-specific immunity as well as basal defense (Botella et al. 1998) Numerous *Hpa* isolates have been found to differentially interact with distinct natural accession of Arabidopsis. Based on these interactions a variety of

avriulence determinants of *Hpa* and their cognate Arabidopsis *R* genes have been defined. In some cases the respective genes have been cloned and characterized at the molecular level (McDowell et al. 2000; Hall et al. 2009). For example the interaction between the H*pa* isolate Hiks1 and the Arabidopsis accession Columbia-0 (Col-0) is incompatible due to specific recognition of a *Hpa*Hiks1 effector by the Arabidopsis *RPP7* (resistance to *Peronospora parasitica*) gene. The *Hpa* isolate Noco2 is recognized by the *R* gene *RPP5*, which is present in the Arabidopsis accession Landsberg erecta (Ler). Col-0 is not equipped with an *R* gene recognizing any virulence effectors from the *Hpa* isolate Noco2. Thus, the Col-0/*Hpa*Noco2 interaction is compatible and is often used as a model for studies basal defense.

Additional common model pathosystems are those of Arabidopsis or tomato interacting with different strains of the pathogenic bacterium *Pseudomonas syringae*. In particular, Arabidopsis is highly suitable for studies on the plant immune system as it is an excellent experimental system for classical and molecular genetics and genomics. The availability of wide array of genetic tools, fully sequenced genome, small genome size, short generation time and small plant size make Arabidopsis as a good model plant (Nishimura and Dangl 2010).

Also a plethora of well-characterized Arabidopsis mutants allow for the fine dissection of plant immunity (Krasileva et al. 2011; Badel et al. 2013;

Goritschnig et al. 2012; Coates and Beynon 2010; McDowell et al. 2005). During the past 25 years numerous Arabidopsis mutants with defined defects in plant immunity have been identified and characterized (Glazebrook et al. 2003; Stael et al. 2015; Bernoux et al. 2011; Dodds and Rathjen 2010). In my research, I used the *Hpa*/Arabidopsis pathosystem and other model interactions to reveal the role of novel synthetic elicitors.

Synthetic Elicitors

The term “plant activator” generally refers to molecules that can protect plants from diseases by inducing immune responses. Plant activators can be either natural elicitors or synthetic elicitors. Natural elicitors are derived from plants or other living organisms and can be used to induce plant immunity. Examples for well-studied natural elicitors are the non-protein amino acid beta-amino-butyric acid (BABA) (Zimmerli et al. 2001; Ton and Mauch-Mani 2004; Hamiduzzaman et al. 2005; Ton et al. 2005), the bacterial protein flagellin (Boller and Felix 2009) or chitin ((Wan et al. 2008b; Wan et al. 2008a). Natural elicitors can trigger plant immunity by serving as activating ligands or agonists for PRR, R-proteins or signal transduction components involved in defense signaling. Synthetic elicitors are small molecules that can activate plant immune responses and are structurally distinct from natural plant defense inducers. Synthetic elicitors may induce plant defense signaling by mimicking interactions signaling

molecules with their respective cognate plant receptors or by interfering with other defense signaling components. They can be used as tools to identify new components of the plant defense network in Arabidopsis as well as mediate protection of crop plants against diseases. Synthetic elicitors can be classified as functional SA analogs, imprimatins, sulfonamides, adipic acid derivatives or jasmonic acid analogs. (Bektas and Eulgem 2015). While some of them were used in basic research, others have been effectively used in crop protection.

The frequently used SA analogs 2,6-dichloro-isonicotinic acid (INA) and benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester (BTH) have been successfully used in research on plant immunity. Interactions of these two compounds with plant defense system have been well characterized and they have been abundantly used as defense triggers in basic and applied studies on plant immunity In addition BTH, which has been marketed by Syngenta under the name BION® or Actigard®. Furthermore some of other SA analogs (Probenazole, Tiadinil, Isotianil) are used as commercialized agrochemicals to protect plants against disease resistance (Bektas & Eulgem, 2015).

I reviewed the literature on synthetic elicitors in detail in chapter one (Bektas and Eulgem 2015).

Hormesis

Hormesis is a pharmacological concept describing biphasic dose-response relationships. While the exposure of biological systems to high doses of detrimental chemical agent or environmental factors has negative effects on their performance, exposure to low doses of the same stimulus can be beneficial (Mattson et al. 2010; Calabrese and Baldwin 2001).

Hormesis often is typically characterized by “inverse U"-shaped dose-response curves instead of the sigmoid curves predicted by standard pharmacological threshold models. Such phenomena have been described in various biological systems including humans and plants in response to many different “stressors”. These stressors include chemicals, environmental stress factors, increased energy expenditure such as running, cognitive challenges, reduced energy availability etc. (Calabrese 2008b, a; Calabrese and Blain 2005; Mattson et al. 2010). In 1880, Hugo Schulz, a professor of pharmacology at the University of Greifswald in northern Germany, discovered the concept of hormesis. He observed that while chemical disinfectants stimulated the metabolism of yeast at low doses, they inhibited it at higher doses (Calabrese 2008b; Calabrese 2015a). Over the years, it has been shown and cataloged thousands of examples of hormetic dose-responses in the fields of biology, toxicology, and medicine (Calabrese and Blain 2005; Calabrese and Blain 2009, 2011).

Hormetic responses are know to occur at multiple levels of biological organization, such as the cellular, organ, individual or in population levels (Calabrese 2008b). This response has been suggested to be generally based on compensatory processes following an initial disruption in homeostasis (Mattson et al. 2010). To survive and reproduce, organisms must be able to tolerate various hazards of their environments and limitations of energy sources. Thus, at least in some cases adaptive evolutionary responses of organisms to their environments seem to explain the occurrence of hormetic phenomena (Mattson 2008; Mattson et al. 2010).

An example of hormesis is responses triggered by the neurotransmitter glutamate. Glutamate is critical for the transfer of electrical activity from one nerve cell to another in mammalian brains. Relatively low amounts of glutamate are released at synapses when the brain is involved in sensory processing, motor responses, learning and memory, or generating emotional behaviors. Low glutamate doses also activate adaptive cellular stress response pathways (ACSRPs). ACSRPs involve the activation of transcription factors that induce expression of cytoprotective proteins such as antioxidant enzymes, protein chaperons and growth factors. This way ACSRPs benefit the nerve cells, promote their growth and survival. However, excessive amounts of glutamate can damage or kill nerve cells with excitotoxicity that is occur in patient with epilepsy, stroke, traumatic brain (Mattson et al. 2010). Thus, the understanding of hormesis can lead to novel approach for preventing and treating human

diseases (Calabrese et al. 2015).

Not only endogenous factors of the organism, but also exogenous factors such as environmental stressors or chemicals can also exhibit hormesis in organism (Calabrese and Baldwin 2001). For example, radioactive radiation, which is a powerful mutagen, metabolic inhibitors, toxic heavy metals or carcinogenic chemicals, such as dioxins, are known to trigger hormetic effects (Mattson et al. 2010; Calabrese 2008b; Calabrese and Dhawan 2013). Hormesis seems to be as common among plants as it is among animals. In plants, herbicides, natural phytotoxins and radioactivity were shown to be potent stimuli of plant hormesis. In the vast majority of cases "growth" or "metabolic rate" were found to be endpoints stimulated by low doses of hormetic agents in plants (Calabrese and Blain 2009; Calabrese 2015b; Velini et al. 2008). Despite the potential significance of hormesis for enhancement for commercial crop production, the genetic and biochemical basis of hormesis in plants is completely unclear. As a model plant, Arabidopsis can serve as a starting point for more extended studies on the mechanistic basis underlying hormesis phenomena in plants.

Goals of this study

My project focuses mainly on synthetic elicitors. In chapter one, I reviewed the literature of the synthetic plant defense elicitors. In chapter two and three I described worked on the functional characterization of two types of novel synthetic elicitors. Previously, by high-throughput screening, our lab identified over 100 drug-like compounds that induce expression of the pathogen-responsive *CaBP22*$^{-333}$*::GUS* reporter gene in Arabidopsis (Knoth et al. 2009) (Rodriguez-Salus, Bektas., et al. 2015, submitted). I worked on two of the synthetic elicitors (BHTC and DPMP) that were identified from this screening. I worked on the characterization of their activity and mode-of action. I used an established pathosystems, pathogenic oomycete *Hpa* and bacterial pathogen *Pseudomonas syringae* pathovar *tomato* strain DC3000 (*Pst*) and their plant host Arabidopsis. In addition to plant defense responses these compounds trigger at higher doses, I have also demonstrated that they induced hormetic root elongation in Arabidopsis plants at low doses. To elucidate both plant immune responses as well as hormesis I performed mRNAseq analysis on root and shoot tissues treated with low- and high doses of BHTC or DPMP. These analyses clearly showed that the transcriptional profiles associated with low- and high dose responses of BTHC are completely different. In addition, experiments with plant defense mutants revealed that WRKY70 links plant defense responses to hormesis on BHTC-induced hormesis. This project highlights the importance of identifying novel synthetic elicitors useful for the identification of novel

components of the plant defense network and hormesis as well as development of novel type of pesticide alternatives.

References

Abramovitch RB, Martin GB (2004) Strategies used by bacterial pathogens to suppress plant defenses. Current opinion in plant biology 7 (4):356-364. doi:10.1016/j.pbi.2004.05.002

An C, Mou Z (2014) Salicylic Acid and Defense Responses in Plants In: Tran L-SP, Pal S (eds) Phytohormones: A Window to Metabolism, Signaling and Biotechnological Applications. Springer Science+Business Media, New York, pp 191-219

Asai T, Tena G, Plotnikova J, Willmann MR, Chiu W-L, Gomez-Gomez L, Boller T, Ausubel FM, Sheen J (2002) MAP kinase signalling cascade in Arabidopsis innate immunity. Nature 415 (6875):977-983. doi:10.1038/415977a

Atamian HS, Eulgem T, Kaloshian I (2012) SlWRKY70 is required for Mi-1-mediated resistance to aphids and nematodes in tomato. Planta 235 (2):299-309. doi:10.1007/s00425-011-1509-6

Badel JL, Piquerez SJM, Greenshields D, Rallapalli G, Fabro G, Ishaque N, Jones JDG (2013) In Planta Effector Competition Assays Detect Hyaloperonospora arabidopsidis Effectors That Contribute to Virulence and Localize to Different Plant Subcellular Compartments. Molecular plant-microbe interactions : MPMI 26 (7):745-757. doi:10.1094/MPMI-06-12-0154-R

Baker B (1997) Signaling in Plant-Microbe Interactions. Science 276 (5313):726-733. doi:10.1126/science.276.5313.726

Balbi V, Devoto A (2008) Jasmonate signalling network in *Arabidopsis thaliana*: crucial regulatory nodes and new physiological scenarios. New Phytologist 177 (2):301-318. doi:10.1111/j.1469-8137.2007.02292.x

Bektas Y, Eulgem T (2015) Synthetic plant defense elicitors. Frontiers in plant science 5

Bent AF, Mackey D (2007) Elicitors, effectors, and R genes: the new paradigm and a lifetime supply of questions. Annual review of phytopathology 45:399-436. doi:10.1146/annurev.phyto.45.062806.094427

Bernoux M, Ellis JG, Dodds PN (2011) New insights in plant immunity signaling activation. Current opinion in plant biology 14 (5):512-518. doi:10.1016/j.pbi.2011.05.005

Boller T, Felix G (2009) A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. Annual review of plant biology 60:379-406. doi:10.1146/annurev.arplant.57.032905.105346

Bonardi V, Dangl JL (2012) How complex are intracellular immune receptor signaling complexes? Frontiers in plant science 3:1-9

Botella MA, Parker JE, Frost LN, Bittner-Eddy PD, Beynon J, Daniels MJ, Holub EB, Jones JGS (1998) Three genes of the Arabidopsis RPP1 complex resistance locus recognize distinct Peronospora parasitica avirulence determinants. The Plant Cell 10:1847-1860

Browse J (2009) Jasmonate passes muster: a receptor and targets for the defense hormone. Annual review of plant biology 60:183-205. doi:10.1146/annurev.arplant.043008.092007

Calabrese EJ (2008a) Hormesis and medicine. British journal of clinical pharmacology 66 (5):594-617. doi:10.1111/j.1365-2125.2008.03243.x

Calabrese EJ (2008b) Hormesis: why it is important to toxicology and toxicologists. Environmental toxicology and chemistry / SETAC 27 (7):1451-1474. doi:10.1897/07-541

Calabrese EJ (2015a) Historical foundations of hormesis. Homeopathy : the journal of the Faculty of Homeopathy 104 (2):83-89. doi:10.1016/j.homp.2015.01.001

Calabrese EJ (2015b) Hormesis within a mechanistic context. Homeopathy : the journal of the Faculty of Homeopathy 104 (2):90-96. doi:10.1016/j.homp.2015.01.002

Calabrese EJ, Baldwin La (2001) Hormesis: a generalizable and unifying hypothesis. Critical reviews in toxicology 31 (4-5):353-424. doi:10.1080/20014091111730

Calabrese EJ, Blain R (2005) The occurrence of hormetic dose responses in the toxicological literature, the hormesis database: an overview. Toxicology and applied pharmacology 202 (3):289-301. doi:10.1016/j.taap.2004.06.023

Calabrese EJ, Blain RB (2009) Hormesis and plant biology. Environmental pollution (Barking, Essex : 1987) 157 (1):42-48. doi:10.1016/j.envpol.2008.07.028

Calabrese EJ, Blain RB (2011) The hormesis database: the occurrence of hormetic dose responses in the toxicological literature. Regulatory toxicology and pharmacology : RTP 61 (1):73-81. doi:10.1016/j.yrtph.2011.06.003

Calabrese EJ, Dhawan G (2013) The historical use of radiotherapy in the treatment of sinus infections. Dose Response 11:469-479. doi:10.2203/dose-response.13-004.Calabrese

Calabrese EJ, Shamoun DY, Hanekamp JC (2015) Cancer risk assessment: Optimizing human health through linear dose-response models. Food and Chemical Toxicology 81:137-140. doi:10.1016/j.fct.2015.04.023

Cao H, Bowling SA, Gordon AS, Dong X (1994) Characterization of an Arabidopsis Mutant That Is Nonresponsive to Inducers of Systemic Acquired Resistance. The Plant Cell 6:1583-1592

Chinchilla D, Zipfel C, Robatzek S, Kemmerling B, Nürnberger T, Jones JDG, Felix G, Boller T (2007) A flagellin-induced complex of the receptor FLS2 and BAK1 initiates plant defence. Nature 448 (7152):497-500. doi:10.1038/nature05999

Chisholm ST, Coaker G, Day B, Staskawicz BJ (2006) Host-microbe interactions: shaping the evolution of the plant immune response. Cell 124 (4):803-814. doi:10.1016/j.cell.2006.02.008

Coates ME, Beynon JL (2010) Hyaloperonospora Arabidopsidis as a pathogen model. Annual review of phytopathology 48:329-345. doi:10.1146/annurev-phyto-080508-094422

Cui H, Tsuda K, Parker JE (2015) Effector-Triggered Immunity: From Pathogen Perception to Robust Defense. Annual Review of Plant Biology, Vol 66 66:487-511. doi:10.1146/annurev-arplant-050213-040012

Delaney TP, Uknes S, Vernooij B, Friedrich L, Weymann K, Negrotto D, Gaffney T, Gut-Rella M, Kessmann H, Ward E, Ryals J (1994) A central role of salicylic Acid in plant disease resistance. Science (New York, NY) 266 (5188):1247-1250. doi:10.1126/science.266.5188.1247

Despres C, Chubak C, Rochon A, Clark R, Bethune T, Desveaux D, Fobert PR (2003) The Arabidopsis NPR1 disease resistance protein is a novel cofactor that confers redox regulation of DNA binding activity to the basic domain/leucine zipper transcription factor TGA1. Plant Cell 15 (9):2181-2191

Despres C, DeLong C, Glaze S, Liu E, Fobert PR (2000) The Arabidopsis NPR1/NIM1 protein enhances the DNA binding activity of a subgroup of the TGA family of bZIP transcription factors. Plant Cell 12 (2):279-290

Dodds PN, Rathjen JP (2010) Plant immunity: towards an integrated view of plant-pathogen interactions. Nature reviews Genetics 11 (8):539-548. doi:10.1038/nrg2812

Dong X (2004) NPR1, all things considered. Current opinion in plant biology 7 (5):547-552. doi:10.1016/j.pbi.2004.07.005

Durrant WE, Dong X (2004) Systemic acquired resistance. Annu Rev Phytopathol 42:185-209. doi:10.1146/annurev.phyto.42.040803.140421

Eulgem T (2006) Dissecting the WRKY web of plant defense regulators. PLoS pathogens 2 (11):e126-e126. doi:10.1371/journal.ppat.0020126

Eulgem T, Rushton PJ, Robatzek S, Somssich IE (2000) The WRKY superfamily of plant transcription factors. Trends in plant science 5 (5):199-206

Eulgem T, Somssich IE (2007) Networks of WRKY transcription factors in defense signaling. Current opinion in plant biology 10 (4):366-371. doi:10.1016/j.pbi.2007.04.020

Eulgem T, Weigman VJ, Chang H-s, McDowell JM, Holub EB (2004) Gene Expression Signatures from Three Genetically Separable Resistance Gene Signaling Pathways for Downy Mildew Resistance 1 [w]. 135 (June):1129-1144. doi:10.1104/pp.104.040444.lecular

Flor HH (1971) Current status of the gene-fob-gene concept.275-296

Friedrich L, Vernooij B, Gaffney T, Morse A, Ryals J (1995) Characterization of tobacco plants expressing a bacterial salicylate hydroxylase gene. Plant Mol Biol 29 (5):959-968

Fu ZQ, Dong X (2013) Systemic acquired resistance: turning local infection into global defense. Annual review of plant biology 64:839-863. doi:10.1146/annurev-arplant-042811-105606

Fu ZQ, Yan S, Saleh A, Wang W, Ruble J, Oka N, Mohan R, Spoel SH, Tada Y, Zheng N, Dong X (2012) NPR3 and NPR4 are receptors for the immune signal salicylic acid in plants. Nature 486 (7402):228-232. doi:10.1038/nature11162

Gaffney T, Friedrich L, Vernooij B, Negrotto D, Nye G, Uknes S, Ward E, Kessmann H, Ryals J (1993) Requirement of salicylic Acid for the induction of systemic acquired resistance. Science 261 (5122):754-756. doi:10.1126/science.261.5122.754

Glazebrook J (2005) Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. Annual review of phytopathology 43:205-227. doi:10.1146/annurev.phyto.43.040204.135923

Glazebrook J, Chen W, Estes B, Chang HS, Nawrath C, Metraux JP, Zhu T, Katagiri F (2003) Topology of the network integrating salicylate and jasmonate signal transduction derived from global expression phenotyping. Plant J 34 (2):217-228

Göhre V, Robatzek S (2008) Breaking the barriers: microbial effector molecules subvert plant immunity. Annual review of phytopathology 46:189-215. doi:10.1146/annurev.phyto.46.120407.110050

Gómez-Gómez L, Boller T (2002) Flagellin perception: a paradigm for innate immunity. Trends in plant science 7 (6):251-256

Goritschnig S, Krasileva KV, Dahlbeck D, Staskawicz BJ (2012) Computational prediction and molecular characterization of an oomycete effector and the cognate Arabidopsis resistance gene. PLoS genetics 8 (2):e1002502-e1002502. doi:10.1371/journal.pgen.1002502

Hall SA, Allen RL, Baumber RE, Baxter A, Fisher K, Bittner-Eddy PD, Rose LE, Holub EB, Beynon JL (2009) Maintenance of genetic variation in plants and pathogens involves complex networks of gene-for-gene interactions. molecular plant pathology 10:449-457

Hamiduzzaman MM, Jakab G, Barnavon L, Neuhaus JM, Mauch-Mani B (2005) beta-Aminobutyric acid-induced resistance against downy mildew in grapevine acts through the potentiationof callose formation and jasmonic acid signaling. MPMI 18:819-829

He Y, Fukushige H, Hildebrand DF, Gan S (2002) Evidence supporting a role of jasmonic acid in Arabidopsis leaf senescence. Plant Physiol 128 (3):876-884. doi:10.1104/pp.010843

Howe G (2010) Jasmonates. In: Davies P (ed) Plant Hormones. Springer Netherlands, pp 646-680. doi:10.1007/978-1-4020-2686-7_28

Hu Y, Dong Q, Yu D (2012) Arabidopsis WRKY46 coordinates with WRKY70 and WRKY53 in basal resistance against pathogen Pseudomonas syringae.

Plant science : an international journal of experimental plant biology 185-186:288-297. doi:10.1016/j.plantsci.2011.12.003

Jones JDG, Dangl JL (2006) The plant immune system. Nature 444 (7117):323-329. doi:10.1038/nature05286

Knoth C, Eulgem T (2014) High-Throughput Screening of Small-Molecule Libraries for Inducers of Plant Defense Responses. In: Hicks GR, Robert S (eds) Plant Chemical Genomics, vol 1056. Methods in Molecular Biology. Humana Press, pp 45-49. doi:10.1007/978-1-62703-592-7_5

Knoth C, Ringler J, Dangl JL, Eulgem T (2007) Arabidopsis WRKY70 is required for full RPP4-mediated disease resistance and basal defense against Hyaloperonospora parasitica. Molecular plant-microbe interactions : MPMI 20 (2):120-128. doi:10.1094/MPMI-20-2-0120

Knoth C, Salus MS, Girke T, Eulgem T (2009) The synthetic elicitor 3,5-dichloroanthranilic acid induces NPR1-dependent and NPR1-independent mechanisms of disease resistance in Arabidopsis. Plant physiology 150 (1):333-347. doi:10.1104/pp.108.133678

Krasileva KV, Zheng C, Leonelli L, Goritschnig S, Dahlbeck D, Staskawicz BJ (2011) Global analysis of Arabidopsis/downy mildew interactions reveals prevalence of incomplete resistance and rapid evolution of pathogen recognition. PloS one 6 (12):e28765-e28765. doi:10.1371/journal.pone.0028765

Li J, Brader G, Kariola T, Palva ET (2006) WRKY70 modulates the selection of signaling pathways in plant defense. The Plant journal : for cell and molecular biology 46 (3):477-491. doi:10.1111/j.1365-313X.2006.02712.x

Lozano-Duran R, Zipfel C (2015) Trade-off between growth and immunity: role of brassinosteroids. Trends in Plant Science 20 (1):12-19. doi:10.1016/j.tplants.2014.09.003

Macho AP, Zipfel C (2014) Plant PRRs and the Activation of Innate Immune Signaling. Molecular Cell 54 (2):263-272. doi:10.1016/j.molcel.2014.03.028

Mattson MP (2008) Awareness of hormesis will enhance future research in basic and applied neuroscience. Critical reviews in toxicology 38 (7):633-639. doi:10.1080/10408440802026406

Mattson MP, Calabrese EJ, Son TG, Cutler RG, Camandola S, Chadwick W, Maudsley S, Stranahan AM, Martin B, Ji S, White CM (2010) Hormesis A revolution in biology, toxicology and medicine. springer, New York, NY

McDowell JM, Cuzick a, Can C, Beynon J, Dangl JL, Holub EB (2000) Downy mildew (Peronospora parasitica) resistance genes in Arabidopsis vary in functional requirements for NDR1, EDS1, NPR1 and salicylic acid accumulation. The Plant journal : for cell and molecular biology 22 (6):523-529

McDowell JM, Dangl JL (2000) Signal transduction in the plant immune response. Trends in biochemical sciences 25 (2):79-82

McDowell JM, Williams SG, Funderburg NT, Eulgem T, Dangl JL (2005) Genetic analysis of developmentally regulated resistance to downy mildew (Hyaloperonospora parasitica) in Arabidopsis thaliana. Molecular plant-microbe interactions : MPMI 18 (11):1226-1234. doi:10.1094/MPMI-18-1226

Monte I, Hamberg M, Chini A, Gimenez-Ibanez S, García-Casado G, Porzel A, Pazos F, Boter M, Solano R (2014) Rational design of a ligand-based antagonist of jasmonate perception. Nat Chem Biol 10 (8):671-676. doi:10.1038/nchembio.1575

http://www.nature.com/nchembio/journal/v10/n8/abs/nchembio.1575.html - supplementary-information

Mou Z, Fan W, Dong X (2003) Inducers of plant systemic acquired resistance regulate NPR1 function through redox changes. Cell 113 (7):935-944

Nimchuk Z, Eulgem T, Holt BF, Dangl JL (2003) Recognition and response in the plant immune system. Annual review of genetics 37:579-609. doi:10.1146/annurev.genet.37.110801.142628

Nishimura MT, Dangl JL (2010) Arabidopsis and the plant immune system. Plant J 61 (6):1053-1066

Ntoukakis V, Saur IML, Conlan B, Rathjen JP (2014) The changing of the guard: the Pto/Prf receptor complex of tomato and pathogen recognition. Current Opinion in Plant Biology 20:69-74. doi:10.1016/j.pbi.2014.04.002

Oh Y, Baldwin IT, Galis I (2013) A jasmonate ZIM-domain protein NaJAZd regulates floral jasmonic acid levels and counteracts flower abscission in Nicotiana attenuata plants. PloS one 8 (2):e57868

Pieterse CMJ, Leon-Reyes A, Van der Ent S, Van Wees SCM (2009) Networking by small-molecule hormones in plant immunity. Nature chemical biology 5 (5):308-316. doi:10.1038/nchembio.164

Pieterse CMJ, van der Does D, Zamioudis C, Leon-Reyes A, van Wees SCM (2012) Hormonal Modulation of Plant Immunity. Annual review of cell and developmental biology (April):1-33. doi:10.1146/annurev-cellbio-092910-154055

Rafiqi M, Bernoux M, Ellis JG, Dodds PN (2009) In the trenches of plant pathogen recognition: Role of NB-LRR proteins. Seminars in cell & developmental biology 20 (9):1017-1024. doi:10.1016/j.semcdb.2009.04.010

Robert-Seilaniantz A, Grant M, Jones JDG (2011) Hormone crosstalk in plant disease and defense: more than just jasmonate-salicylate antagonism. Annual review of phytopathology 49:317-343. doi:10.1146/annurev-phyto-073009-114447

Roux M, Schwessinger B, Albrecht C, Chinchilla D, Jones A, Holton N, Malinovsky FG, Tör M, de Vries S, Zipfel C (2011) The Arabidopsis leucine-rich repeat receptor-like kinases BAK1/SERK3 and BKK1/SERK4 are required for innate immunity to hemibiotrophic and biotrophic pathogens. The Plant cell 23 (6):2440-2455. doi:10.1105/tpc.111.084301

Santino A, Taurino M, De Domenico S, Bonsegna S, Poltronieri P, Pastor V, Flors V (2013) Jasmonate signaling in plant development and defense response to multiple (a)biotic stresses. Plant Cell Rep 32 (7):1085-1098. doi:10.1007/s00299-013-1441-2

Segonzac C, Zipfel C (2011) Activation of plant pattern-recognition receptors by bacteria. Current opinion in microbiology 14 (1):54-61. doi:10.1016/j.mib.2010.12.005

Slusarenko AJ, Schlaich NL (2003) Downy mildew of Arabidopsis thaliana caused by Hyaloperonospora parasitica (formerly Peronospora parasitica). Molecular plant pathology 4 (3):159-170. doi:10.1046/j.1364-3703.2003.00166.x

Spanu PD (2012) The Genomics of Obligate (and Nonobligate) Biotrophs. Annual review of phytopathology (April):1-19. doi:10.1146/annurev-phyto-081211-173024

Spoel SH, Dong X (2012) How do plants achieve immunity? Defence without specialized immune cells. Nature reviews Immunology 12 (2):89-100. doi:10.1038/nri3141

Stael S, Kmiecik P, Willems P, Van Der Kelen K, Coll NS, Teige M, Van Breusegem F (2015) Plant innate immunity - sunny side up? Trends in Plant Science 20 (1):3-11. doi:10.1016/j.tplants.2014.10.002

Svoboda J, Boland W (2010) Plant defense elicitors: analogues of jasmonoyl-isoleucine conjugate. Phytochemistry 71 (13):1445-1449. doi:10.1016/j.phytochem.2010.04.027

Tada Y, Spoel SH, Pajerowska-Mukhtar K, Mou Z, Song J, Wang C, Zuo J, Dong X (2008) Plant immunity requires conformational changes [corrected] of NPR1 via S-nitrosylation and thioredoxins. Science (New York, NY) 321 (5891):952-956. doi:10.1126/science.1156970

Thilmony R, Underwood W, He SY (2006) Genome-wide transcriptional analysis of the Arabidopsis thaliana interaction with the plant pathogen Pseudomonas syringae pv. tomato DC3000 and the human pathogen Escherichia coli O157:H7. Plant J 46 (1):34-53. doi:10.1111/j.1365-313X.2006.02725.x

Ton J, Jakab G, Toquin V, Flors V, Lavicoli A, Maeder MN, Metraux JP, Mauch-Mani B (2005) Dissecting the b -Aminobutyric Acid – Induced Priming Phenomenon in Arabidopsis. The Plant Cell 17:987-999. doi:10.1105/tpc.104.029728

Ton J, Mauch-Mani B (2004) Beta-amino-butyric acid-induced resistance against necrotrophic pathogens is based on ABA-dependent priming for callose. The Plant Journal 38:119-130. doi:10.1111/j.1365-313X.2004.02028.x

Tsuda K, Sato M, Stoddard T, Glazebrook J, Katagiri F (2009) Network properties of robust immunity in plants. PLoS Genet 5 (12):e1000772. doi:10.1371/journal.pgen.1000772

Ulker B, Shahid Mukhtar M, Somssich IE (2007) The WRKY70 transcription factor of Arabidopsis influences both the plant senescence and defense signaling pathways. Planta 226 (1):125-137. doi:10.1007/s00425-006-0474-y

van der Biezen Ea, Freddie CT, Kahn K, Parker JE, Jones JDG (2002) Arabidopsis RPP4 is a member of the RPP5 multigene family of TIR-NB-LRR genes and confers downy mildew resistance through multiple

signalling components. The Plant journal : for cell and molecular biology 29 (4):439-451

Van der Biezen Ea, Jones JD (1998) Plant disease-resistance proteins and the gene-for-gene concept. Trends in biochemical sciences 23 (12):454-456

van der Hoorn RA, Kamoun S (2008) From Guard to Decoy: a new model for perception of plant pathogen effectors. Plant Cell 20 (8):2009-2017. doi:10.1105/tpc.108.060194

Velini ED, Alves E, Godoy MC, Meschede DK, Souza RT, Duke SO (2008) Glyphosate applied at low doses can stimulate plant growth. 496 (January):489-496. doi:10.1002/ps

Vlot aC, Dempsey DMA, Klessig DF (2009) Salicylic Acid, a multifaceted hormone to combat disease. Annual review of phytopathology 47:177-206. doi:10.1146/annurev.phyto.050908.135202

Wan J, Zhang X, Neece D, Ramonell KM, Clough S, Kim S, Stacey MG, Stacey G (2008a) A LysM receptor-like kinase plays a critical role in chitin signaling and fungal resistance in Arabidopsis. The Plant Cell 20:471-481

Wan J, Zhang XC, Stacey G (2008b) Chitin signaling and plant disease resistance. Plant signal Behav 3:831-833

Wiermer M, Feys BJ, Parker JE (2005) Plant immunity: the EDS1 regulatory node. Current opinion in plant biology 8 (4):383-389. doi:10.1016/j.pbi.2005.05.010

Wildermuth MC (2006) Variations on a theme: synthesis and modification of plant benzoic acids. Curr Opin Plant Biol 9 (3):288-296. doi:10.1016/j.pbi.2006.03.006

Zhang S, Klessig DF (2001) MAPK cascades in plant defense signaling. Trends Plant Sci 6 (11):520-527

Zhang Y, Turner JG (2008) Wound-induced endogenous jasmonates stunt plant growth by inhibiting mitosis. PLoS One 3 (11):e3699

Zimmerli L, Metraux JP, Mauch-Mani B (2001) Beta-Aminobutyric acid-induced protection of Arabidopsis against the necrotrophic fungus Botrytis cinerea. Plant Physiology 126:517-523

Zipfel C (2008) Pattern-recognition receptors in plant innate immunity. Current opinion in immunology 20 (1):10-16. doi:10.1016/j.coi.2007.11.003

Zipfel C (2014) Plant pattern-recognition receptors. Trends in Immunology 35 (7):345-351. doi:10.1016/j.it.2014.05.004

Zipfel C, Kunze G, Chinchilla D, Caniard A, Jones JDG, Boller T, Felix G (2006) Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts Agrobacterium-mediated transformation. Cell 125 (4):749-760. doi:10.1016/j.cell.2006.03.037

Zipfel C, Robatzek S, Navarro L, Oakeley EJ, Jones JDG, Felix G, Boller T (2004) Bacterial disease resistance in Arabidopsis through flagellin perception. Nature 428 (6984):764-767. doi:10.1038/nature02485

CHAPTER 1: A Literature Review on Synthetic Plant Defense Elicitors

Previously published under the title “Synthetic Plant Defense Elicitors” by Bektas Y and Eulgem T (2015), *Front. Plant Sci.* 5:804. doi: 10.3389/fpls.2014.00804

Abstract

To defend themselves against invading pathogens plants utilize a complex regulatory network that coordinates extensive transcriptional and metabolic reprogramming. Although many of the key players of this immunity-associated network are known, the details of its topology and dynamics are still poorly understood. As an alternative to forward and reverse genetic studies, chemical genetics-related approaches based on bioactive small molecules have gained substantial popularity in the analysis of biological pathways and networks. Use of such molecular probes can allow researchers to access biological space that was previously inaccessible to genetic analyses due to gene redundancy or lethality of mutations. Synthetic elicitors are small drug-like molecules that induce plant defense responses, but are distinct from known natural elicitors of plant immunity. While the discovery of some synthetic elicitors had already been reported in the 1970s, recent breakthroughs in combinatorial chemical synthesis now allow for inexpensive high-throughput

screens for bioactive plant defense-inducing compounds. Along with powerful reverse genetics tools and resources available for model plants and crop systems, comprehensive collections of new synthetic elicitors will likely allow plant scientists to study the intricacies of plant defense signaling pathways and networks in an unparalleled fashion. As synthetic elicitors can protect crops from diseases, without the need to be directly toxic for pathogenic organisms, they may also serve as promising alternatives to conventional biocidal pesticides, which often are harmful for the environment, farmers and consumers. Here we are discussing various types of synthetic elicitors that have been used for studies on the plant immune system, their modes-of-action as well as their application in crop protection.

Introduction

The plant immune system

Plants serve as a source of nutrients for a wide variety of heterotrophic microorganisms that can cause diseases in their hosts. Physical barriers, such as a waxy cuticular layer and rigid cell walls, as well as preformed antimicrobial chemicals can provide some protection against attacking phytopathogens (Nürnberger and Lipka, 2005). In addition, plants have evolved an inducible immune system that is based on the specific recognition of pathogen-derived molecules (Chisholm et al., 2006; Jones and Dangl, 2006). Two classes of plant immune receptors are critical for defense activation (Jones and Dangl, 2006; Dodds and Rathjen, 2010). Pattern recognition receptors (PRRs) directly interact with highly conserved microbe associated molecular patterns (MAMPs) activating pattern-triggered immunity (PTI) (Gómez-Gómez and Boller, 2002; Zipfel et al., 2004; Segonzac and Zipfel, 2011). PTI can be attenuated or blocked by effector molecules that are secreted into plant cells by microbial pathogens that are well- adapted to their hosts (Abramovitch and Martin, 2004). The remaining weakened host immunity operating during such compatible plant/pathogen interactions (a state also referred to as effector-triggered susceptibility, ETS) is called basal defense (Glazebrook et al., 2003; Chisholm et al., 2006; Jones and Dangl, 2006). While basal defense can limit

the spread of virulent pathogens in their hosts, it is typically insufficient to prevent disease.

A second class of plant immune receptors, encoded by disease *resistance* (*R*)-genes, recognize the presence or activity of effectors and induce effector-triggered immunity (ETI), a manifestation of the well-described phenomenon of gene-for-gene resistance or race-specific resistance which leads to incompatible interactions (Flor, 1971; Nimchuk et al., 2003; Jones and Dangl, 2006; Elmore et al., 2011). ETI is a strong immune response that efficiently protects plants from avirulent pathogens and is often associated with the hypersensitive reaction (HR), a form of programmed death of plant cells at infection sites. Purified molecules or crude biochemical preparations from pathogens triggering PTI have also been referred to as general elicitors, while those triggering ETI, or race-specific resistance, have been termed race-specific elicitors (Wevelsiep et al., 1991). Numerous studies have shown that ETI, basal defense and PTI utilize a common set of signaling components including multiple regulatory proteins, reactive oxygen intermediates (ROI) as well as the phytohormones salicylic acid (SA), ethylene (ET) and jasmonic acid (JA) (Nimchuk et al., 2003; Glazebrook, 2005; Spanu, 2012). Levels of ROI, SA, ET or JA often increase in plant tissues after pathogen infections. While basal defense seems mainly to be a weakened form of PTI, ETI has been proposed to result from boosted basal defense- or PTI-associated responses (Tao et al., 2003; Jones and Dangl, 2006; Shen et al.,

2007). Inducible immune responses are tightly associated with extensive transcriptional- and metabolic–reprogramming controlled by a complex regulatory network (Glazebrook et al., 2003; Stockwell, 2004; Tsuda et al., 2009). While historically ten classes of pathogenesis-related (*PR*) genes had been recognized, which exhibit transcriptional up- regulation in defense-related biological situations (Kombrink and Somssich, 1997), more recent genome-wide transcript profiling studies have revealed that hundreds to thousands of genes typically respond to defense induction by transiently altered transcript levels. Numerous signal transducers and transcription factors have been implicated in the plant defense network (Katagiri, 2004; Eulgem, 2005; Jones and Dangl, 2006). This network can be subdivided into various defined sectors that can interact with each other (Tsuda et al., 2009; Sato et al., 2010). For example, distinct defense signaling sectors dependent on early MAMP-activated MAP kinases or the hormones 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 et al., 2009). The latter phenomenon seems to allow for compensatory effects if a defined sector is disabled due to interferences with pathogen effectors. Inducible immune responses are tightly associated with extensive transcriptional- and metabolic–reprogramming controlled by a complex regulatory network (Glazebrook et al., 2003; Stockwell, 2004; Tsuda et al., 2009). While historically ten classes of pathogenesis-related (*PR*) genes

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The complexity of this network is likely the result of two separate co-directional evolutionary pressures. Firstly, the asymmetrical arms race between plants and pathogens/pests manifested in continuous co-evolution of effectors and their host targets may have resulted in an ever-increasing diversity of plant defense regulators and regulatory circuits. Secondly, the need to fine-tune defense outputs appropriate for the respective attacker(s), which may exhibit biotrophic, hemibiotrophic or nectrotrophic lifestyles, requires a complex

regulatory system that allows for extensive crosstalk and compensatory interactions (Tsuda et al., 2009). An additional level of complexity likely arose from the need to link effector recognition mechanisms, which appear to be of recent evolutionary origin to more ancient regulatory processes mediating PTI (Chisholm et al., 2006; Holub, 2008).

While PTI, basal defense and ETI are transient local responses limited to pathogen infected tissues, plants can also activate long-lasting systemic immunity. Such systemic immunity can be initiated by local compatible or incompatible interactions resulting in systemic acquired resistance (SAR) or triggered by certain strains of nonpathogenic plant growth-promoting rhizobacteria (PGPR) leading to induced systemic resistance (ISR) (Pieterse et al., 1998; van Wees et al., 2000). SAR mediates long-lasting broad- spectrum resistance to a wide range of pathogens in uninfected tissues and organs (Ward et al., 1991; Fu and Dong, 2013). In addition to local pathogen infections, exogenous application of SA or SA analogs (see below) can induce SAR-like responses (White, 1979; Metraux et al., 1991; Ward et al., 1991). SAR and related systemic immune responses have been demonstrated in several plant systems, such as cucumber, watermelon, tobacco and *Arabidopsis thaliana* (Arabidopsis) (White, 1979; Kuc, 1982; Metraux et al., 1991; Ward et al., 1991). Typically SAR is associated with a local and systemic increase of SA levels that conditions enhanced expression of several classical *PR* genes (Rasmussen et al., 1991; Ward et al., 1991; Vernooij et al., 1994; Wildermuth et al., 2001;

Durrant and Dong, 2004). Some of these *PR* genes, such as *PR1*, *PR2* and *PR5* serve as robust markers for this systemic immune response (Kombrink and Somssich, 1997).

While local and systemic accumulation of SA is critical for SAR induction, this hormone seems not to serve as a mobile signal mediating immunity in uninfected distal tissues. Several other small molecules have been proposed to fulfill such a role, such as methyl- salicylic acid (MeSA), azelaic acid, glycerol-3-phosphate, the abi-etane diterpenoid dehydroabietinal, JA, and the amino acid-derivative pipecolic acid (Park et al., 2007; Fu and Dong, 2013). A central regulator of SAR is the transcriptional co-factor NON- EXPRESSOR OF PR GENES1 (NPR1) (Dong, 2004). By interacting with TGA bZIP transcription factors, NPR1 seems to mediate up-regulation of the vast majority of SAR-associated genes (Fu and Dong, 2013). NPR1 activity has been proposed to be controlled by the SA-binding proteins NPR3 and NPR4, which can physically bind to NPR1 in a SA-concentration-dependent manner (Fu et al., 2012).

In contrast to SAR, induction of ISR is not associated with the accumulation of SA and *PR* transcripts (Sticher et al., 1997; van Wees et al., 2000). ISR has been shown to be triggered by the *Pseudomonas fluorescens* strain WCS417r (WCS417r) and other non- pathogenic rhizobacteria in several plant species including Arabidopsis (Wei et al., 1996; Sticher et al., 1997; Pieterse et al., 1998; Yan et al., 2002; Vallad and Goodman, 2004). In Arabidopsis, WCS417r-induced ISR acts against *Pseudomonas syringae* pv.

tomato, is dependent on JA and ET signaling, but does not require SA. Intriguingly, ISR is blocked in the Arabidopsis *npr1* mutant. Thus, *NPR1* also plays an important role in the ISR signaling pathway (Pieterse et al., 1998; Glazebrook, 2001).

Upon perception of several exogenous defense-related stimuli, plants can establish an enhanced capacity to activate immune responses. This sensitization process, which is called priming, can be triggered by treatment of plants with necrotizing pathogens, beneficial microorganisms, wounding or with various natural and synthetic compounds (Conrath et al., 2002; Conrath, 2006; Conrath et al., 2006; Beckers and Conrath, 2007; Goellner and Conrath, 2008). Once a pathogen infects primed plants, defense responses are activated faster and more robustly (Conrath et al., 2006; Goellner and Conrath, 2008). Although this phenomenon has been known for years, its molecular basis is still only partly understood (Conrath, 2006; Conrath et al., 2006; Conrath, 2011).

Chromatin modifications, accumulation of dormant mitogen-activated protein kinases and alterations of primary metabolism have been shown to be associated with this process (Conrath et al., 2002; Conrath et al., 2006; Beckers et al., 2009; Conrath, 2011; Jaskiewicz et al., 2011).

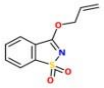
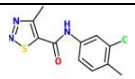
A brief history of synthetic elicitors

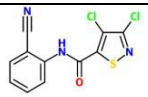
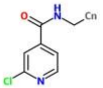
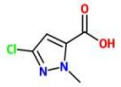
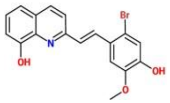
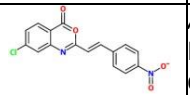
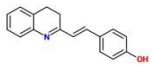
Synthetic elicitors are small molecules that can induce plant immune responses and are structurally distinct from natural plant defense inducers, such as general or race-specific elicitors or endogenous plant defense signaling molecules. Synthetic elicitors may trigger defense reactions by mimicking interactions of natural elicitors or defense signaling molecules with their respective cognate plant receptors or by interfering with other defense signaling components. Often the term “plant activators” is used for molecules that can protect plants from diseases by inducing immune responses. However, this term does not discriminate between synthetic and natural elicitors. One of the first synthetic elicitors was identified in 1974 by Kassanis and White, who found Polyacrylic acid derivatives of 3500 Da or lower molecular weights to mediate resistance of tobacco (*Nicotiana tabacum*) against tobacco mosaic virus (TMV) or tobacco necrosis virus (TNV) and to activate *PR1* gene expression in tobacco (Gianinazzi and Kassanis, 1974; Kassanis and White, 1975). At the same time, 2,2- dichloro-3,3-dimethyl-cyclopropane carboxylic acid (WL28325) was described as a compound suitable for controlling rice blast in rice. WL28325 affects the phenol metabolism of rice plants by enhancing peroxidase activities (Langcake and Wickins, 1975a; Langcake and Wickins, 1975b). Two years later, 3-allyloxy-1,2-benzisothia-zole- 1,1-dioxide, widely called Probenazole (PBZ), was described. It activates defense- related enzymes and triggers dramatic increases of tolerance against rice blast in rice. It has

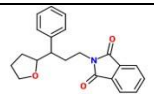
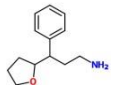
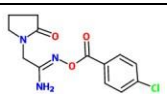
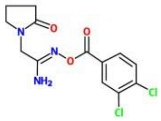
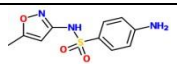
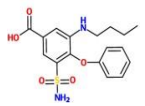
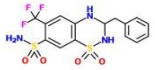
effectively been used in agriculture for over three decades against rice blast (Watanabe et al., 1977; Schreiber and Desveaux, 2008).

In 1979, exogenous application of SA and other benzoic acid derivatives, such as acetylsalicylic acid (Aspirin), was reported to induce resistance of tobacco against TMV and to cause the accumulation of PR-proteins (White, 1979). This discovery was a major breakthrough and paved the way for the identification of more potent related compounds by the Switzerland-based pharmaceutical corporation Ciba-Geigy (now Syngenta). Ciba-Geigy researchers reported 2,6-dichloro isonicotinic acid (INA) and its ester derivative CGA 41397 as potent SAR-inducers in 1987. They also identified benzo(1,2,3)thiadiazole-7-carbothioic acid S-methyl ester (BTH), which has similar effects as INA, but was later found to be more suitable for applications in crop protection (Metraux et al., 1990; Ward et al., 1991; Friedrich et al., 1996; Görlach et al., 1996; Lawton et al., 1996; Uknes et al., 1996). As INA and BTH mimic the defense-associated effects of SA, but are less phytotoxic and more efficient than this natural plant defense hormone, they have been abundantly used as defense triggers in basic and applied studies on plant immunity. As outlined in detail below, these two compounds have been among the most frequently used synthetic elicitors in research for the past 15 – 20 years. However, recent improvements in combinatorial chemistry (Blackwell and Zhao, 2003; Stockwell, 2004; Dean, 2005; Raikhel and Pirrung, 2005) have enabled scientists outside the private sector to perform systematic

screens for synthetic elicitors. Thus, a plethora of new compounds with defense-inducing properties distinct from INA and BTH or other established synthetic elicitors is currently emerging (Table 1.1). Such second- generation synthetic elicitors will equip researchers with an extensive repertoire of new chemical tools to dissect the plant defense network in an unprecedented fashion and to explore their use as active ingredients of novel types of pesticides and other agrochemicals.

Chemical names	Chemical	Biotic interactions*	Application	Concentrations**	References
3-allyloxy-l,2-benzisothia- zole-1,1-dioxide (Probenazole, PBZ)		*Oryza sativa* - *Magnaporthe grisea*	Root drench	896 µM (200 ppm)	(Watanabe et al., 1977)
2,6-dichloro-isonicotinic acid (INA)		Cucumber (*Cucumis sativus*) - *Colletotrichum lagenarium*	Foliar spray	104 µM (20 ppm)	(Metraux et al., 1991; Ward et al., 1991; Uknes et al., 1992)
		Nicotiana tabacum -*Tobacco mosaic virus* (TMV)	injection into leaves	1000 µM	
		Arabidopsis thaliana (Ler) - *Hyaloperonospora arabidopsidis*	Soil drench	52 µM	
		A. thaliana (Col-0) - *Pseudomonas syringae* pv 'tomato' DC3000	Foliar spray	650 µM	
benzo(l,2,3)thiadiazole-7- carbothioic acid S-methyl ester (BTH)		*A. thaliana* (Col-0) - *P. syringae* pv 'tomato' DC3000	Foliar spray	300 µM	(Friedrich et al., 1996; Görlach et al., 1996; Lawton et al., 1996)
		A. thaliana (Col-0) - *H. arabidopsidis*	Foliar spray	300 µM	
		A. thaliana (Col-0) - *Turnip crinkle virus*	Foliar spray	300 µM	
		N. tabacum - *Cercospora nicotianae*	Foliar spray	1200 µM	
		N. tabacum - *Erwinia carotovora*	Foliar spray	1200 µM	
		N. tabacum - *Phytophthora parasitica*	Foliar spray	1200 µM	
		N. tabacum - *P. syringae* pv. tabaci	Foliar spray	1200 µM	
		N. tabacum - TMV	Foliar spray	1200 µM	
N-(3-chloro-4-methylphenyl)-4-methyl-1,2,3-thiadiazole-5- carboxamide (Tiadinil, TDL)		*N. tabacum* -TMV	Root drench	1 mg / pot	(Yasuda et al., 2004)

Isotianil		*O. sativa - M. grisea*	Foliar spray	840 µM (250 ppm)	(Ogava et al., 2011)
N-cyanomethyl-2-chloroisonicotinamide (NCI)		*O. sativa - Pyricularia oryzae*	Root drench	240 g a.i (active ingredient) / 10a (are)	(Yoshida et al., 1990b)
3-chloro-1-methyl-1H-pyrazole-5-carboxylic acid (CMPA)		*O. sativa - P. oryzae*	Root drench	0.05 mg / pot	(Nishioka et al., 2003; Nishioka et al., 2005)
3,5-dichloroanthranilic acid (DCA)		*A. thaliana - H. arabidopsidis*	Foliar spray	100 µM	(Knoth et al., 2009)
2-[(E)-2-(2-bromo-4-hydroxy-5-methoxyphenyl)ethenyl] quinolin-8-ol) (I*mprimatin A1)*		*A. thaliana-P. syringae pv tomato DC3000 avrRpm1* or *A.thaliana-P. syringae pv. tomato DC3000*	Root drench	100 µM	(Noutoshi et al., 2012d; Noutoshi et al., 2012f; Noutoshi et al., 2012e)
7-chloro-2-[(E)-2- (4-itrophenyl)ethenyl]-4H-3,1- benzoxazin-4-one) *(Imprimatin A2)*		*A. thaliana - P. syringae* pv tomato DC3000 avrRpm1 or *A. thaliana - P. syringae* pv. tomato DC3000	Root drench	100 µM	(Noutoshi et al., 2012c, Noutoshi et al., 2012b, Noutoshi et al., 2012a)
4-[(E)-2-(quinolin-2-yl)ethenyl]phenol) (Imprimatin A3)		*A.thaliana - P. syringae* pv tomato DC3000 avrRpm1 or *A. thaliana - P. syringae* pv. tomato DC3000	Root drench	100 µM	(Noutoshi et al., 2012c, Noutoshi et al., 2012b, Noutoshi et al., 2012a)

2-(3-(2-furyl)-3-phenylpropyl) benzo[c]azoline-1,3-dione) *(Imprimatin B1)*		*A.thaliana* - *P. syringae* pv tomato DC3000 avrRpm1 or *A.thaliana* - *P. syringae* pv. tomato DC3000	Root drench	100 µM	(Noutoshi et al., 2012c, Noutoshi et al., 2012b, Noutoshi et al., 2012a)
3-(2-furyl)-3-phenylpropylamine) *(Imprimatin B2)*		*A.thaliana* - *P. syringae* pv tomato DC3000 avrRpm1 or *A.thaliana* - *P. syringae* pv. tomato DC3000	Root drench	100 µM	(Noutoshi et al., 2012c, Noutoshi et al., 2012b, Noutoshi et al., 2012a)
[(E)-[1-amino-2-(2-oxopyrrolidin- 1-yl)ethylidene]amino] 4-chlorobenzoate) (*Imprimatin C1)*		*A.thaliana* - *P. syringae* pv tomato DC3000 avrRpm1 or *A.thaliana* - *P. syringae* pv. tomato DC3000	Root drench	100 µM	(Noutoshi et al., 2012c)
[(E)-[1-amino-2-(2-oxopyrrolidin- 1-yl)ethylidene]amino]3,4-dichlorobenzoate) (*Imprimatin C2)*		*A.thaliana* - *P. syringae* pv tomato DC3000 avrRpm1 Or *A.thaliana* - *P. syringae* pv. tomato DC3000	Root drench	100 µM	(Noutoshi et al., 2012c)
Sulfamethoxazole (Smex)		*A. thaliana* - *P. syringae* pv. tomato DC3000	Foliar spray	100 µM	(Schreiber et al., 2008)
3-(Butylamino)-4-phenoxy-5-sulfamoylbenzoic acid (bumatanide)		*A.thaliana* - *P. syringae* pv. tomato DC3000 (Pst) avrRpm1	Root drench	100 µM	(Noutoshi et al., 2012b)
3-benzyl-1,1-dioxo-6-(trifluoromethyl)-3,4-dihydro-2*H*- 1,2,4-benzothiadiazine-7-sulfonamide (bendroflumethiazide)		*A. thaliana* - *P. syringae* pv. tomato DC3000 (Pst) avrRpm1	Root drench	100 µM	(Noutoshi et al., 2012a)

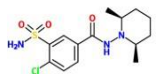
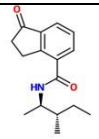
4-chloro-*N*-(2,6-dimethyl-1- piperidyl)-3-sulfamoyl-benzamide (clopamide)		*A. thaliana* - *P. syringae* pv. tomato DC3000 (Pst) avrRpm1	Root drench	100 µM	(Noutoshi et al., 2012a)
1-oxo-indanoyl-L-isoleucine methyl ester (Ind-Ile-Me)		*Pennisetum glaucum* - *Sclerospora graminicola*	Seeds are soaked with chemical	75 µM	(Deepak et al., 2007)

Table 1.1: Synthetic elicitors discussed in the main text.

*Biotic interactions tested and affected by compound

**Converted to molarity if information on concentration available

Functional analogs of salicylic acid

The natural plant defense hormone SA (2-hydroxybenzoic acid) serves as an endogenous signal to activate certain immune responses and to establish disease resistance. Various defense-related stimuli have been shown to trigger enhanced SA levels in local and systemic plant tissues. Exogenous application of SA can induce ROI production, *PR* gene expression and immunity against various pathogens with biotrophic or hemibiotrophic lifestyles (Glazebrook, 2005; Vlot et al., 2009).

In plants, SA can be synthesized from the shikimate pathway-derived primary metabolite chorismate either via phenlypropanoid derivatives in the cytoplasm or via isochorismic acid in chloroplasts (Pieterse et al., 2012; An and Mou, 2014). Although both metabolic pathways are not fully understood, several of their enzymes have been identified. The production of SA and its levels are normally tightly regulated (Wildermuth, 2006). Critical for the production of the majority of defense-associated SA in Arabidopsis is isochorismate synthase 1 (ICS1), which is transcriptionally induced by defense-related stimuli (Wildermuth et al., 2001). Two distinct forms of SA glucosyltransferase (SAGT) enzymes convert most of the produced SA to either salicyloyl glucose ester (SGE) or SA O-β-glucoside (SAG), which is stored in the vacuole. Additional SA derivatives are known in plants, such as MeSA. SAG, SGE and MeSA are likely biologically inactive (Vlot et al., 2009; Fu

and Dong, 2013).

SA plays a pivotal role in defense signaling and several proteins have been proposed to bind to SA and to potentially serve as SA receptors. The first putative SA-binding protein reported in the literature was SABP1 from tobacco, a potential catalase (Chen et al., 1993). It was proposed that SA inhibits its ability to convert H_2O_2 to O_2 and H_2O (Conrath et al., 1995; Du and Klessig, 1997; Vlot et al., 2009). However, this claim is controversial, as much higher SA-concentrations seem to be needed for catalase inhibition than observed in defense-activated plants (Chamnongpol et al., 1996; Tenhaken and Rubel, 1997). Similarly, it was shown that SA can also bind to ascorbate peroxidase (APX) and inhibit its activity upon application of high concentrations of exogenous SA (Durner and Klessig, 1995; Vlot et al., 2009). An additional tobacco SA- binding protein, SABP2, functions as a MeSA esterase. SABP2 shows a high binding affinity for SA, which inhibits its esterase activity (Kumar and Klessig, 2003; Forouhar et al., 2005). SABP2 seems to play an important role in the activation of SAR in tobacco by catalyzing the release of SA from the transport metabolite MeSA in systemic tissues (Park et al., 2007). Another SA-binding protein, SABP3, a tobacco chloroplastic carbonic anhydrase, is involved in HR and has antioxidant function (Slaymaker et al., 2002; Vlot et al., 2009). However, it remains to be determined whether this function can affect plant defense.

In Arabidopsis, NPR1 plays a critical role in the interpretation of the SA

signal. NPR1 is responsible for activating a large set of defense genes in response to SA-related signals (Dong, 2004; Fu and Dong, 2013). Moreover, the NPR1 paralogues NPR3 and NPR4 function as SA receptors, and their interactions with NPR1 are directly regulated by binding to SA (Fu et al., 2012). In addition, NPR1 itself has also been shown to be capable of binding SA independently of NPR3 and NPR4 and to respond to interactions with this ligand by conformational changes (Wu et al., 2012).

With several proteins capable of binding to SA, defense mechanisms controlled by this phytohormone feature a set of “drug-able” targets potentially interfering with SA-related synthetic molecules. Consequently, some synthetic elicitors have been found to mimic a subset of known SA functions; likely by directly interfering with known or unknown receptors of this defense hormone. Besides such SA agonists, which molecularly mimic SA, other synthetic elicitors may trigger transcriptional and physiological responses related to those induced by SA without directly interfering with SA targets. For this review we consider both types of SA mimics as functional SA analogs. Synthetic elicitors of this type are described in the section below.

Probenazole (PBZ)

Several biologically active 1,2-benzisothiazole derivatives have been found to exhibit a broad spectrum of pharmacological activities and to serve as

antibacterials, fungicides and anti-inflammatory agents (De, 1981; Trapani et al., 1985; Zani et al., 1996; Vicini et al., 2002). Some of them also show auxin-like activity and have been used a s herbicides (Giannella et al., 1971; Branca et al., 1975). Inspired by the potency of some of these compounds, researchers of Meiji Seika Kaisha Ltd. in Japan performed systematic tests with representatives of this class of molecules (Watanabe et al., 1977). They found 3-allyloxy-1,2-benzisothiazole-1,1-dioxide (now widely known as Probenazole; PBZ), to efficiently control rice blast (*Magnaporthe oryzae*; anamorph: *Pyricularia oryzae*) infections in rice (*Oryza sativa*) (Watanabe et al., 1977; Schreiber and Desveaux, 2008). This compound showed remarkable effects in suppressing rice blast at a dose of 896 µM (200 ppm) when applied by drenching roots (Watanabe et al., 1977) and has been commercially used under the name Oryzemate® for more than 30 years in the field protecting rice from rice blast fungus and bacterial leaf blight as well as corn from southern corn leaf blight (Iwata, 2001; Oostendorp et al., 2001). PBZ does not influence the growth of various tested crops, such as tomato, cucumber, Chinese cabbage, kidney bean or rice, when sprayed at a concentration of 2240 µM (500 ppm), but at 4480 µM (1000 ppm) some abnormalities in plant development can be observed (Watanabe et al., 1977).

PBZ affects various stages of the blast fungus infection cycle and inhibits hyphal penetration into the host tissue, lesion expansion and sporulation (Watanabe et al., 1977). From PBZ-treated rice plants anti-conidial germination

substances were isolated and characterized as toxic against fungi. These antifungal plant metabolites included a mixture of fatty acids, such as octadecatrienoic acid, palmitic acid, linoleic acid and linolenic acid (Sekizawa et al., 1981; Shimura et al., 1983). Moreover, activities of defense-related enzymes, such as peroxidase, polyphenoloxidase, PAL, tyrosine ammonia-lyase and catechol-O-methyltransferase, increased dramatically in rice upon treatment with PBZ, as they do in response to infection with rice blast fungus (Midoh and Iwata, 1996; Iwata, 2001).

A PBZ-induced cDNA termed *PBZ-responsive gene* (*PBZ1*) has been cloned from rice. PBZ1 transcript accumulation was found to serve as a robust marker for responses to this synthetic elicitor. PBZ-induced PBZ1 mRNA accumulates in a dose-dependent manner. *PBZ1* expression is also induced by rice blast fungus, but not wounding. *PBZ1* belongs to the *PR-10* family of classical *PR* genes. One of the metabolites of PBZ, 1,2- benzisothiazole-3(2H)-one-1,1-dioxide (BIT) was found to be as potent in inhibiting rice blast as PBZ, but does not induce the accumulation of the PBZ1 transcripts (Midoh and Iwata, 1996; Nakashita et al., 2001; Yoshioka et al., 2001; Nakashita et al., 2002b). Thus, induced *PBZ1* expression seems not to be needed for rice blast resistance.

Microarray and RT-PCR analysis revealed up-regulation of UDP-glucose:SA glucosyltransferase (*Os*SGT1) transcripts in response to PBZ treatment in rice (Umemura et al., 2009). RNAi-mediated *Os*SGT1 knockdown

in transgenic rice plants resulted in reduced PBZ-mediated resistance against blast. Although mechanistic details of its role in defense induction are unclear, *Os*SGT1 appears to be critical for PBZ-mediated defense induction (Umemura et al., 2009).

In Arabidopsis, both PBZ and its metabolite BIT stimulate expression of *PR* genes and induce SA accumulation and SAR. PBZ and BIT do not activate plant immunity in *npr1* mutants or *nahG* plants. Thus, SA and NPR1 seem to be required for PBZ- and BIT- mediated defense responses and both compounds mimic effects of SA (Yoshioka et al., 2001; Nakashita et al., 2002b). However, in contrast to INA, BTH and DCA, which are likely authentic SA agonists (see below), PBZ and BIT appear to interfere with defense signaling steps upstream from SA accumulation and not to interact with downstream targets of SA.

2,6-dichloro-isonicotinic acid (INA)

In 1987, Ryals and coworkers of Ciba-Geigy screened a large number of compounds for activation of resistance in cucumber (*Cucumis sativus*) against the fungal pathogen *Colletotrichum lagenarium* and identified 2,6-dichloro-isonicotinic acid (INA) and its ester derivative CGA41397 (Kunz et al., 1988; Metraux et al., 1991). High levels of protection of cucumber against *C. lagenarium*, were achieved by foliar-spray application of 104 µM (20 ppm) INA

or CGA41397 as well as root drench application of 10-fold lower concentrations of each compound. In these chemically-treated plants, responses were similar to those observed in systemic tissues of plants whose lower leaves were inoculated with TNV *or C. lagenarium* that induce SAR in upper leaves. Under field conditions, INA provided pathogen resistance in pear, pepper and rice (Kuc, 1982; Metraux et al., 1991). INA was also shown to induce SAR in tobacco and Arabidopsis *(Ward et al., 1991; Uknes et al., 1992)* and provide significant protection of tobacco against TMV, *Cercospora nicotianae, Peronospora tabacina, Phytophthora parasitica* var nicotianae, and *P. syringae* pv. tabaci *(*Ward *et al., 1991).*

In Arabidopsis INA can trigger long-lasting *PR* gene expression and disease resistance. In this species it can reduce susceptibility to virulent strains of the oomycete *Hyaloperonospora arabidopsidis (Hpa*) or *P. syringae* pv. tomato DC3000 without directly affecting viability of these pathogens (Uknes et al. 1992; Knoth, et al. 2009). As injection of 1mM INA into tobacco leaves induces transcript accumulation of the same characteristic set of *PR* genes as SA application, it is considered a functional SA analog. Although INA partially mimics defense-associated effects of SA, it does not trigger any changes of SA levels and, unlike SA or PBZ, induces SAR in *nahG* transgenic tobacco and Arabidopsis plants (Delaney et al., 1994; Vernooij et al., 1995). Thus, INA must be interfering with targets that operate downstream from SA accumulation and are likely involved in the interpretation of SA levels. Consistent with this

assumption, INA has been reported to mimic several proposed biochemical and physiological effects of SA, such as inhibition of catalase and APX activity or the induction of cellular H_2O_2 accumulation (Chen and Klessig, 1991; Chen et al., 1993; Chen et al., 1995; Conrath et al., 1995; Durner and Klessig, 1995). The modulation of ROI levels seems to be a critical aspect of INA activity, since antioxidants can block the INA-dependent induction of *PR* gene expression (Chen et al., 1995; Durner and Klessig, 1995).

Through mutant screens to identify genes required for SAR in Arabidopsis, the *npr1/nim1* (*non-expresser of PR genes 1, no immunity 1*) mutants that are insensitive to SA and INA were discovered (Cao et al., 1994; Delaney et al., 1995). Both biologically- and INA-induced SAR as well as basal defense were found to be compromised in either one of these mutants. The *npr1* and *nim1* mutants are in different Arabidopsis accessions, but were found to be allelic and to have defects in the same gene (Cao et al., 1994; Cao et al., 1997; Ryals et al., 1997). A large body of literature has reported on molecular roles of NPR1 as a transcriptional cofactor, since its identification as a major regulator of SAR. These studies have been summarized in several excellent reviews (Dong, 2004; Durrant and Dong, 2004; Fu and Dong, 2013). Most importantly, NPR1, together with NPR3 or NPR4, have been found to serve as SA receptors (Fu et al., 2012; Fu and Dong, 2013). NPR3 can bind to NPR1 in a SA dose-dependent manner, while NPR4-NPR1 interactions are constitutive and inhibited by SA. In yeast two-hybrid assays, in addition to SA,

INA can promote NPR1–NPR3 interactions. INA can also reduce the binding affinity of SA to NPR3 and NPR4 by competing with this defense hormone (Fu et al., 2012). Thus, INA appears to be a true SA agonist.

In addition to *npr1* mutants, triple or quadruple mutants of closely related TGA-bZIP transcription factors, which are known to physically interact with NPR1, are also blocked in INA-induced *PR* gene expression and pathogen resistance (Zhang et al., 2003; Wang et al., 2006). Thus, INA seems to mediate its defense-related effects upon interactions with NPR1-related proteins, which control several TGA transcription factors. Interactions with other SA-binding proteins, such as SABP1 and SABP2 may also to contribute to the activity of this SA analog. So far, INA has been applied to many plant species and was found to induce resistance against a wide variety of pathogens (Hijwegen and Verhaar, 1993; Conrath et al., 1995; Van Kan et al., 1995; Han et al., 2000; Lee et al., 2009). However, because INA and its derivatives have phytotoxic side effects in crops, none of these compounds has been commercialized as agrochemicals (Oostendorp et al., 2001). Still, INA is being continually used as an efficient chemical tool to study SAR.

Benzothiadiazole (BTH)

The similar SAR-inducer screening that led to the discovery of INA, with a large number of benzo[1,2,3]thiadiazole-7-carboxylic acid derivatives resulted in the identification of benzo(1,2,3)-thiadiazole-7-carbothioic acid S-methyl-ester (benzothiadiazole, BTH, acibenzolar-S- methyl (ASM), CGA245704) as a potent inducer of plant immune responses (Schurter et al., 1993; Kunz et al., 1997; Oostendorp et al., 2001). BTH was subsequently shown to trigger in various plant species resistance against a wide variety of pathogens, such as TMV, *Cercospora nicotianae, Erwinia carotovora, Phytophthora parasitica* and *P. syringae* pv. tabaci (Friedrich et al., 1996; Görlach et al., 1996; Lawton et al., 1996; Kunz et al., 1997). As BTH did not show any direct effect on a number of plant pathogens *in vitro*, BTH is not antimicrobial (Friedrich et al., 1996). In Arabidopsis, BTH triggers *NPR1*-dependent SAR (Lawton et al., 1996).

At the molecular level, BTH induces the same characteristic set of SAR-related responses that are induced by pathogens or SA, including up-regulation of *PR* genes. Thus, like INA, BTH appears to be a functional analog of SA (Friedrich et al., 1996; Wendehenne et al., 1998). INA and BTH share several characteristic functional features. Both compounds do not induce accumulation of SA in plants (Vernooij et al., 1995; Friedrich et al., 1996) and share the ability to induce SAR and *PR* gene expression in transgenic *nahG* lines (Vernooij et al., 1995; Lawton et al., 1996). Thus, both INA and BTH seem to activate SA-response mechanisms by interfering as

SA agonists with targets operating downstream from SA accumulation. Like SA and INA, BTH was also proposed to inhibit both APX and catalase functions (Du and Klessig, 1997; Wendehenne et al., 1998). However, BTH is a much more effective inhibitor of catalase than SA and the catalase inhibition mechanisms of BTH and SA are different. While SA seems to inhibit catalase function in an H_2O_2- and time-dependent manner, BTH inhibits this activity independently from time and H_2O_2. INA was not included in these experiments. For APX inhibition, however, BTH and SA exhibit similar dose-response curves (Wendehenne et al., 1998).

Recent data suggested that BTH is converted into acibenzolar by SABP2 and this product is critical for SAR induction. When BTH was sprayed on SABP2-silenced tobacco plants, it failed to induce PR1 protein expression and SAR. On the contrary, when the same transgenic plants were treated with acibenzolar, SAR was fully induced (Tripathi et al., 2010).

In rice, it was shown that the *Os*WRKY45 transcription factor plays a pivotal role in BTH-induced defense responses against rice blast disease. This BTH-triggered defense mechanism seems independent of NH1, a rice ortholog of *Arabidopsis thaliana* NPR1 (Shimono et al., 2007). WRKY45 knockdown lines exhibited strongly reduced levels of BTH-induced resistance to the fungal pathogen *Magnaporthe oryzae* and the bacterial pathogen *Xanthomonas oryzae* pv. oryzae (Xoo) (Shimono et al., 2007). Interestingly, *Os*WRKY45 is an ortholog of *At*WRKY70, which also can act in an NPR1-

independent manner in SA signaling in Arabidopsis (Li et al., 2004; Knoth et al., 2007; Knoth et al., 2009). In addition to BTH, PBZ and Tiadinil (TDL) (see below) partly induced blast resistance in rice through a WRKY45-dependent pathway (Shimono et al., 2012). Recently, WRKY45-regulated BTH-responsive genes were identified by microarrays (Nakayama et al., 2013).

BTH can also prime plant defense reactions. Low doses of BTH that is insufficient to trigger detectable levels of defense responses, can prime parsley cells and increase their sensitivity for MAMP-triggered coumarin phytoalexin secretion. This effect is associated with potentiated activation of genes encoding phenylalanine ammonia-lyase (PAL), which is critical for coumarin biosynthesis. In addition to BTH, also SA and INA can prime parsley cells for the activation of coumarin secretion by low MAMP doses (Kauss et al., 1992; Katz et al., 1998; Thulke and Conrath, 1998; Conrath et al., 2002). BTH can also prime Arabidopsis plants for enhanced pathogen-responsiveness of *PAL* gene expression. BTH-mediated defense priming in Arabidopsis is dependent on NPR1 (Kohler et al., 2002; Goellner and Conrath, 2008). An interesting mechanism involving two known defense-associated MAP kinases (MAPKs), MPK3 and MPK6, seems to contribute to this priming phenomenon in Arabidopsis. BTH induces the accumulation of mRNA and inactive protein forms of both MAPKs. Subsequent stress treatment results in phosphorylation and activation of MPK3 and MPK6 (Beckers et al., 2009). In addition, epigenetic chromatin marks appear to be involved in defense-priming processes. The

AtWRKY29, *AtWRKY6* and *AtWRKY53* genes showed a typical priming response and were strongly transcribed after stress application following pre-treatment with BTH. BTH pre-treatment also triggered in these experiments various histone modifications that are typically found at actively transcribed genes, such as H3K4me3, H3K4me2, H3ac or H4ac at *AtWRKY29* and H3K4me3 or H3K4me2 at *AtWRKY6* and *AtWRKY53*. BTH- induced trimethylation of H3K4 is reduced in the priming-deficient *npr1* mutant. On the contrary, the constitutively primed *cpr1* and *sni1* mutants exhibit high levels of H3K4me3 in the absence of BTH treatment. Thus, elevated H3K4me3 levels are closely associated with BTH-induced defense gene priming (Jaskiewicz et al., 2011).

In contrast to INA, BTH was found to be suitable for agricultural crop protection. It became a commercial product under the trade name of BION® (in Europe) in 1989 and Actigard® (in the US) in 1990 (Schurter et al., 1993; Kunz et al., 1997; Oostendorp et al., 2001). BTH activates very wide spectrum of resistances of various plant species against fungal, bacterial, or viral pathogens and several insects and nematodes.

N-(3-chloro-4-methylphenyl)-4-methyl-1,2,3-thiadiazole-5-carboxamide (Tiadinil, TDL)

Thiadiazoles are known to have many pharmacological activities (Camoutsis et al., 2010; Chaudhary et al., 2010; Kharb et al., 2011; Singh et al., 2011). Tests of various 1,2,3-thiadiazole derivatives for their ability to control rice blast disease by Nihon Nohyaku Co., Ltd. (Japan) resulted in the discovery of N-(3-chloro-4-methylphenyl)-4- methyl-1,2,3-thiadiazole-5-carboxamide (Tiadinil, TDL), which provided protection against this disease without exhibiting any antimicrobial activity (Tsubata et al., 2006). Since 2003, this compound has been commercially available under the trade name V-GET® in Japan. Its metabolite 4-methyl-1,2,3-thiadiazole-5-carboxylic acid (SV-03), exhibited similar levels of anti-rice blast activity as TDL (Tsubata et al., 2006; Toquin et al., 2012). In addition to rice blast, TDL is also used to control the pathogenic fungi *Colletotrichum theaesinensis* and *Pestalotiopsis longiseta* on tea leaves (Yoshida et al., 2010).

In tobacco, TDL and SV-03 induce SAR and increased local resistance to TMV, the virulent bacterial pathogen *P. syringae* pv. tabaci and powdery mildew (*Oidium lycopersici*) without affecting these pathogens directly. Both compounds also induce *PR1*, *PR2* and *PR5* gene expression in Arabidopsis and enhance basal resistance of this species to *P. syringae* pv. tomato DC3000 (Yasuda et al., 2004; Yasuda et al., 2006; Yasuda, 2007). TDL or SV-03 treatment does not induce accumulation of SA in tobacco. Moreover,

TDL or SV-03-treated *nahG* transgenic tobacco plants exhibit enhanced resistance to TMV and *P. syringae* pv. tabaci and induced *PR* gene expression. However, TDL- or SV-03-triggered defense responses are blocked in Arabidopsis *npr1* mutants. Taken together, these results suggest that, similar to BTH and INA, TDL and SV-03 trigger disease resistance by interfering with signaling steps downstream of SA (Yasuda et al., 2006; Yasuda, 2007).

The thiadiazole derivative, 1,3,4-oxadiazole, has also been shown to exhibit antifungal and antibacterial activities (Kharb et al., 2011; Singh et al., 2011). By combining different heterocyclic thiadiazole-related moieties, including oxadiazoles, new compounds were designed and evaluated regarding their performance in crop disease protection. Although only three out of the 23 tested compounds elicited SAR more efficiently than TDL, combining thiazole- and oxadiazole moieties may be a promising approach in designing new crop protectants (Fan et al., 2009).

Isotianil

As a result of a comprehensive search for isothiazole-based compounds, Isotianil was discovered by Bayer AG (now Bayer CropScience AG) in Germany in 1997 and developed jointly with the Japanese company Sumitomo Chemical Co., Ltd. as a crop protectant against rice blast and bacterial leaf blight in rice. It also activates defense responses against a wide

range of additional pathogens in various plants. Moreover, Isotianil does not show any direct antimicrobial activity against bacteria and fungi (Ogava et al., 2011; Toquin et al., 2012). In 2010, it was registered under the name Stout® in Japan and China, where it substantially increased rice production (Ogava et al., 2011; Brozek et al., 2012; Yoshida et al., 2013). Its efficiency against rice blast seems unusually high, as lower dosages of Isotianil are needed than of any other existing plant defense activator, such as PBZ and TDL (Ogava et al., 2011).

At the molecular level, Isotianil treatment triggers accumulation of defense-related enzymes such as lipoxygenase or PAL in rice. Affymetrix whole genome microarray analysis revealed that Isotianil treatment induces some defense-related genes, including *OsWRKY45,* that are involved in SA signaling (Ogava et al., 2011; Toquin et al., 2012). Further microarray analyses showed that Isotianil likely primes rice for more intense defense activation in response to pathogen infections. At this point no published information on its mode-of-action is available.

N- cyanomethyl-2-chloroisonicotinamide (NCI)

A screen of 2-chloroisonicotinamide derivatives for effective rice blast control agents were performed by Nihon Nohyaku Co., Ltd. (Japan), resulted in the identification of N- cyanomethyl-2-chloroisonicotinamide (NCI) as a potent

defense inducer (Yoshida et al., 1989; Yoshida et al., 1990a; Yoshida et al., 1990b). NCI showed one of the highest anti- blast activities compared to other N-alkyl-2-chloroisonicotinamides and its efficacy was equal to that of PBZ. It does not show antifungal activity against rice blast *in vitro* at concentrations as high as 1100 µM (500 ppm). Its activity is long-lasting, as it was found to be still effective against rice blast 30 days after a single application. NCI treatment inhibits mycelial development of *P. oryzae* at inner epidermal cells and increases the number of small brownish lesions that are correlated with active immunity of rice. These results suggest that NCI efficiently induces plant defense mechanisms (Yoshida et al., 1990a).

In tobacco, NCI can induce SAR and mediate local resistance to TMV, *Oidium lycopersici* and *P. syringae* pv. tabaci. It also induces expression of *PR1*, *PR2* and *PR5* and is active in transgenic *nahG* tobacco plants. Thus, it does not require SA for activation of defense (Nakashita et al., 2002a). In Arabidopsis, NCI reduces growth of virulent *P. syringae* and induces resistance independently from SA accumulation, ET and JA, but requires NPR1. Thus, like INA and BTH, NCI seems to interfere with defense signaling steps operating between SA and NPR1 (Yasuda et al., 2003a; Yasuda, 2007).

3-chloro-1-methyl-1H-pyrazole-5-carboxylic acid (CMPA)

A screen by Nishioka and co-workers (Nishioka et al., 2003) targeting new chemicals to control blast disease in rice resulted in the discovery of pyrazolecarboxylic acid derivatives as potent inducers of systemic immunity. The most efficient anti-blast compound identified in this screen was 3-chloro-1-methyl-1H-pyrazole-5-carboxylic acid (CMPA). CMPA does not directly affect pathogen viability up to a concentration of 623 µM (100 ppm), while it can significantly induce rice blast resistance at 10-fold lower concentrations. Thus, its anti-blast activity is not dependent on antimicrobial activity and this compound seems to activate systemic plant defense mechanisms (Nishioka et al., 2003). Although, CMPA, BTH and PBZ trigger rice blast resistance with similar efficacies, CMPA induces PBZ1 transcript accumulation in rice at levels lower than PBZ or BTH (Nishioka et al., 2005).

In tobacco, CMPA enhances resistance to *P. syringae* pv. tabaci and *Oidium sp*.. CMPA also induces expression of *PR1*, *PR2* and *PR5* in wild-type as well as *nahG* transgenic tobacco. Therefore, CMPA seems not to require SA to induce SAR-like disease resistance and may interfere with defense signaling downstream from SA. Consistent with this assumption, CMPA was found to act through NPR1 in Arabidopsis (Yasuda et al., 2003b; Yasuda, 2007).

3,5-dichloroanthranilic acid (DCA)

The compound 3,5-dichloroanthranilic acid (DCA) is one of 114 synthetic elicitor candidates that were identified by a comprehensive screening of 60,000 diverse compounds for inducers of the pathogen-responsive *CaBP22::GUS* reporter gene in Arabidopsis (Knoth et al., 2009; Knoth and Eulgem, 2014). DCA efficiently triggers resistance of Arabidopsis against virulent strains of the oomycete *Hpa* and *P. syringae* DC3000. It up-regulates transcript levels of various known SA-responsive defense- related genes, such as *PR1*, *WRKY70* and *CaBP22*. Like INA and BTH, its activity does not require accumulation of SA. However, unlike these well-characterized SA analogs, DCA-mediated immunity is not fully blocked in *npr1* Arabidopsis mutants. DCA-triggered immune responses are to a large extent independent from NPR1, but partially blocked in *wrky70* mutants. Thus DCA partially targets a WRKY70-dependent branch of the defense signaling network that does not require NPR1 (Knoth et al., 2009).

Microarray analyses revealed that DCA, INA and BTH trigger partially overlapping transcriptional responses in Arabidopsis (Wang et al., 2006; Knoth et al., 2009; Bhattarai et al., 2010). For example, transcripts of a set of 202 genes were found to be commonly up-regulated by each one of these three synthetic elicitors. However, DCA, INA and BTH also induce unique transcriptional changes. Taken together, these and other observations suggest that each of these SA analogs interferes with targets in the SA

response pathway in a unique manner.

Additional functional analogs of SA

Besides the functional analogs of SA that are discussed above, additional derivatives of this defense hormone were tested (Conrath et al., 1995; Knoth et al., 2009). This includes 3,5-dichlorosalicylic acid, 4-chlorosalicylic acid and 5-chlorosalicylic acid, which mimic SA, induce *PR1* gene expression and enhance disease resistance to TMV infection in tobacco (Conrath et al., 1995). Furthermore, 3-chlorobenzoic acid and 3,5- dichlorobenzoic acid induce basal defense against *Hpa* as well as *CaBP22::GUS* expression in Arabidopsis (Knoth et al., 2009). In contrast, the SA-related compounds benzoic acid, 2-aminobenzoic acid, 3-hydroxybenzoic acid, 4-hydroxybenzoic acid, 2,3-dihydroxybenzoic acid, 2,4-dihydroxybenzoic acid, 2,5-dihydroxybenzoic acid and 4- amino-SA did not show any defense-inducing activity (Chen and Klessig, 1991; Conrath et al., 1995; Durner and Klessig, 1995).

Furthermore, several agonists of the peroxisome proliferator-activated receptor were found to mimic effects of SA in local HR responses, but not *PR* gene expression or SAR, in soybean. The latter finding suggested that the roles of SA in local and systemic defense induction are distinct (Tenhaken et al., 2001).

Imprimatins

A screen of 10,000 small molecules to identify plant immune priming compounds by Noutoshi and coworkers resulted in the identification of three distinct classes of compounds that can prime Arabidopsis cells to exhibit enhanced immunity against virulent and avirulent *P. syringae* (Noutoshi et al., 2012d). These immune-priming compounds were termed Imprimatins. Based on structural similarities they were classified as Imprimatin A, - B or - C, representatives, respectively (Table 1.2) (Noutoshi et al., 2012c; Noutoshi et al., 2012d; Noutoshi et al., 2012e; Noutoshi et al., 2012f).

Main type	Common name	Systematic name
Imprimatin A	Imprimatin A_1	2-[(E)-2-(2-bromo-4-hydroxy-5-methoxyphenyl)ethenyl] quinolin-8-ol)
	Imprimatin A_2	7-chloro-2-[(E)-2-(4-nitrophenyl)ethenyl]-4H-3,1-benzoxazin-4-one)
	Imprimatin A_3	4-[(E)-2-(quinolin-2-yl)ethenyl]phenol)
Imprimatin B	Imprimatin B_1	2-(3-(2-furyl)-3-phenylpropyl) benzo[c]azoline-1,3-dione)
	Imprimatin B_2	3-(2-furyl)-3-phenylpropylamine)
Imprimatin C	Imprimatin C_1	[(E)-[1-amino-2-(2-oxopyrrolidin-1-yl)ethylidene]amino] 4- chlorobenzoate)
	Imprimatin C_2	[(E)-[1-amino-2-(2-oxopyrrolidin-1-yl)ethylidene]amino]3,4-dichlorobenzoate)

Table 1.2: Imprimatins

A common feature of Imprimatin A and Imprimatin B compounds is that they only prime plants for enhanced defense reactions and cannot directly induce immune responses (Noutoshi et al., 2012f; Noutoshi et al., 2012e). Application of each of these compounds increases levels of endogenous SA and decreases levels of the inactive SA metabolite SAG suggesting they inhibit SAGTs (Noutoshi et al., 2012f; Noutoshi et al., 2012e). Supporting this view, single and double knockout mutants of the Arabidopsis SAGT genes *UGT74F1* and *UGT76B1* showed increased disease resistance and free SA levels and resemble in this respect wild-type Arabidopsis plants treated with Imprimatins A1, A2, A 3, B1 or B2 (Noutoshi et al., 2012e). The enzymatic activities of UGT74F1 and UGT76B1 were also blocked *in vitro* by each of these Imprimatins at concentrations effective for immune priming. These results

suggest that Imprimatin A and - B representatives have a unique mode-of-action in defense priming and specifically inhibit SAGTs (Noutoshi et al., 2012e; Noutoshi et al., 2012f).

Two members of class C of Imprimatins, C1 and C2, were found to be SA analogs, as they activate downstream SA signaling steps and induce expression of known SA- responsive genes. However, their defense-inducing activity is weaker than that of SA. Further structure-function analyses suggested that these compounds may be converted in Arabidopsis to 4-chlorobenzoic acid and 3,4-chlorobenzoic acid, which can mimic the defense-related effects of Imprimatins C1 and C2 (Noutoshi et al., 2012c).

Sulfonamides

Sulfanilamides

In order to identify small molecules that reduce susceptibility of Arabidopsis to virulent *P. syringae*, a small collection of 200 molecules from the LATCA library (Library of Active Compounds in Arabidopsis) (Zhao et al., 2007) was screened for candidates that reduce cotyledon bleaching in liquid grown seedlings. *P. syringae* induced bleaching of Arabidopsis cotyledons is a robust disease symptom that develops within 4-5 days post- inoculation with this

pathogen (Schreiber et al., 2008). Among other candidates, the sulfanilamide compounds, sulfamethoxazole (Smex), sulfadiazine (Sdiz) and sulfapyridine (Spyr) were found to reduce this bleaching phenotype. Although, sulfanilamides have been widely used as antibiotics, the authors showed that these three candidates did not directly reduce bacterial viability and growth at concentrations that suppress their virulence. Thus, these compounds seem to act by inducing plant immune responses (Schreiber et al., 2008).

Smex was found to be the most potent one of the three identified sulfanilamides. Smex can prevent cotyledon bleaching at a concentration of 100 µM. Interestingly, Smex does not induce *PR1* expression and is active in *npr1* mutants. Thus, Smex is likely to induce defense mechanisms unrelated to the canonical SA defense pathway. Smex-mediated disease protection is also independent from JA, ET, and ABA signaling and does not require an oxidative burst (Schreiber et al., 2008; Schreiber and Desveaux, 2008).

Sulfanilamides are structural analogues of *p*-aminobenzoic acid (PABA), which can inhibit dihydropteroate synthase, an enzyme that catalyzes an important step in the folate biosynthetic pathway. Smex-mediated inhibition of folate biosynthesis may induce plant defense mechanism independently from *PR1* expression (Schreiber et al., 2008; Schreiber et al., 2012). A screen performed by the same lab to identify compounds that protect Arabidopsis against the fungal pathogen *Fusarium graminearum* resulted, besides Smex, in the identification of the indole alkaloid gramine as a plant defense inducer.

Both gramine and Smex reduced severity of *F. graminearum* infection in wheat as well (Schreiber et al., 2011).

Other sulfonamides

In 2012, additional sulfonamide compounds were also reported to induce disease resistance in plants (Noutoshi et al., 2012a). By using the same chemical screening strategy that was used for Imprimatins, chemical libraries representing 2677 bioactive molecules and small natural compounds were screened to identify immune-priming molecules. Four different sulfonamide compounds, sulfameter (SFM), sulfamethoxypyridazine (SMP), sulfabenzamide (SBA), and sulfachloropyridazine (SCP) were identified in this screening and further characterized. They increased the occurrence of cell death of Arabidopsis suspension cell cultures infected by an avirulent

P. syringae strain and were classified as immune-priming compounds. However, unlike Smex, these compounds can induce *PR1* gene expression and, unlike Imprimatin A or B representatives, they do not inhibit SAGTs (Noutoshi et al., 2012a).

Diuretics

Diuretics are pharmaceutical drugs that are widely used in clinical medicine, especially to treat hypertensive and oedematous states (Plant, 2003). Three diuretics, 3- (butylamino)-4-phenoxy-5-sulfamoylbenzoic acid

(Bumetanide), 3-benzyl-1,1-dioxo-6- (trifluoromethyl)-3,4dihydro-2*H*-1,2,4-benzothiadiazine-7-sulfonamide (Bendroflumethiazide) and 4-chloro-*N*-(2,6-dimethyl-1-piperidyl)-3-sulfamoyl-benzamide (Clopamide) (McNeil et al., 1987; Breyer and Jacobson, 1990; Pacifici, 2012) were identified as plant immune-priming compounds through the screening of a chemical library of 2000 known bioactive compounds (Noutoshi et al., 2012b). They stimulate pathogen-induced cell death in Arabidopsis in a concentration-dependent manner. In Arabidopsis they can enhance disease resistance to both avirulent and virulent *P. syringae* strains. Effects of 100 µM diuretic on defense induction are comparable to those triggered by 50 µM SA and they do not directly inhibit bacterial growth up to concentration of 200 µM. Application of these diuretics significantly decreases the growth of avirulent bacteria compared to mock treatment and mediates enhanced *PR1* gene expression after infection with *P. syringae*. These compounds potentiate disease resistance by enhancing plant defense responses, but, unlike SA and its analogs, do not induce *PR1* expression in the absence of pathogen infection (Noutoshi et al., 2012b).

Diuretics exhibit pharmacological effects in humans by acting on proteins of the SLC12A family, which are sodium-coupled chloride co-transporters that are located along the renal tubule of the kidney nephron. Diuretics inhibit these co-transporters by binding to their Cl^- binding site (Breyer and Jacobson, 1990; Gamba, 2005). The Arabidopsis genome encodes only a single protein

closely related to SLC12A, *At*1g30450 (*At*CCC1). Thus, diuretics-triggered defense priming may be mediated via *At*CCC1. However, no results regarding this possible role of *At*CCC1 have been reported.

Interestingly, diuretics contain a sulfonamide moiety similar to those identified in the defense-inducing sulfanilamide compounds sulfamethoxazole, sulfadiazine and sulfapyridine (Schreiber et al., 2008). Both diuretics and sulfanilamides can decrease bacterial growth *in planta.* The presence of sulfonamide moieties seems to be essential for their ability to induce defense reactions, as diuretics without sulfonamide groups do not exhibit this activity (Schreiber et al., 2008; Noutoshi et al., 2012b). Further studies with diuretics and sulfanilamides are needed to uncover their modes-of-action.

Adipic acid derivatives

In order to identify chemical mixtures that can delay senescence and induce immunity in plants, various mixtures of adipic acid monoethyl ester derivatives were tested. Application of a mixture of furfurylamine and 1,2,3,4-tetra-O-acetyl-β-D-glucopy-ranose (FGA) increased chlorophyll content, cell wall sugar content and delayed the chlorophyll degrading rate along with senescence in tomato and pepper (Flors et al., 2001). FGA also increased PAL activity as well as the concentration of flavonoids and phenolic compounds and strengthened plant immunity against various different

pathogens such as *Phytophthora citrophthora* and *Altemaria solani* in tomato (*Solanum lycopersicum L*.) as well as *Alternaria solani* in pepper (*Capsicum annuum L.)* (Flors et al., 2001). Individual application of three novel amides of adipic acid, 5-carbamoil ethyl pentanoate (N1), 5-(2-furfurylmethylcarbamoil) ethyl pentanoate (N2) and 5-(3- aminopropylcarbamoil) ethyl pentanoate (N3) was shown to strongly induce resistance against *Alternaria solani* in pepper. However, many other adipic acid derivatives were most effective when used as a mixture (Flors et al., 2003a; Flors et al., 2 0 0 3 b). Although these chemicals reduced pathogen growth in their hosts, many of them did not show any direct antimicrobial effect to pathogens and, therefore, likely induce plant immune responses (Flors et al., 2001; Flors et al., 2003a; Flors et al., 2003b; Flors et al., 2004). However, the mode-of-action underlying this function remains unresolved.

Jasmonic acid analogs

Jasmonic acid (JA) and its methylester, methyl-jasmonate (MeJA), are important members of the family of jasmonates which are biologically active fatty-derived cyclopentanones, that are broadly present in the plant kingdom. They are synthesized rapidly by the octadecanoid (and possibly hexadecanoid) biosynthesis pathways upon pathogen or insect attack and activate defense responses (Howe, 2010; Wasternack and Hause, 2013). Jasmonates are

known to control stress responses against nectrotrophic pathogens, herbivores and wounding, but are also known to perform various important roles in plant development related to leaf senescence, growth inhibition and floral development (He et al., 2002; Balbi and Devoto, 2008; Zhang and Turner, 2008; Oh et al., 2013; Santino et al., 2013). Upon synthesis, JA can either be metabolized to MeJA or conjugated to L-isoleucine leading to jasmonoyl-isoleucine (JA- Ile), which is an active form of JA (Svoboda and Boland, 2010; Pieterse et al., 2012).

Together with Jasmonate ZIM-domain (JAZ)-type transcriptional repressors, the F-box protein Coronatine Insensitive1 (COI1) functions as JA-Ile receptors. Recruitment of JAZ proteins into COI1-containing SKP1-Cullin-F-box (SCF^{COI1}) complexes results in proteasome-mediated degradation of these transcriptional repressors. Consequently expression of a large number of JA-responsive genes is de-repressed and defense responses are activated (Browse, 2009; Pieterse et al., 2012; Monte et al., 2014). Jasmonates typically promote defense responses against necrotrophic microbial pathogens. For example, exogenous application of JA or MeJA was shown to protect barley against *Erysiphe graminis* f.sp. hordei (Schweizer et al., 1993). In Arabidopsis, MeJA up-regulates transcript levels of the *PDF1.2* gene family along with hundreds of additional genes (Schenk et al., 2000; Jung et al., 2007; Scranton et al., 2013) and enhances resistance to various necrotrophic pathogens, such as the fungi *Alternaria brassicicola* and *Botrytis*

cinerea (Thomma et al., 1998; Seo et al., 2001; Rowe et al., 2010).

Systematic structural modifications of JA revealed the minimal structural requirements required for its bioactivity allowing for the synthesis of JA-mimics (Svoboda and Boland, 2010). The synthetic JA mimic coronalon (2-[(6-ethyl-1-oxo-indane-4-carbonyl)-amino]- 3-methyl-pentanoic acid methyl ester) mediated induction of stress responses in various plant species (Schüler et al., 2004). In addition, coronalon and its unsubstituted form (1- oxo-indanoyl-L-isoleucine methyl ester) increased levels of nicotine and trypsin proteinase inhibitors which are known MeJA-activated defense products in *Nicotiana attenuata.* They also triggered transcriptional up-regulation of the majority of genes that are known to be responsive to MeJA (Pluskota et al., 2007). The compound 1-oxo- indanoyl-L-isoleucine methyl ester was also shown to enhance activity of defense- related enzymes such as PAL or peroxidases and to induce resistance against downy mildew (Deepak et al., 2007). Additional synthetic JA mimics were shown to induce jasmonate signaling and immune responses in various plant species (Krumm et al., 1995; Fliegmann et al., 2003; Pluskota et al., 2007). However, none of these compounds were studied at the molecular level and nothing is known about their modes-of-action.

Conclusions and Perspectives

In this review article we have provided an overview of the discovery and functional characteristics of synthetic elicitors as well as their potential for basic research and crop protection. In our opinion, three major observations stand out.

(1) The vast majority of known synthetic elicitors belongs to the large group of functional SA analogs and mimics roles of this messenger molecule in defense induction. Many of these compounds are structurally related to SA. This strong trend may be partially due to a bias in the used compound screening strategies, most of which were based on the use of known SA-triggered immune responses as an indicator of defense induction. However, the dominance of functional SA analogs among known synthetic elicitors may also reflect that the SA-response pathway is particularly enriched for drug-able targets (which often have natural ligand binding pockets) and may involve more than just one type of SA receptor. This is consistent with the fact that responses triggered by different SA analogs do often not fully overlap and are partly unique. Thus, many functional SA analogs may constitute selective SA agonists, each of which interferes in a distinct manner with natural SA targets.

(2) Synthetic elicitors can be successfully applied in crop protection. Several examples illustrate the utility of plant immune-stimulants or -inducers in agriculture. Most likely more examples will follow, providing attractive

alternatives to conventional biocidal agrochemicals.

(3) Synthetic elicitors can also serve as potent tools in basic research approaches expanding our knowledge of plant immunity. A particularly prominent example highlighting their potency in this respect is the role of INA in the discovery of NPR1 as a central regulator of SA-dependent immune responses.

While additional screens for synthetic elicitors that are more potent and possibly distinct from those that are known are desirable, a rich arsenal of interesting plant defense- inducing compounds is already at hand. What is missing at this point, is a comprehensive systematic comparison of their functional characteristics in a single plant system, such as Arabidopsis. We anticipate specific interactions of many of these compounds with the plant immune system to define distinct “points of reference”, that can be probed and further examined with each compound. A next critical step will be the identification of direct synthetic elicitor targets and their roles in plant defense. This may lead to the discovery of so far unknown components of the plant immune system and reveal novel regulatory interactions controlling plant defense reactions. Furthermore, innovative screening designs are needed to complement the set of available compounds. A greater diversity of synthetic elicitors will not only be beneficial for basic research, but may also be necessary for the design of innovative multifunctional crop protectants that

stimulate multiple aspects of the plant defense system and can provide resistance against a broader spectrum of plant pathogens.

Acknowledgements

Yasemin Bektas collected, consolidated and interpreted all published information used in this manuscript. She also wrote a full draft of this manuscript, which was edited by Thomas Eulgem. Yasemin Bektas was supported by the Turkish Republic Ministry of National Education. I further thank Mercedes Schroeder for critical reading of this manuscript.

References

Abramovitch, R. B. and Martin, G. B. (2004). Strategies Used by Bacterial Pathogens to Suppress Plant Defenses. *Curr Opin Plant Biol,* 7**,** 356-64.

An, C. and Mou, Z. (2014). Salicylic Acid and Defense Responses in Plants *In:* Tran, L.-S. P. and Pal, S. (eds.) *Phytohormones: A Window to Metabolism, Signaling and Biotechnological Applications.* New York: Springer Science+Business Media.

Balbi, V. and Devoto, A. (2008). Jasmonate Signalling Network in *Arabidopsis Thaliana*: Crucial Regulatory Nodes and New Physiological Scenarios. *New Phytologist,* 177**,** 301-318.

Beckers, G. J. and Conrath, U. (2007). Priming for Stress Resistance: From the Lab to the Field. *Curr Opin Plant Biol,* 10**,** 425-31.

Beckers, G. J., Jaskiewicz, M., Liu, Y., Underwood, W. R., He, S. Y., Zhang, S. and Conrath, U. (2009). Mitogen-Activated Protein Kinases 3 and 6 are Required for Full Priming of Stress Responses in *Arabidopsis Thaliana*. *Plant Cell,* 21**,** 944-53.

Bhattarai, K. K., Atamian, H. S., Kaloshian, I. and Eulgem, T. (2010). WRKY72-Type Transcription Factors Contribute to Basal Immunity in Tomato and Arabidopsis as Well as Gene-for-Gene Resistance Mediated by the Tomato R Gene Mi-1. *Plant J,* 63**,** 229-40.

Blackwell, H. E. and Zhao, Y. (2003). Chemical Genetic Approaches to Plant Biology. *Plant Physiol,* 133**,** 448-55.

Branca, C., Plazzi, V., Marina, V., Bordi, F., Fracassini, D. S. and Nello, B. (1975). Auxin-Like Activity of 1,2- Benzisothiazole Derivatives. *Phytochemistry,* 14**,** 2545-2550.

Breyer, J. and Jacobson, H. R. (1990). Molecular Mechanisms of Diuretic Agents. *Annual review of medicine,* 41**,** 265-275.

Browse, J. (2009). Jasmonate Passes Muster: A Receptor and Targets for the Defense Hormone. *Annu Rev Plant Biol,* 60**,** 183-205.

Brozek, V., Dias, L. M., Hadano, H., Münks, K. W., Sawada, H., Sirven, C. and Toquin, V. (2012). Use of Isothiazolecarboxamides to Create Latent Host Defenses in a Plant. Google Patents.

Camoutsis, C., Geronikaki, A., Ciric, A., Soković, M., Zoumpoulakis, P. and Zervou, M. (2010).

Sulfonamide-1,2,4-Thiadiazole Derivatives as Antifungal and Antibacterial Agents: Synthesis, Biological Evaluation, Lipophilicity, and Conformational Studies. *Chem Pharm Bull (Tokyo),* 58**,** 160-7.

Cao, H., Bowling, S. A., Gordon, A. S. and Dong, X. (1994). Characterization of an Arabidopsis Mutant that Is Nonresponsive to Inducers of Systemic Acquired Resistance. *The Plant Cell Online,* 6**,** 1583- 1592.

Cao, H., Glazebrook, J., Clarke, J. D., Volko, S. and Dong, X. (1997). The Arabidopsis NPR1 Gene That Controls Systemic Acquired Resistance Encodes a Novel Protein Containing Ankyrin Repeats. *Cell,* 88**,** 57-63.

Chamnongpol, S., Willekens, H., Langebartels, C., Van Montagu, M., Inzé, D. and Van Camp, W. (1996). Transgenic Tobacco with a Reduced Catalase Activity Develops Necrotic Lesions and Induces Pathogenesis-Related Expression under High Light. *The Plant Journal,* 10**,** 491-503.

Chaudhary, S., Chattopadhyay, P., Wahi, A. K., Didel, M. and Dahiya, V. (2010). Synthesis and Study of Antibacterial and Antifungal Activity of Novel 2-(5-Substituted Methylamino-1,3,4-Thiadiazol- 2yl)Phenols. *Journal of Chemical and Pharmaceutical Research,* 2**,** 47-52.

Chen, Z. and Klessig, D. F. (1991). Identification of a Soluble Salicylic Acid-Binding Protein that May Function in Signal Transduction in the Plant Disease-Resistance Response. *Proceedings of the National Academy of Sciences,* 88**,** 8179-8183.

Chen, Z., Malamy, J., Henning, J., Conrath, U., Sánchez-Casas, P., Silva, H., Ricigliano, J. and Klessig, D. K. (1995). Induction, Modification, and Transduction of the Salicylic Acid Signal in Plant Defense Responses. *Proc Natl Acad Sci U S A,* 92**,** 4134-7.

Chen, Z., Ricigliano, J. W. and Klessig, D. F. (1993). Purification and Characterization of a Soluble Salicylic Acid-Binding Protein from Tobacco. *Proc Natl Acad Sci U S A,* 90**,** 9533-7.

Chisholm, S. T., Coaker, G., Day, B. and Staskawicz, B. J. (2006). Host-Microbe Interactions: Shaping the Evolution of the Plant Immune Response. *Cell,* 124**,** 803-14.

Conrath, U. (2006). Systemic Acquired Resistance. *Plant Signal Behav,* 1**,** 179-84. Conrath, U. (2011). Molecular Aspects of Defence Priming. *Trends Plant Sci,* 16**,** 524-31.

Conrath, U., Beckers, G. J., Flors, V., García-Agustín, P., Jakab, G., Mauch, F., Newman, M. A., Pieterse, C. M., Poinssot, B., Pozo, M. J., Pugin, A., Schaffrath, U., Ton, J., Wendehenne, D., Zimmerli, L. and Mauch-Mani, B. (2006). Priming: Getting Ready for Battle. *Mol Plant Microbe Interact,* 19**,** 1062- 71.

Conrath, U., Chen, Z., Ricigliano, J. R. and Klessig, D. F. (1995). Two Inducers of Plant Defense Responses, 2,6-Dichloroisonicotinec Acid and Salicylic Acid, Inhibit Catalase Activity in Tobacco. *Proc Natl Acad Sci U S A,* 92**,** 7143-7.

Conrath, U., Pieterse, C. M. and Mauch-Mani, B. (2002). Priming in Plant-Pathogen Interactions. *Trends Plant Sci,* 7**,** 210-6.

De, A. (1981). 4 Biologically Active 1,2-Benzisothiazole Derivatives. *In:* Ellis, G. P. and West, G. B. (eds.) *Progress in Medicinal Chemistry.* Elsevier.

Dean, P. (2005). Computer-Aided Design of Small Molecules for Chemical Genomics. *In:* Zanders, E. (ed.) *Chemical Genomics.* Humana Press.

Deepak, S., Niranjan-Raj, S., Shailasree, S., Kini, R. K., Boland, W., Shetty, H. S. and Mithöfer, A. (2007). Induction of Resistance against Downy Mildew Pathogen in Pearl Millet by a Synthetic Jasmonate Analogon. *Physiological and Molecular Plant Pathology,* 71**,** 96-105.

Delaney, T. P., Friedrich, L. and Ryals, J. A. (1995). Arabidopsis Signal Transduction Mutant Defective in Chemically and Biologically Induced Disease Resistance. *Proc Natl Acad Sci U S A,* 92**,** 6602-6.

Delaney, T. P., Uknes, S., Vernooij, B., Friedrich, L., Weymann, K., Negrotto, D., Gaffney, T., Gut-Rella, M., Kessmann, H., Ward, E. and Ryals, J. (1994). A Central Role of Salicylic Acid in Plant Disease Resistance. *Science,* 266**,** 1247-50.

Dodds, P. N. and Rathjen, J. P. (2010). Plant Immunity: Towards an Integrated View of Plant-Pathogen Interactions. *Nat Rev Genet,* 11**,** 539-48.

Dong, X. (2004). NPR1, All Things Considered. *Curr Opin Plant Biol,* 7**,** 547-52.

Du, H. and Klessig, D. F. (1997). Identification of a Soluble, High-Affinity Salicylic Acid-Binding Protein in Tobacco. *Plant Physiol,* 113**,** 1319-1327.

Durner, J. and Klessig, D. F. (1995). Inhibition of Ascorbate Peroxidase by Salicylic Acid and 2,6- Dichloroisonicotinic Acid, Two Inducers of Plant Defense Responses. *Proc Natl Acad Sci U S A,* 92**,** 11312-6.

Durrant, W. E. and Dong, X. (2004). Systemic Acquired Resistance. *Annu Rev Phytopathol,* 42**,** 185-209. Elmore, J. M., Lin, Z. J. and Coaker, G. (2011). Plant NB-LRR Signaling: Upstreams and Downstreams. *Curr Opin Plant Biol,* 14**,** 365-71.

Eulgem, T. (2005). Regulation of the Arabidopsis Defense Transcriptome. *Trends Plant Sci,* 10**,** 71-8. Fan, Z., Shi, Z., Zhang, H., Liu, X., Bao, L., Ma, L., Zuo, X., Zheng, Q. and Mi, N. (2009). Synthesis and

Biological Activity Evaluation of 1,2,3-Thiadiazole Derivatives as Potential Elicitors with Highly Systemic Acquired Resistance. *J Agric Food Chem,* 57**,** 4279-86.

Fliegmann, J., Schüler, G., Boland, W., Ebel, J. and Mithöfer, A. (2003). The Role of Octadecanoids and Functional Mimics in Soybean Defense Responses. *Biol Chem,* 384**,** 437-46.

Flor, H. H. (1971). Current Status of the Gene for Gene Concept. *annu Rev Phytopathol,* 9**,** 275-296.

Flors, V., Miralles, C., Cerezo, M., González-Bosch, C. and García-Agustín, P. (2001). Effect of a Novel Chemical Mixture on Senescence Processes and Plant-Fungus Interaction in Solanaceae Plants. *J Agric Food Chem,* 49**,** 2569-75.

Flors, V., Miralles, M. C., González-Bosch, C., Carda, M. and García-Agustín, P. (2003a). Induction of Protection against the Necrotrophic Pathogens *Phytophthora Citrophthora* and *Alternaria Solani* in *Lycopersicon Esculentum* Mill. by a Novel Synthetic Glycoside Combined with Amines. *Planta,* 216**,** 929-38.

Flors, V., Miralles, M. C., Varas, E., Company, P., González-Bosch, C. and García-Agustín, P. (2004). Effect of Analogues of Plant Growth Regulators on in Vitro Growth of Eukaryotic Plant Pathogens. *Plant Pathology,* 53**,** 58-64.

Lors,. C., Miralles, C., Gon le - osc , C., Carda, M. and Garc a -A us , P. (2003b). Three Novel Synthetic Amides of Adipic Acid Protect *Capsicum Anuum* Plants against the Necrotrophic Pathogen *Alternaria Solani*. *Physiological and Molecular Plant Pathology,* 63**,** 151-158.

Forouhar, F., Yang, Y., Kumar, D., Chen, Y., Fridman, E., Park, S. W., Chiang, Y., Acton, T. B., Montelione,

G. T., Pichersky, E., Klessig, D. F. and Tong, L. (2005). Structural and Biochemical Studies Identify Tobacco SABP2 as a Methyl Salicylate Esterase and Implicate It in Plant Innate Immunity. *Proc Natl Acad Sci U S A,* 102**,** 1773-8.

Friedrich, L., Lawton, K., Ruess, W., Masner, P., Specker, N., Rella, M. G., Meier, B., Dincher, S., Staub, T., Uknes, S., Métraux, J.-P., Kessmann, H. and Ryals, J. (1996). A Benzothiadiazole Derivative Induces Systemic Acquired Resistance in Tobacco. *The Plant Journal,* 10**,** 61-70.

Fu, Z. Q. and Dong, X. (2013). Systemic Acquired Resistance: Turning Local Infection into Global Defense. *Annu Rev Plant Biol,* 64**,** 839-63.

Fu, Z. Q., Yan, S., Saleh, A., Wang, W., Ruble, J., Oka, N., Mohan, R., Spoel, S. H., Tada, Y., Zheng, N. and Dong, X. (2012). NPR3 and NPR4 Are Receptors for the Immune Signal Salicylic Acid in Plants. *Nature,* 486**,** 228-32.

Gamba, G. (2005). Molecular Physiology and Pathophysiology of Electroneutral Cation-Chloride Cotransporters. *Physiol Rev,* 85**,** 423-93.

Gianinazzi, S. and Kassanis, B. (1974). Virus Resistance Induced in Plants by Polyacrylic Acid. *Journal of General Virology,* 23**,** 1-9.

Giannella, M., Gualtieri, F. and Melchiorre, C. (1971). Benzisoxazole and Benzisothiazole Analogs of Auxin. *Phytochemistry,* 10**,** 539-544.

Glazebrook, J. (2001). Genes Controlling Expression of Defense Responses in Arabidopsis--2001 Status. *Curr Opin Plant Biol,* 4**,** 301-8.

Glazebrook, J. (2005). Contrasting Mechanisms of Defense against Biotrophic and Necrotrophic Pathogens. *Annu Rev Phytopathol,* 43**,** 205-27.

Glazebrook, J., Chen, W., Estes, B., Chang, H. S., Nawrath, C., Metraux, J. P., Zhu, T. and Katagiri, F. (2003). Topology of the Network Integrating Salicylate and Jasmonate Signal Transduction Derived from Global Expression Phenotyping. *Plant J,* 34**,** 217-28.

Goellner, K. and Conrat, U. (2008). Primin: It's All t e World to Induced Disease Resistance. *European Journal of Plant Pathology,* 121**,** 233-242.

Gómez-Gómez, L. and Boller, T. (2002). Flagellin Perception: A Paradigm for Innate Immunity. *Trends Plant Sci,* 7**,** 251-6.

Görlach, J., Volrath, S., Knauf-Beiter, G., Hengy, G., Beckhove, U., Kogel, K.-H., Oostendorp, M., Staub, T., Ward, E., Kessmann, H. and Ryals, J. (1996). Benzothiadiazole, a Novel Class of Inducers of Systemic Acquired Resistance, Activates Gene Expression and Disease Resistance in Wheat. *Plant Cell,* 8**,** 629-43.

Han, D. Y., Coplin, D. L., Bauer, W. D. and Hoitink, H. a. J. (2000). A Rapid Bioassay for Screening Rhizosphere Microorganisms for Their Ability to Induce Systemic Resistance. *Phytopathology,* 90**,** 327-332.

He, Y., Fukushige, H., Hildebrand, D. F. and Gan, S. (2002). Evidence Supporting a Role of Jasmonic Acid in Arabidopsis Leaf Senescence. *Plant Physiol,* 128**,** 876-84.

Hijwegen, T. and Verhaar, M. A. (1993). Induced Resistance to *Peronospora Parasitica* in Red Cabbage. *Netherlands Journal of Plant Pathology,* 99**,** 103-107.

Holub, E. B. (2008). Natural History of *Arabidopsis Thaliana* and Oomycete Symbioses. *Eur j plant pathol,* 122**,** 91-109.

Howe, G. (2010). Jasmonates. *In:* Davies, P. (ed.) *Plant Hormones.* Springer Netherlands. Iwata, M. (2001). Probenazole - a Plant Defence Activator. *Pesticide Outlook,* 12**,** 28-31.

Jaskiewicz, M., Conrath, U. and Peterhänsel, C. (2011). Chromatin Modification Acts as a Memory for Systemic Acquired Resistance in the Plant Stress Response. *EMBO Rep,* 12**,** 50-5.

Jones, J. D. and Dangl, J. L. (2006). The Plant Immune System. *Nature,* 444**,** 323-9.

Jung, C., Lyou, S. H., Yeu, S., Kim, M. A., Rhee, S., Kim, M., Lee, J. S., Choi, Y. D. and Cheong, J. J. (2007). Microarray-Based Screening of Jasmonate-Responsive Genes in *Arabidopsis Thaliana*. *Plant Cell Rep,* 26**,** 1053-63.

Kassanis, B. and White, R. F. (1975). Polyacrylic Acid -Induced Resistance to Tobacco Mosaic Virus in Tobacco Cv. Xanthi. *Annals of Applied Biology,* 79**,** 215-220.

Katagiri, F. (2004). A Global View of Defense Gene Expression Regulation--a Highly Interconnected Signaling Network. *Curr Opin Plant Biol,* 7**,** 506-11.

Katz, V. A., Thulke, O. U. and Conrath, U. (1998). A Benzothiadiazole Primes Parsley Cells for Augmented Elicitation of Defense Responses. *Plant Physiol,* 117**,** 1333-9.

Kauss, H., Theisinger-Hinkel, E., Mindermann, R. and Conrath, U. (1992). Dichloroisonicotinic and Salicylic Acid, Inducers of Systemic Acquired Resistance, Enhance Fungal Elicitor Responses in Parsley Cells. *The Plant Journal,* 2**,** 655-660.

Kharb, R., Kaur, P., Sharma, P. C. and Yar, M. S. (2011). Significance of Thiadiazole Derivatives as Antimicrobial Agents. *Int. J. Res. Pharm. Biomed. Sci,* 2**,** 1520-1540.

Knoth, C. and Eulgem, T. (2014). High-Throughput Screening of Small-Molecule Libraries for Inducers of Plant Defense Responses. *In:* Hicks, G. R. and Robert, S. (eds.) *Plant Chemical Genomics.* Humana Press.

Knoth, C., Ringler, J., Dangl, J. L. and Eulgem, T. (2007). Arabidopsis WRKY70 Is Required for Full RPP4- Mediated Disease Resistance and Basal Defense against *Hyaloperonospora Parasitica*. *Molecular Plant-Microbe Interactions,* 20**,** 120-128.

Knoth, C., Salus, M. S., Girke, T. and Eulgem, T. (2009). The Synthetic Elicitor 3,5-Dichloroanthranilic Acid Induces NPR1-Dependent and NPR1-Independent Mechanisms of Disease Resistance in Arabidopsis. *Plant Physiol,* 150**,** 333-47.

Kohler, A., Schwindling, S. and Conrath, U. (2002). Benzothiadiazole-Induced Priming for Potentiated Responses to Pathogen Infection, Wounding, and Infiltration of Water into Leaves Requires the NPR1/NIM1 Gene in Arabidopsis. *Plant Physiol,* 128**,** 1046-56.

Kombrink, E. and Somssich, I. E. (1997). Pathogenesis-Related Proteins and Plant Defense. *In:* Carroll, G. and Tudzynski, P. (eds.) *Plant Relationships.* Springer Berlin Heidelberg.

Krumm, T., Bandemer, K. and Boland, W. (1995). Induction of Volatile Biosynthesis in the Lima Bean (*Phaseolus Lunatus*) by Leucine- and Isoleucine Conjugates of 1-Oxo- and 1-Hydroxyindan-4- Carboxylic Acid: Evidence for Amino Acid Conjugates of Jasmonic Acid as Intermediates in the Octadecanoid Signalling Pathway. *FEBS Lett,* 377**,** 523-9.

Kuc, J. (1982). Induced Immunity to Plant Disease. Bioscience*,* 32**,** 854-860.

Kumar, D. and Klessig, D. F. (2003). High-Affinity Salicylic Acid-Binding Protein 2 Is Required for Plant Innate Immunity and Has Salicylic Acid-Stimulated Lipase Activity. *Proc Natl Acad Sci U S A,* 100**,** 16101-6.

Kunz, W., Schurter, R. and Maetzke, T. (1997). The Chemistry of Benzothiadiazole Plant Activators. *Pesticide Science,* 50**,** 275-282.

Kunz, W., Staub, T., Metraux, J.-P., Hoegerle, K., Nyfeler, R. and Ahl, P. (1988). *A Method for Protecting Plants against Diseases*.

Langcake, P. and Wickins, S. G. A. (1975a). Studies on the Action of the Dichlorocyclopropanes on the Host-Parasite Relationship in the Rice Blast Disease. *Physiological Plant Pathology,* 7**,** 113-126.

Langcake, P. and Wickins, S. G. A. (1975b). Studies on the Mode of Action of the Dichlorocyclopropane Fungicides : Effects of 2,2-Dichloro- 3,3-Dimethyl Cyclopropane Carboxylic Acid on the Growth of *Piricularia Oryzae* Cav. *journal of general microbiology,* 88**,** 295-306.

Lawton, K. A., Friedrich, L., Hunt, M., Weymann, K., Delaney, T., Kessmann, H., Staub, T. and Ryals, J. (1996). Benzothiadiazole Induces Disease Resistance in Arabidopsis by Activation of the Systemic Acquired Resistance Signal Transduction Pathway. *Plant J,* 10**,** 71-82.

Lee, S., Hong, J. C., Jeon, W. B., Chung, Y. S., Sung, S., Choi, D., Joung, Y. H. and Oh, B. J. (2009). The Salicylic Acid-Induced Protection of Non-Climacteric Unripe Pepper Fruit against *Colletotrichum Gloeosporioides* is Similar to the Resistance of Ripe Fruit. *Plant Cell Rep,* 28**,** 1573-80.

Li, J., Brader, G. and Palva, E. T. (2004). The WRKY70 Transcription Factor: A Node of Convergence for Jasmonate-Mediated and Salicylate-Mediated Signals in Plant Defense. *The Plant Cell Online,* 16**,** 319-331.

Mcneil, J. J., Conway, E. L., Drummer, O. H., Howes, L. G., Christophidis, N. and Louis, W. J. (1987).

Clopamide: Plasma Concentrations and Diuretic Effect in Humans. *Clin Pharmacol Ther,* 42**,** 299- 304.

Metraux, J. P., Ahlgoy, P., Staub, T., Speich, J., Steinemann, A., Ryals, J. and Ward, E. (1991). Induced Systemic Resistance in Cucumber in Response to 2,6-Dichloro-Isonicotinic Acid and Pathogens. *In:* Hennecke, H. and Verma, D. (eds.) *Advances in Molecular Genetics of Plant-Microbe Interactions Vol. 1.* Springer Netherlands.

Metraux, J. P., Signer, H., Ryals, J., Ward, E., Wyss-Benz, M., Gaudin, J., Raschdorf, K., Schmid, E., Blum, W. and Inverardi, B. (1990). Increase in Salicylic Acid at the Onset of Systemic Acquired Resistance in Cucumber. *Science,* 250**,** 1004-6.

Midoh, N. and Iwata, M. (1996). Cloning and Characterization of a Probenazole-Inducible Gene for an Intracellular Pathogenesis-Related Protein in Rice. *Plant Cell Physiol,* 37**,** 9-18.

Monte, I., Hamberg, M., Chini, A., Gimenez-Ibanez, S., García-Casado, G., Porzel, A., Pazos, F., Boter, M. and Solano, R. (2014). Rational Design of a Ligand-Based Antagonist of Jasmonate Perception. *Nat Chem Biol,* 10**,** 671-676.

Nakashita, H., Yasuda, M., Nishioka, M., Hasegawa, S., Arai, Y., Uramoto, M., Yoshida, S. and Yamaguchi, I. (2002a). Chloroisonicotinamide Derivative Induces a Broad Range of Disease Resistance in Rice and Tobacco. *Plant and cell physiology,* 43**,** 823-831.

Nakashita, H., Yoshioka, K., Takayama, M., Kuga, R., Midoh, N., Usami, R., Horikoshi, K., Yoneyama, K. and Yamaguchi, I. (2001). Characterization of PBZ1, a Probenazole-Inducible Gene, in Suspension-Cultured Rice Cells. *Biosci Biotechnol Biochem,* 65**,** 205-8.

Nakashita, H., Yoshioka, K., Yasuda, M., Nitta, T., Arai, Y., Yoshida, S. and Yamaguchi, I. (2002b).

Probenazole Induces Systemic Acquired Resistance in Tobacco through Salicylic Acid Accumulation. *Physiological and Molecular Plant Pathology,* 61**,** 197-203.

Nakayama, A., Fukushima, S., Goto, S., Matsushita, A., Shimono, M., Sugano, S., Jiang, C. J., Akagi, A., Yamazaki, M., Inoue, H. and Takatsuji, H. (2013). Genome-Wide Identification of WRKY45-

Regulated Genes That Mediate Benzothiadiazole-Induced Defense Responses in Rice. *BMC Plant Biol,* 13**,** 150.

Nimchuk, Z., Eulgem, T., Holt, B. F., 3rd and Dangl, J. L. (2003). Recognition and Response in the Plant Immune System. *Annu Rev Genet,* 37**,** 579-609.

Nishioka, M., Nakashita, H., Suzuki, H., Akiyama, S., Yoshida, S. and Yamaguchi, I. (2003). Induction of Resistance against Rice Blast Disease by a Novel Class of Plant Activators, Pyrazolecarboxylic Acid Derivatives. *j. pestic. sci.,* 28**,** 416-421.

Nishioka, M., Nakashita, H., Yasuda, M., Yoshida, S. and Yamaguchi, I. (2005). Induction of Resistance against Rice Bacterial Leaf Blight by 3-Chloro-1-Methyl-1h-Pyrazole-5-Carboxylic Acid. *j. pestic. sci.,* 30**,** 47-49.

Noutoshi, Y., Ikeda, M., Saito, T., Osada, H. and Shirasu, K. (2012a). Sulfonamides Identified as Plant Immune-Priming Compounds in High-Throughput Chemical Screening Increase Disease Resistance in *Arabidopsis Thaliana*. *Front Plant Sci,* 3**,** 245.

Noutoshi, Y., Ikeda, M. and Shirasu, K. (2012b). Diuretics Prime Plant Immunity in *Arabidopsis Thaliana*. *PLoS One,* 7**,** e48443.

Noutoshi, Y., Jikumaru, Y., Kamiya, Y. and Shirasu, K. (2012c). ImprimatinC1, a Novel Plant Immune- Priming Compound, Functions as a Partial Agonist of Salicylic Acid. *Sci Rep,* 2**,** 705.

Noutoshi, Y., Okazaki, M., Kida, T., Nishina, Y., Morishita, Y., Ogawa, T., Suzuki, H., Shibata, D., Jikumaru, Y., Hanada, A., Kamiya, Y. and Shirasu, K. (2012d). Novel Plant Immune-Priming Compounds Identified Via High-Throughput Chemical Screening Target Salicylic Acid Glucosyltransferases in Arabidopsis. *Plant Cell,* 24**,** 3795-804.

Noutoshi, Y., Okazaki, M. and Shirasu, K. (2012e). Imprimatins A and B: Novel Plant Activators Targeting Salicylic Acid Metabolism in *Arabidopsis Thaliana*. *Plant Signal Behav,* 7**,** 1715-7.

Noutoshi, Y., Okazaki, M. and Shirasu, K. (2012f). Isolation and Characterization of the Plant Immune- Priming Compounds Imprimatin B3 and -B4, Potentiators of Disease Resistance in *Arabidopsis Thaliana*. *Plant Signal Behav,* 7**,** 1526-8.

Nürnberger, T. and Lipka, V. (2005). Non-Host Resistance in Plants: New Insights into an Old Phenomenon. *Mol Plant Pathol,* 6**,** 335-45.

Ogava, M., Kadowaki, A., Yamada, T. and Kadooka, O. (2011). Applied Development of a Novel Fungicide Isotianil (Stout®). Sumitomo Chemical Co., Ltd.

Oh, Y., Baldwin, I. T. and Galis, I. (2013). A Jasmonate ZIM-Domain Protein Najazd Regulates Floral Jasmonic Acid Levels and Counteracts Flower Abscission in *Nicotiana Attenuata* Plants. *PloS one,* 8**,** e57868.

Oostendorp, M., Kunz, W., Dietrich, B. and Staub, T. (2001). Induced Disease Resistance in Plants by Chemicals. *European Journal of Plant Pathology,* 107**,** 19-28.

Pacifici, G. M. (2012). Clinical Pharmacology of the Loop Diuretics Furosemide and Bumetanide in Neonates and Infants. *Paediatr Drugs,* 14**,** 233-46.

Park, S. W., Kaimoyo, E., Kumar, D., Mosher, S. and Klessig, D. F. (2007). Methyl Salicylate is a Critical Mobile Signal for Plant Systemic Acquired Resistance. *Science,* 318**,** 113-6.

Pieterse, C. M., Van Der Does, D., Zamioudis, C., Leon-Reyes, A. and Van Wees, S. C. (2012). Hormonal Modulation of Plant Immunity. *Annu Rev Cell Dev Biol,* 28**,** 489-521.

Pieterse, C. M., Van Wees, S. C., Van Pelt, J. A., Knoester, M., Laan, R., Gerrits, H., Weisbeek, P. J. and Van Loon, L. C. (1998). A Novel Signaling Pathway Controlling Induced Systemic Resistance in Arabidopsis. *Plant Cell,* 10**,** 1571-80.

Plant, L. (2003). Clinical Use of Diuretics. *Clinical medicine,* 3**,** 517-520.

Pluskota, W. E., Qu, N., Maitrejean, M., Boland, W. and Baldwin, I. T. (2007). Jasmonates and Its Mimics Differentially Elicit Systemic Defence Responses in *Nicotiana Attenuata*. *J Exp Bot,* 58**,** 4071-82.

Raikhel, N. and Pirrung, M. (2005). Adding Precision Tools to the Plant Biologists' Toolbox with Chemical Genomics. *Plant Physiol,* 138**,** 563-4.

Rasmussen, J. B., Hammerschmidt, R. and Zook, M. N. (1991). Systemic Induction of Salicylic Acid Accumulation in Cucumber after Inoculation with *Pseudomonas Syringae* Pv Syringae. *Plant Physiol,* 97**,** 1342-7.

Rowe, H. C., Walley, J. W., Corwin, J., Chan, E. K., Dehesh, K. and Kliebenstein, D. J. (2010). Deficiencies in Jasmonate-Mediated Plant Defense Reveal Quantitative Variation in *Botrytis Cinerea* Pathogenesis. *PLoS Pathog,* 6**,** e1000861.

Ryals, J., Weymann, K., Lawton, K., Friedrich, L., Ellis, D., Steiner, H. Y., Johnson, J., Delaney, T. P., Jesse, T., Vos, P. and Uknes, S. (1997). The Arabidopsis NIM1 Protein Shows Homology to the Mammalian Transcription Factor Inhibitor I kappa B. *Plant Cell,* 9**,** 425-39.

Santino, A., Taurino, M., De Domenico, S., Bonsegna, S., Poltronieri, P., Pastor, V. and Flors, V. (2013). Jasmonate Signaling in Plant Development and Defense Response to Multiple (a) Biotic Stresses. *Plant Cell Rep,* 32**,** 1085-98.

Sato, M., Tsuda, K., Wang, L., Coller, J., Watanabe, Y., Glazebrook, J. and Katagiri, F. (2010). Network Modeling Reveals Prevalent Negative Regulatory Relationships between Signaling Sectors in Arabidopsis Immune Signaling. *PLoS Pathog,* 6**,** e1001011.

Schenk, P. M., Kazan, K., Wilson, I., Anderson, J. P., Richmond, T., Somerville, S. C. and Manners, J. M. (2000). Coordinated Plant Defense Responses in Arabidopsis Revealed by Microarray Analysis. *Proc Natl Acad Sci U S A,* 97**,** 11655-60.

Schreiber, K., Ckurshumova, W., Peek, J. and Desveaux, D. (2008). A High-Throughput Chemical Screen for Resistance to *Pseudomonas Syringae* in Arabidopsis. *Plant J,* 54**,** 522-31.

Schreiber, K. and Desveaux, D. (2008). Message in a Bottle: Chemical Biology of Induced Disease Resistance in Plants. *The Plant Pathology Journal,* 24**,** 245-268.

Schreiber, K. J., Austin, R. S., Gong, Y., Zhang, J., Fung, P., Wang, P. W., Guttman, D. S. and Desveaux, D. (2012). Forward Chemical Genetic Screens in Arabidopsis Identify Genes that Influence Sensitivity to the Phytotoxic Compound Sulfamethoxazole. *BMC Plant Biol,* 12**,** 226.

Schreiber, K. J., Nasmith, C. G., Allard, G., Singh, J., Subramaniam, R. and Desveaux, D. (2011). Found in Translation: High-Throughput Chemical Screening in *Arabidopsis Thaliana* Identifies Small Molecules That Reduce Fusarium Head Blight Disease in Wheat. *Mol Plant Microbe Interact,* 24**,** 640-8.

Schüler, G., Mithöfer, A., Baldwin, I. T., Berger, S., Ebel, J., Santos, J. G., Herrmann, G., Hölscher, D., Kramell, R., Kutchan, T. M., Maucher, H., Schneider, B., Stenzel, I., Wasternack, C. and Boland, W. (2004). Coronalon: A Powerful Tool in Plant Stress Physiology. *FEBS Lett,* 563**,** 17-22. Schurter, R., Kunz, W. and Nyfeler, R. (1993). Process and a Composition for Immunizing Plants against diseases. US patent 5190928.

Schweizer, P., Gees, R. and Mosinger, E. (1993). Effect of Jasmonic Acid on the Interaction of Barley (*Hordeum Vulgare* L.) with the Powdery Mildew *Erysiphe Graminis* F. Sp. Hordei. *Plant physiology,* 102**,** 503-511.

Scranton, M. A., Fowler, J. H., Girke, T. and Walling, L. L. (2013). Microarray Analysis of Tomato's Early and Late Wound Response Reveals New Regulatory Targets for Leucine Aminopeptidase A. *PLoS One,* 8**,** e77889.

Segonzac, C. and Zipfel, C. (2011). Activation of Plant Pattern-Recognition Receptors by Bacteria. *Curr Opin Microbiol,* 14**,** 54-61.

Sekizawa, Y., Shimura, M., Suzuki, A. and Iwata, M. (1981). Anti-Conidial Germination Factors Induced in the Presence of Probenazole in Infected Host Leaves. Ii. Structural Elucidation of the Major Component (Substance B). *Agricultural and Biological Chemistry,* 45**,** 1437-1439.

Seo, H. S., Song, J. T., Cheong, J. J., Lee, Y. H., Lee, Y. W., Hwang, I., Lee, J. S. and Choi, Y. D. (2001). Jasmonic Acid Carboxyl Methyltransferase: A Key Enzyme for Jasmonate-Regulated Plant Responses. *Proc Natl Acad Sci U S A,* 98**,** 4788-93.

Shen, Q. H., Saijo, Y., Mauch, S., Biskup, C., Bieri, S., Keller, B., Seki, H., Ulker, B., Somssich, I. E. and Schulze-Lefert, P. (2007). Nuclear Activity of MLA Immune Receptors Links Isolate-Specific and Basal Disease-Resistance Responses. *Science,* 315**,** 1098-103.

Shimono, M., Koga, H., Akagi, A., Hayashi, N., Goto, S., Sawada, M., Kurihara, T., Matsushita, A., Sugano, S., Jiang, C. J., Kaku, H., Inoue, H. and Takatsuji, H. (2012). Rice WRKY45 Plays Important Roles in Fungal and Bacterial Disease Resistance. *Mol Plant Pathol,* 13**,** 83-94.

Shimono, M., Sugano, S., Nakayama, A., Jiang, C. J., Ono, K., Toki, S. and Takatsuji, H. (2007). Rice WRKY45 Plays a Crucial Role in Benzothiadiazole-Inducible Blast Resistance. *Plant Cell,* 19**,** 2064- 76.

Shimura, M., Mase, S., Iwata, M., Suzuki, A., Watanabe, T., Sekizawa, Y., Sasaki, T., Furihata, K., Seto, H. and Otake, N. (1983). Anti-Conidial Germination Factors Induced in the Presence of Probenazole in Infected Host Leaves. III. Structural Elucidation of Substances A and C. *Agricultural and Biological Chemistry,* 47**,** 1983-1989.

Singh, A. K., Mishra, G. and Jyoti, K. (2011). Review on Biological Activities of 1, 3, 4-Thiadiazole Derivatives.

Slaymaker, D. H., Navarre, D. A., Clark, D., Del Pozo, O., Martin, G. B. and Klessig, D. F. (2002). The Tobacco Salicylic Acid-Binding Protein 3 (SABP3) Is the Chloroplast Carbonic Anhydrase, Which Exhibits Antioxidant Activity and Plays a Role in the Hypersensitive Defense Response. *Proc Natl Acad Sci U S A,* 99**,** 11640-5.

Spanu, P. D. (2012). The Genomics of Obligate (and Nonobligate) Biotrophs. *Annu Rev Phytopathol,* 50**,** 91-109.

Sticher, L., Mauch-Mani, B. and Metraux, J. P. (1997). Systemic Acquired Resistance. *Annu Rev Phytopathol,* 35**,** 235-70.

Stockwell, B. R. (2004). Exploring Biology with Small Organic Molecules. *Nature,* 432**,** 846-54. Svoboda, J. and Boland, W. (2010). Plant Defense Elicitors: Analogues of Jasmonoyl-Isoleucine Conjugate. *Phytochemistry,* 71**,** 1445-9.

Tao, Y., Xie, Z., Chen, W., Glazebrook, J., Chang, H. S., Han, B., Zhu, T., Zou, G. and Katagiri, F. (2003). Quantitative Nature of Arabidopsis Responses During Compatible and Incompatible Interactions with the Bacterial Pathogen *Pseudomonas Syringae*. *Plant Cell,* 15**,** 317-30.

Tenhaken, R., Anstätt, C., Ludwig, A. and Seehaus, K. (2001). WY-14,643 and Other Agonists of the Peroxisome Proliferator-Activated Receptor Reveal a New Mode of Action for Salicylic Acid in Soybean Disease Resistance. *Planta,* 212**,** 888-895.

Tenhaken, R. and Rubel, C. (1997). Salicylic Acid is Needed in Hypersensitive Cell Death in Soybean but Does Not Act as a Catalase Inhibitor. *Plant physiology,* 115**,** 291-298.

Thomma, B. P. H. J., Eggermont, K., Penninckx, I. a. M. A., Mauch-Mani, B., Vogelsang, R., Cammue, B. P. A. and Broekaert, W. F. (1998). Separate Jasmonate-Dependent and Salicylate-Dependent Defense-Response Pathways in Arabidopsis are Essential for Resistance to Distinct Microbial Pathogens. *Proceedings of the National Academy of Sciences,* 95**,** 15107-15111.

Thulke, O. and Conrath, U. (1998). Salicylic Acid has a Dual Role in the Activation of Defence-Related Genes in Parsley. *Plant J,* 14**,** 35-42.

Toquin, V., Sirven, C., Assmann, L. and Sawada, H. (2012). Host Defense Inducers. *Modern Crop Protection Compounds.* Wiley-VCH Verlag GmbH & Co. KGaA.

Trapani, G., Reho, A., Morlacchi, F., Latrofa, A., Marchini, P., Venturi, F. and Cantalamessa, F. (1985).

Synthesis and Antiinflammatory Activity of Various 1,4-Benzothiazine Derivatives. *Farmaco Sci,* 40**,** 369-76.

Tripathi, D., Jiang, Y. L. and Kumar, D. (2010). SABP2, a Methyl Salicylate Esterase is Required for the Systemic Acquired Resistance Induced by Acibenzolar-S-Methyl in Plants. *FEBS Lett,* 584**,** 3458- 63.

Tsubata, K., Kuroda, K., Yamamoto, Y. and Yasokawa, N. (2006). Development of a Novel Plant Activator for Rice Diseases, Tiadinil. *Journal of Pesticide Science,* 31**,** 161-162.

Tsuda, K., Sato, M., Stoddard, T., Glazebrook, J. and Katagiri, F. (2009). Network Properties of Robust Immunity in Plants. *PLoS Genet,* 5**,** e1000772.

Uknes, S., Mauch-Mani, B., Moyer, M., Potter, S., Williams, S., Dincher, S., Chandler, D., Slusarenko, A., Ward, E. and Ryals, J. (1992). Acquired Resistance in Arabidopsis. *Plant Cell,* 4**,** 645-56.

Uknes, S., Morris, S., Vernooij, B. and Ryals, J. (1996). The Role of Benzoic Acid Derivatives in Systemic Acquired Resistance. *In:* Romeo, J., Saunders, J. and Barbosa, P. (eds.) *Phytochemical Diversity and Redundancy in Ecological Interactions.* Springer US.

Umemura, K., Satou, J., Iwata, M., Uozumi, N., Koga, J., Kawano, T., Koshiba, T., Anzai, H. and Mitomi, M. (2009). Contribution of Salicylic Acid Glucosyltransferase, OSSGT1, to Chemically Induced Disease Resistance in Rice Plants. *Plant J,* 57**,** 463-72.

Vallad, G. E. and Goodman, R. M. (2004). Systemic Acquired Resistance and Induced Systemic Resistance in Conventional Agriculture. *crop science,* 44**,** 1920-1934.

Van Kan, J. L., Cozijnsen, T., Danhash, N. and De Wit, P. G. M. (1995). Induction of Tomato Stress Protein mRNAs by Ethephon, 2,6-Dichloroisonicotinic Acid and Salicylate. *Plant Molecular Biology,* 27**,** 1205-1213.

Van Wees, S. C., De Swart, E. A., Van Pelt, J. A., Van Loon, L. C. and Pieterse, C. M. (2000). Enhancement of Induced Disease Resistance by Simultaneous Activation of Salicylate- and Jasmonate- Dependent Defense Pathways in *Arabidopsis Thaliana*. *Proc Natl Acad Sci U S A,* 97**,** 8711-6.

Vernooij, B., Friedrich, L., Goy, P. A., Staub, T., Kessmann, H. and Ryals, J. (1995). 2,6- Dicholoroisonicotinic Acid-Induced Resistance to Pathogens without the Accumulation of Saliciylic Acid. *Molecular plant-microbe interactions* 8**,** 228-234.

Vernooij, B., Uknes, S., Ward, E. and Ryals, J. (1994). Salicylic Acid as a Signal Molecule in Plant-Pathogen Interactions. *Curr Opin Cell Biol,* 6**,** 275-9.

Vicini, P., Zani, F., Cozzini, P. and Doytchinova, I. (2002). Hydrazones of 1,2-Benzisothiazole Hydrazides: Synthesis, Antimicrobial Activity and QSAR Investigations. *European Journal of Medicinal Chemistry,* 37**,** 553-564.

Vlot, A. C., Dempsey, D. A. and Klessig, D. F. (2009). Salicylic Acid, a Multifaceted Hormone to Combat Disease. *Annu Rev Phytopathol,* 47**,** 177-206.

Wang, D., Amornsiripanitch, N. and Dong, X. (2006). A Genomic Approach to Identify Regulatory Nodes in the Transcriptional Network of Systemic Acquired Resistance in Plants. *PLoS Pathog,* 2**,** e123.

Ward, E. R., Uknes, S. J., Williams, S. C., Dincher, S. S., Wiederhold, D. L., Alexander, D. C., Ahl-Goy, P., Metraux, J. P. and Ryals, J. A. (1991). Coordinate Gene Activity in Response to Agents that Induce Systemic Acquired Resistance. *Plant Cell,* 3**,** 1085-1094.

Wasternack, C. and Hause, B. (2013). Jasmonates: Biosynthesis, Perception, Signal Transduction and Action in Plant Stress Response, Growth and Development. An Update to the 2007 Review in Annals of Botany. *Ann Bot,* 111**,** 1021-58.

Watanabe, T., Igarashi, H., Matsumoto, K., Seki, S., Mase, S. and Sekizawa, Y. (1977). The Characteristics of Probenazole (Oryzemate) for the Control of Rice Blast. *Journal of Pesticide Science,* 2**,** 291- 296.

Wei, G., Kloepper, J. W. and Tuzun, S. (1996). Induced Systemic Resistance to Cucumber Diseases and Increased Plant Growth by Plant Growth-Promoting Rhizobacteria under Field Conditions. *biological control,* 86**,** 221-224.

Wendehenne, D., Durner, J., Chen, Z. and Klessig, D. F. (1998). Benzothiadiazole, an Inducer of Plant Defenses, Inhibits Catalase and Ascorbate Peroxidase. *Phytochemistry,* 47**,** 651-657.

Wevelsiep, L., Kogel, K.-H. and Knogge, W. (1991). Purification and Characterization of Peptides from *Rhynchosporium Secalis* Inducing Necrosis in Barley. *Physiological and Molecular Plant Pathology,* 39**,** 471-482.

White, R. F. (1979). Acetylsalicylic Acid (Aspirin) Induces Resistance to Tobacco Mosaic Virus in Tobacco. *virology,* 99**,** 410-412.

Wildermuth, M. C. (2006). Variations on a Theme: Synthesis and Modification of Plant Benzoic Acids. *Curr Opin Plant Biol,* 9**,** 288-96.

Wildermuth, M. C., Dewdney, J., Wu, G. and Ausubel, F. M. (2001). Isochorismate Synthase is Required to Synthesize Salicylic Acid for Plant Defence. *Nature,* 414**,** 562-5.

Wu, Y., Zhang, D., Chu, J. Y., Boyle, P., Wang, Y., Brindle, I. D., De Luca, V. and Despres, C. (2012). The Arabidopsis NPR1 Protein is a Receptor for the Plant Defense Hormone Salicylic Acid. *Cell Rep,* 1**,** 639-47.

Yan, Z., Reddy, M. S., Ryu, C. M., Mcinroy, J. A., Wilson, M. and Kloepper, J. W. (2002). Induced Systemic Protection against Tomato Late Blight Elicited by Plant Growth-Promoting Rhizobacteria. *Phytopathology,* 92**,** 1329-33.

Yasuda, M. (2007). Regulation Mechanisms of Systemic Acquired Resistance Induced by Plant Activators. *Journal of Pesticide Science,* 32**,** 281-282.

Yasuda, M., Kusajima, M., Nakajima, M., Akutsu, K., Kudo, T., Yoshida, S. and Nakashita, H. (2006).

Thiadiazole Carboxylic Acid Moiety of Tiadinil, SV-03 , Induces Systemic Acquired Resistance in Tobacco without Salicylic Acid Accumulation. *Journal of Pesticide Science,* 31**,** 329-334.

Yasuda, M., Nakashita, H., Hasegawa, S., Nishioka, M., Arai, Y., Uramoto, M., Yamaguchi, I. and Yoshida,

S. (2003a). N-Cyanomethyl-2-Chloroisonicotinamide Induces Systemic Acquired Resistance in Arabidopsis without Salicylic Acid Accumulation. *Bioscience, biotechnology, and biochemistry,* 67**,** 322-328.

Yasuda, M., Nakashita, H. and Yoshida, S. (2004). Tiadinil, a Novel Class of Activator of Systemic Acquired Resistance, Induces Defense Gene Expression and Disease Resistance in Tobacco. *Journal of Pesticide Science,* 29**,** 46-49.

Yasuda, M., Nishioka, M., Nakashita, H., Yamaguchi, I. and Yoshida, S. (2003b). Pyrazolecarboxylic Acid Derivative Induces Systemic Acquired Resistance in Tobacco. *Biosci Biotechnol Biochem,* 67**,** 2614-20.

Yoshida, H., Konishi, K., Koike, K., Nakagawa, T., Sekido, S. and Yamaguchi, I. (1990a). Effect of N- Cyanomethyl-2-Chloroisonicotinamide for Control of Rice Blast. *Journal of Pesticide Science,* 15**,** 413-417.

Yoshida, H., Konishi, K., Nakagawa, T., Sekido, S. and Yamaguchi, I. (1990b). Characteristics of N- Phenylsulfonyl-2-Chloroisonicotinamide as an Anti-Rice Blast Agent. *Journal of Pesticide Science,* 15**,** 199-203.

Yoshida, H., Shimano, S., Mochizuki, S., Koike, K., Nakagawa, T. and Konishi, K. (1989). *N- Cyanoalkylisonicotinamide Derivatives*. US patent 4804762 A.

Yoshida, K., Ogino, A., Yamada, K. and Sonoda, R. (2010). Induction of Disease Resistance in Tea (*Camellia Sinensis* L.) by Plant Activators. *JARQ,* 44**,** 391-398.

Yoshida, T., Itou, A., Yamamoto, R., Tobino, T., Murakawa, H. and Toda, K. (2013). Determination of Isotianil in Brown Rice and Soil Using Supercritical Fluid Extraction and Gas Chromatography/Mass Spectrometry. *Anal Sci,* 29**,** 919-22.

Yoshioka, K., Nakashita, H., Klessig, D. F. and Yamaguchi, I. (2001). Probenazole Induces Systemic Acquired Resistance in Arabidopsis with a Novel Type of Action. *Plant J,* 25**,** 149-57.

Zani, F., Mingiardi, M., Ca, M. and Mazza, P. (1996). Biological Studies on 1 , 2-Benzisothiazole Derivatives VI Antimicrobial Activity of 1 , 2-Benzisothiazole and 1 , 2-Benzisothiazolin-3-One Derivatives and of Some Corresponding 1,2-Benzisoxazoles. *Farmaco,* 51**,** 707-713.

Zhang, Y., Tessaro, M. J., Lassner, M. and Li, X. (2003). Knockout Analysis of Arabidopsis Transcription Factors TGA2, TGA5, and TGA6 Reveals their Redundant and Essential Roles in Systemic Acquired Resistance. *Plant Cell,* 15**,** 2647-53.

Zhang, Y. and Turner, J. G. (2008). Wound-Induced Endogenous Jasmonates Stunt Plant Growth by Inhibiting Mitosis. *PLoS One,* 3**,** e3699.

Zhao, Y., Chow, T. F., Puckrin, R. S., Alfred, S. E., Korir, A. K., Larive, C. K. and Cutler, S. R. (2007).

Chemical Genetic Interrogation of Natural Variation Uncovers a Molecule that is Glycoactivated. *Nat Chem Biol,* 3**,** 716-21.

Zipfel, C., Robatzek, S., Navarro, L., Oakeley, E. J., Jones, J. D., Felix, G. and Boller, T. (2004). Bacterial Disease Resistance in Arabidopsis through Flagellin Perception. *Nature,* 428**,** 764-7.

CHAPTER 2: Immunity and Hormesis Triggered by the Synthetic Elicitor 2-(5-bromo-2-hydroxy-phenyl)-thiazolidine-4-carboxylic acid (BHTC)

Submitted for publication in Plant Physiology in June 2015 under the title “The synthetic elicitor BHTC (2-(5-bromo-2-hydroxy-phenyl)-thiazolidine-4-carboxylic acid) links plant immunity to hormesis” by Melinda Rodriguez-Salus[*], Yasemin Bektas[*♦], Mercedes Schroeder, Colleen Knoth, Trang Vu, Philip Roberts, Isgouhi Kaloshian and Thomas Eulgem[♦].

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Abstract

Synthetic elicitors are drug-like compounds that induce plant immune responses, but are structurally distinct from natural defense elicitors. Using high-throughput screening we previously identified 114 synthetic elicitors that activate expression of a pathogen-responsive reporter gene in *Arabidopsis thaliana* (Arabidopsis). Here we report on the characterization of one of these compounds, 2-(5-bromo-2-hydroxy-phenyl)-thiazolidine-4-carboxylic acid (BHTC). BHTC induces disease resistance of plants against bacterial, oomycete and fungal pathogens and has a unique mode-of-action and structure. Surprisingly, we found that low doses of BHTC enhanced root growth in Arabidopsis, while high doses of this compound inhibited root growth, besides

inducing defense. These effects are reminiscent of the hormetic response, which is characterized by low-dose stimulatory effects of a wide range of agents that are toxic or inhibitory at higher doses. Like its effects on defense, BHTC-induced hormesis in Arabidopsis roots is partially dependent on the WRKY70 transcription factor. Interestingly, BHTC-induced root hormesis is also affected in the auxin-response mutants, *axr1-3* and *slr1*. By mRNA-seq we uncovered a dramatic difference between transcriptional profiles triggered by low and high doses of BHTC. Only high levels of BHTC induce typical defense-related transcriptional changes. Instead, low BHTC levels trigger a coordinated inter-compartmental transcriptional response manifested in suppression of photosynthesis- and respiration-related genes in the nucleus, chloroplasts and mitochondria as well as induction of development-related nuclear genes. Taken together, our functional characterization of BHTC links defense regulation to hormesis and provides a hypothetical transcriptional scenario for the induction of hormetic root growth.

Introduction

Plant innate immunity against pathogens depends on a network of functionally interconnected genes involved in the regulation and execution of defense reactions (Glazebrook et al., 2003, Sato et al., 2010, Tsuda 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 (Hein et al., 2009, Jones and Dangl, 2006, Zipfel, 2014). MAMP-recognition activates a comprehensive set of defense reactions collectively referred to as pattern triggered immunity (PTI). Adapted pathogens have acquired the ability to attenuate PTI through the secretion of effector molecules, suppressing defense and, thus, enabling infection (effector triggered susceptibility, ETS) (Chisholm et al., 2006). In this case, the pathogen is virulent and the host susceptible. During such compatible interactions plants can still mount a weakened immune response, called basal defense, which limits pathogen spread, but is typically not capable of fully preventing disease (Ahmad et al., 2011, Glazebrook, 2001). As a countermeasure to ETS, plants can recognize effectors by highly specific plant resistance (R) proteins, which mediate effector triggered immunity (ETI) resulting in incompatible interactions and leaving pathogens avirulent (Jones and Dangl, 2006). Numerous studies have shown that ETI, basal defense, and PTI utilize a common set of signaling components including multiple messenger substances, such as reactive oxygen

species (ROS), Ca^{2+}, salicylic acid (SA), ethylene (ET) and jasmonic acid (JA) (Nimchuk 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 et al., 2003).

The plant immune network can be subdivided into various defined sectors that can interact with each other (Sato et al., 2010, Tsuda et al., 2009). For example, distinct defense signaling sectors dependent on early MAMP-activated MAP kinases or the messenger molecules SA or JA, have been described for *Arabidopsis thaliana* (Arabidopsis).

While wild plant species have generally developed highly effective mechanisms to cope with pathogens, contemporary crops often have lost substantial parts of their innate immunity. Consequently, plant diseases cause dramatic losses in crop production. Global agriculture depends heavily on the use of pesticides to control such crop diseases. Pesticides typically rely on direct toxic, anti-pathogenic activity, which leads to undesirable ecological side effects (Casida, 2009). The disquiet over the dangers of pesticides has spawned considerable interest in alternative methods of disease control {Pimentel, 2005 #4948; Hart, 2005 #4949}. The use of plant defense inducing chemicals (plant activators, synthetic elicitors), which protect plants from diseases by activating their innate immune responses without the need of being toxic to pathogens, offers an attractive alternative for disease control regimes that can be environmentally friendly (Bektas and Eulgem, 2014). 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 et al., 1991, Uknes et al., 1992, Ward et al., 1991). Interactions of these compounds with the plant immune system have been well characterized and both INA and BTH are known to trigger a profile of defense-associated responses related to those triggered by SA-dependent signaling mechanisms (Bektas and Eulgem, 2014, Gorlach et al., 1996, Lawton et al., 1996, Ward et al., 1991).

We have initiated a chemical genomics-based approach to identify, characterize and utilize new types of synthetic elicitors for the dissection of the plant immune system and the development of novel types of environmentally safe pesticide alternatives (Knoth et al., 2009). By high-throughput chemical screening we identified 114 drug-like organic compounds that induce the pathogen-responsive *CaBP22*$^{-333}$*::GUS* reporter gene in transgenic Arabidopsis. One of them, 3-5-dicholoroanthranilic acid (DCA) triggered fast, strong and transient disease resistance against the pathogenic oomycete *Hyaloperonospora arabidopsidis* (*Hpa*) and the bacterial pathogen *Pseudomonas syringae* (Knoth et al., 2009). Experiments addressing the defense-inducing activity of DCA in various Arabidopsis defense mutants showed that this synthetic elicitor activates a signaling route partially dependent on the WRKY70 transcription factor. In contrast to INA- and BTH-mediated immunity, which is fully dependent on the transcriptional co-factor and SA co-receptor NPR1 (Cao et al., 1994a, Dong, 2004), DCA-mediated immunity is only weakly NPR1-dependent (Knoth et al.,

2009). In addition, immunity mediated by BTH and INA is long-lasting, while DCA acts transiently (Knoth et al., 2009). Thus, the mode-of action utilized by DCA in defense induction is distinct from that of INA and BTH.

Here we report on another representative of the 114 novel synthetic elicitors we identified, 2-(5-bromo-2-hydroxy-phenyl)-thiazolidine-4-carboxylic acid (BHTC). Like DCA, BHTC also induces disease resistance quickly and transiently. However its mode-of-action is distinct from that of DCA, as it strongly depends on NPR1. In addition, we found that low doses of BHTC enhanced elongation of Arabidopsis roots, while high concentrations inhibited root elongation. These effects are reminiscent of the phenomenon of hormesis, which has been described in various biological systems and which is characterized by enhanced biological performance in response to low doses of a wide range of stimuli that are toxic or otherwise detrimental at higher doses (Calabrese and Baldwin, 2002, Calabrese and Blain, 2011, Mattson and Calabrese, 2010). Interestingly we found transcriptional profiles triggered in Arabidopsis by low and high BHTC doses to be very different. In addition, the *wrky70-3* mutant, which exhibits reduced BHTC-mediated immunity, as well as the auxin-response mutants *axr1-3* and *slr-1* are compromised in BHTC-triggered root hormesis. Taken together, our results link plant defense signaling to hormetic developmental responses and provide a genetic and transcriptional framework for future studies on the mechanistic basis of plant hormesis.

Results

BHTC, a small molecule elicitor of *CaBP22*$^{-333}$::*GUS* expression and transient resistance of Arabidopsis to *Hpa*

We previously identified 114 compounds that reproducibly induce expression of the pathogen-responsive *CaBP22*$^{-333}$::*GUS* reporter gene in Arabidopsis (Knoth et al., 2009). One of them, 2-(5-bromo-2-hydroxy-phenyl)-thiazolidine-4-carboxylic acid (BHTC), has not been reported as a synthetic elicitor and has a chemical structure distinct from DCA or any other so far described plant defense inducers (Bektas and Eulgem, 2014, Knoth et al., 2009, Schreiber and Desveaux, 2008). BHTC activated reporter gene expression in one week-old *CaBP22*$^{-333}$*::GUS* Arabidopsis seedlings submerged in liquid growth medium at a concentration as low as 1 µM (Fig. 2.S1A). To examine if BHTC induces phytotoxicity, we stained *CaBP22*$^{-333}$*::GUS* seedlings after BHTC treatment with trypan blue. We observed dark blue staining indicating cell death in 100% of the seedlings treated for 24 h with 500 µM BHTC (Fig. 2.S1B). No cell death was observed at lower concentrations (1-100 µM), which resulted in *CaBP22*$^{-333}$::*GUS* activation, indicating that BHTC-induced phytotoxicity is not responsible for its effect on expression of this pathogen-responsive reporter gene.

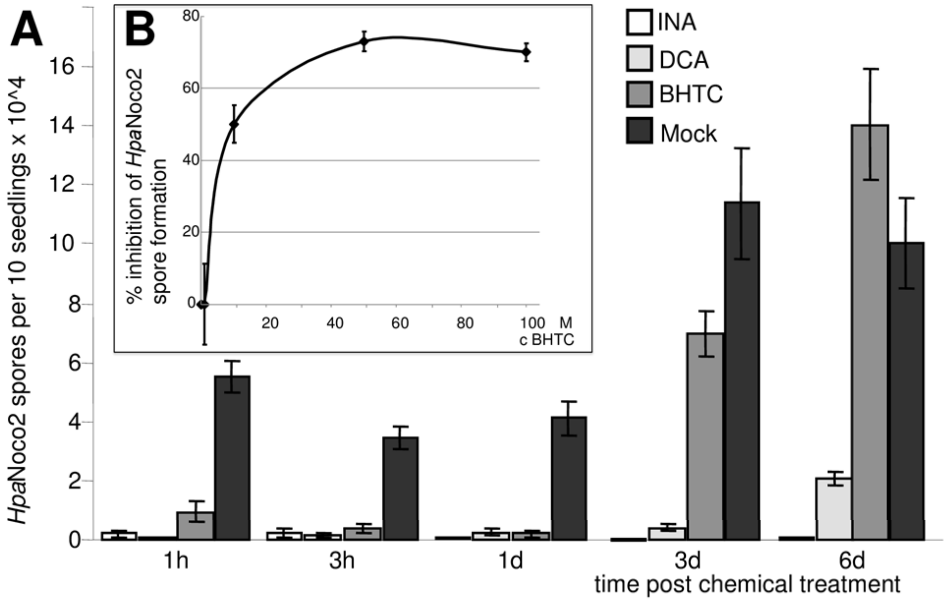


Figure 2.1. Kinetic and dose-response analysis of BHTC-induced immunity of Arabidopsis against *Hpa*.
(A) Kinetic analysis of chemically induced disease resistance. Three-week-old soil-grown Col-0 seedlings sprayed with 100 µM BHTC, DCA, INA, or mock solution (1% DMSO) at the indicated times prior to infection with 2 x 10^4 mL^{-1} *Hpa*Noco2 spores (2 mL per pot). *Hpa* spores were counted 7 days post infection. 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 elicitor treatments relative to the mock-treated control, except for 6 d post treatment with BHTC.
(B) Dose-response curve for BHTC mediated immunity of Arabidopsis against *Hpa*. Plotted is relative inhibition of *Hpa* spore formation versus the concentration of BHTC used in single foliar spray applications.

We further examined if BHTC, like DCA, has the ability to induce pathogen resistance in soil-grown plants. Single foliar-spray application of 100 µM BHTC 1 hour to 1 day prior to infection with the virulent *Hpa* isolate Noco2 significantly reduced numbers of *Hpa* spores by up to 73% (Fig. 2.1A & 2.1B). Maximal levels of immunity against *Hpa*Noco2 were observed with 50 µM to 100 µM BHTC (Fig. 2.1B). We estimated the median effective concentration (EC_{50}) of BHTC regarding its ability to protect Col-0 from *Hpa*Noco2 as 5.5 µM (Fig. 2.S2A). EC_{50}

values represent the concentration of a bioactive compound at which half-maximal biological activity is observed and reflect its potency regarding uptake and/or ability to interact with its target(s). Compounds with lower EC_{50} values are likely more efficiently taken up by biological systems and/or have a higher affinity for their targets than compounds with higher EC_{50} values. While DCA triggered higher levels of immunity, suppressing *Hpa*Noco2 formation in Col-0 by nearly 100% (Knoth et al., 2009), its estimated EC_{50} value of 6.5 µM (Fig. 2.S2B) regarding this response is slightly higher than that of BHTC.

We further compared the kinetics of defense induction in Col-0 seedlings sprayed once with 100 µM of BHTC, DCA or INA at various time points prior to pathogen challenge (Fig. 2.1A). Mock treatment itself diminished spore growth when time points between pathogen challenge and elicitor pre-treatment were less than one day apart. This effect may be due to residual liquid coating Arabidopsis seedlings before being sprayed with the *Hpa* spore suspension. Already at 1 h post treatment (hpt) to 3 hpt all three tested synthetic elicitors strongly suppressed *Hpa* spore production. However, at 3 days post treatment (dpt) BHTC-triggered immunity to *Hpa*Noco2 was reduced and no effect of this compound on immunity was detectable at 6 dpt. As reported previously, DCA also induces plant defense transiently (Knoth et al., 2009), while the activity of INA is long-lasting (Bowling et al., 1997, Gorlach et al., 1996, Métraux et al., 1991). Based on our data, the defense-inducing activity of BHTC is even more

transient than that of DCA. Taken together, BHTC, like DCA, is a fast, potent, but reversible inducer of Arabidopsis immunity against *Hpa*Noco2.

Structure activity analysis with BHTC derivatives

To determine which substituents or moieties of the BHTC molecule are critical for its defense-inducing activity, seven commercially available BHTC derivatives were analyzed that differed only minimally from the original synthetic elicitor structure (Fig. 2.2A). We tested the ability of these compounds, next to DCA and BHTC, to inhibit *Hpa*Noco2 spore development in Col-0 plants after a single foliar-spray application (Fig. 2.2B). DCA and BHTC provided highest protection against *Hpa*Noco2 infection significantly suppressing *Hpa* spore formation at 10 µM and 100 µM and reaching levels of over 70% protection. BTC (2-(5-bromo-phenyl)-thiazolidine-4-carboxylic acid) and BMTC (2-(5-bromo-2-methoxy-phenyl)-thiazolidine-4-carboxylic acid) mediated at one of the tested concentrations significant levels of intermediate spore reduction. Compared to BHTC, BTC lacks the hydroxy group of the phenyl moiety, while this substituent is replaced by a methoxy group in BMTC. HTC (2-(2-hydroxy-phenyl)-thiazolidine-4-carboxylic acid), which lacks the bromine of the phenyl moiety, induced only low, but not significant, levels of spore suppression at 10 µM and 100 µM. However, we observed significant levels of spore suppression with this compound at a concentration of 200 µM (Fig. 2.S3). PTC (2-phenyl-thiazolidine-4-carboxylic acid) did not mediate any protection against *Hpa*Noco2. Thus,

substitution of the phenyl moiety of phenylthiazolidine-4-carboxylic acid derivatives seems to be critical for their ability to induce plant immune responses. The isolated thiazolidine-4-carboxylic acid moiety of BHTC, T4CA (4-carboxy-4-thiazolidinyl), as well as CMP389, which consists of a phenyl moiety with two 4-carboxy-4-thiazolidinyl substituents also did not mediate significant protection against *Hpa*Noco2. Interestingly, 2BP (5-bromo-2hydroxy-phenyl), which consists only of the substituted phenyl moiety of BHTC, was sufficient to trigger some protection against *Hpa*Noco2. However, significant levels of immunity were only observed at one of the tested concentrations (1 µM) and levels of *Hpa* spore suppression did not exceed 50%. Except for 2BP, all other tested BHTC derivatives that induced significant protection of Col-0 against *Hpa*Noco2, also triggered GUS expression in our *CaBP22*$^{-333}$*::GUS* reporter gene assays at 100 µM or lower concentrations (not shown). Thus, 2BP seems to be a weaker and less reliable plant defense inducer than BHTC. Compared to its tested derivatives, BHTC seems to be the most robust and efficient synthetic elicitor. Therefore, we used this compound as a representative for the new class of 2-phenyl-thiazolidine-4-carboxylic acid (PTC) synthetic elicitors for all further experiments in this study.

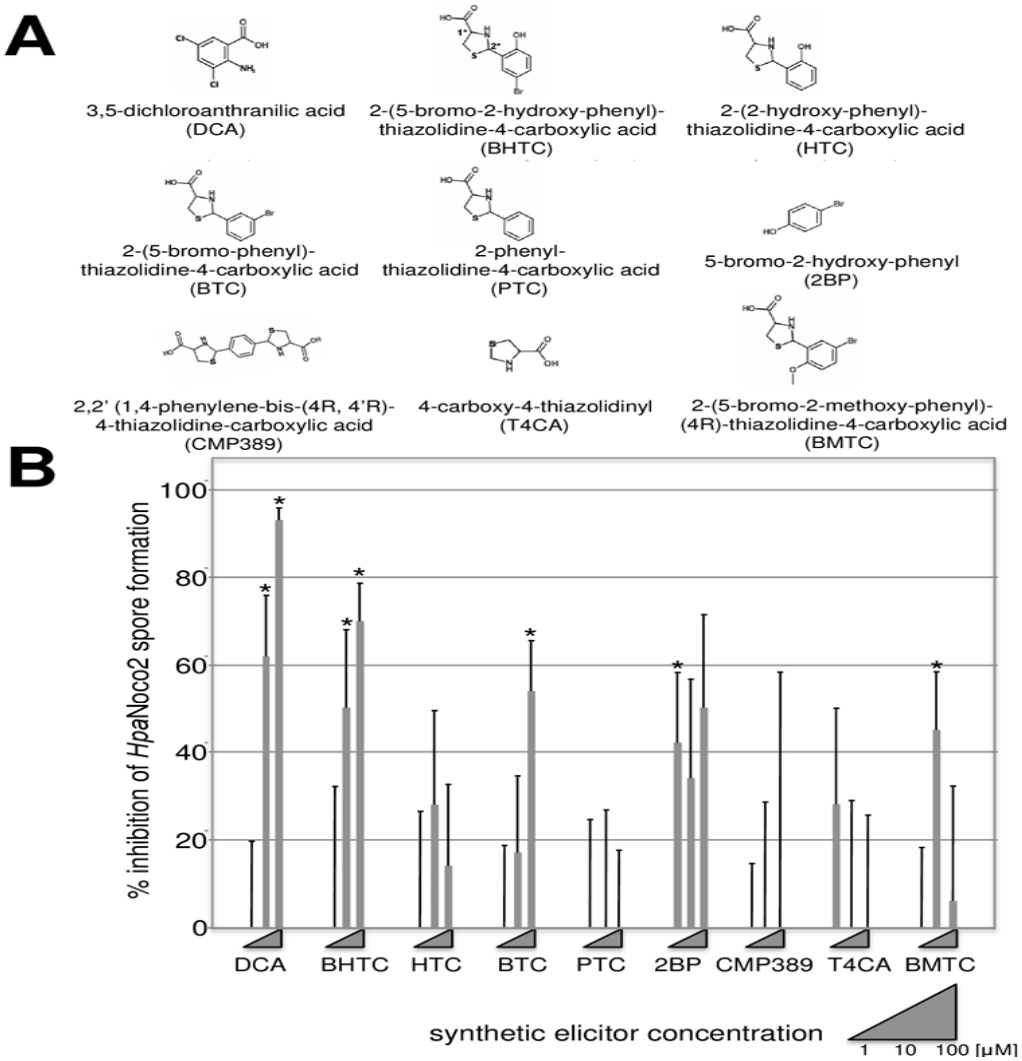


Figure 2.2. Structure-activity analysis of BHTC analogs.
(**A)** Chemical structures of DCA, BHTC and tested BHTC derivatives. Chiral centers of the BHTC skeleton are indicated by "1*" and "2*" in HTC.
(B) *Hpa*Noco2 spore inhibition assay. Three-week-old soil-grown Col-0 seedlings were spray-infected 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 those three replicates is shown above. Significant differences of compound-treated compared to mock-treated seedlings determined by Student's *t*-tests ($p<0.05$) are marked by asterisks.

BHTC is functionally distinct from DCA

DCA and BHTC differ regarding the timing of their defense-inducing activity. While both synthetic elicitors trigger transient protection of Col-0 against *Hpa*Noco2, immunity mediated by BHTC is of even shorter duration than that triggered by DCA (Fig. 2.1A). To genetically establish whether the mode-of-action of BHTC differs from that of DCA, we tested the defense-inducing activity of this new synthetic elicitor in various Arabidopsis mutants after a single foliar-spray application. We previously reported that full immunity mediated by DCA requires both NPR1 and the WRKY70 transcription factor (Knoth et al., 2007, Knoth et al., 2009). However, the dependency of DCA on WRKY70 is more pronounced than that on NPR1. While BHTC triggered significant levels of immunity against *Hpa*Noco2 in Col-0 plants as well as the *sid2-2*, *pad4-1* and *wrky72-2* mutants, no significant protection of the *npr1-3* and *wrky70-3* mutants against this pathogen was observed (Fig. 2.3A). The *sid2-2* and *pad4-1* mutants are compromised in the defense-associated accumulation of SA (Feys et al., 2001; Wildermuth et al., 2001). We previously reported the *wrky72-2* mutant to be deficient in signaling processes that seem independent of SA (Bhattarai et al., 2010). Based on this, BHTC, like DCA (Knoth et al., 2009), appears to interfere with signaling processes operating downstream from SA and to require NPR1 as well as WRKY70 for defense induction. A critical difference between BHTC and DCA, however, seems to be their level of NPR1 dependency. In contrast to DCA, which can trigger significant levels of immunity against *Hpa*Noco2 in the *npr1-3*

mutant (Knoth et al., 2009), BHTC is unable to provide significant protection against this pathogen in *npr1-3* plants (Fig. 2.3B).

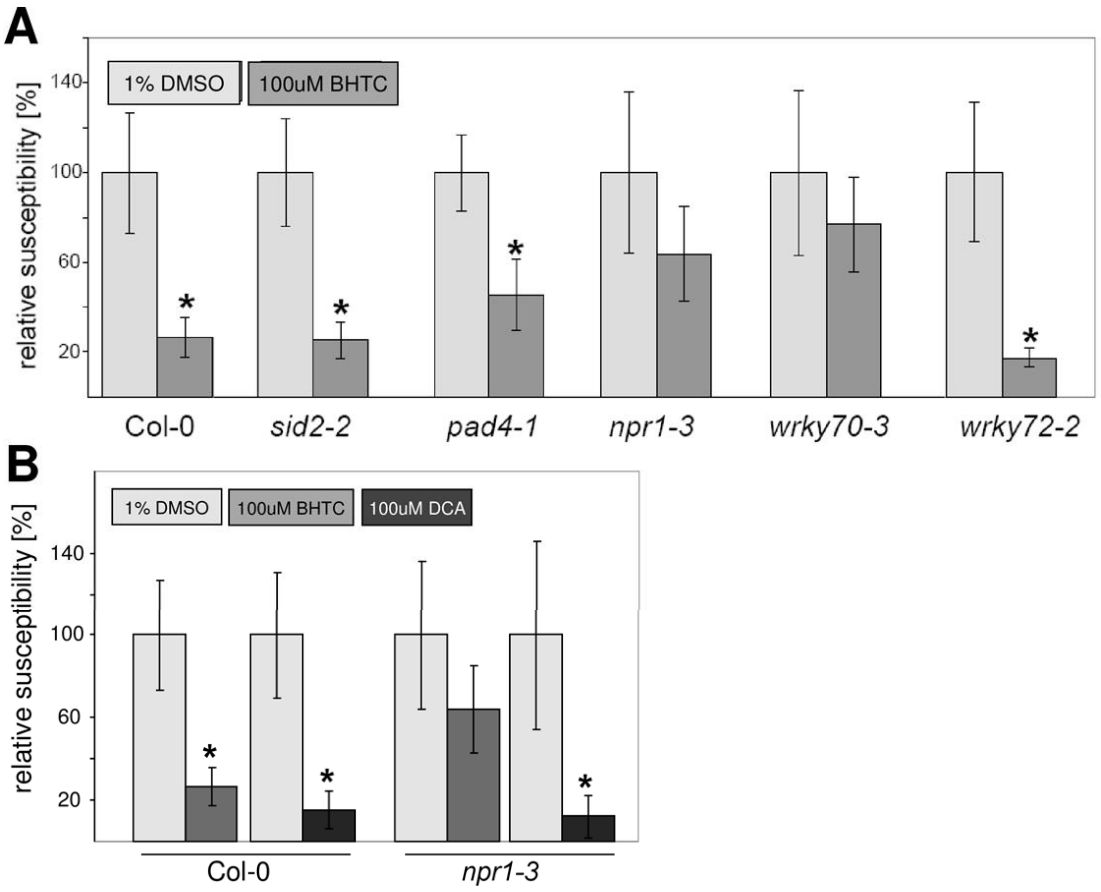


Figure 2.3. Analysis of BHTC activity in known Arabidopsis defense mutants.
(A, B) Analysis of BHTC activity in Arabidopsis Col-0 or Col-0 defense mutants. Experiments were conducted with three-week-old soil-grown seedlings sprayed with 100 µM BHTC, 100 µM DCA, or mock-solution (1% DMSO) 24 h prior to infection with 3 x 10^4 virulent *Hpa*Noco2 spores mL^{-1} (2 mL per pot). Spores were counted at 7 dpi. Shown are relative numbers of spores per seedling compared to values obtained with the respective mock-treated controls. Error bars represent the standard error of the mean based on at least 6 independent biological replicates. Spore numbers that are significantly reduced based on Student's *t*-tests (p<0.05) are marked by asterisks.

BHTC can provide disease protection in a variety of plant pathogen interactions

We further tested if BHTC can mediate disease protection in additional plant pathogen interactions. Like DCA and INA, BHTC significantly reduced growth of the virulent bacterial pathogen *P. syringae* pv. *tomato* (*Pst*) strain DC3000 growth in Arabidopsis after a single foliar spray application at a concentration of 100 µM (Fig. 2.4A). To determine the direct antibacterial activity of BHTC, we monitored the growth of *Pst* in liquid medium containing 100 µM BHTC, DCA, INA or the antibiotic hygromycin (Fig. 2.4B). None of the tested synthetic elicitors reduced bacterial growth, while hygromycin completely eliminated growth of the bacteria. Taken together, these data show that BHTC can protect Arabidopsis against *Pst* by inducing plant defense reactions and not by direct toxicity against these bacteria.

We further tested the effects of BHTC on the compatible interaction of tomato (*Solanum lycopersicum)* cv. Moneymaker with *Pst*. At 3 dpi, tomato plants treated with 50 µM BHTC exhibited significantly reduced numbers of colony-forming units of *Pst* compared to mock-treated control plants (Fig. 2.4C). Similarly, BHTC mildly, but significantly suppressed the development of disease symptoms in the legume cowpea (*Vigna unguiculata)* cv. California Blackeye 5 (CB5) after infection with the fungal pathogen *Fusarium oxysporum* f. sp. *tracheiphilum* race 3 (*Fot*3) (Fig. 2.4D).

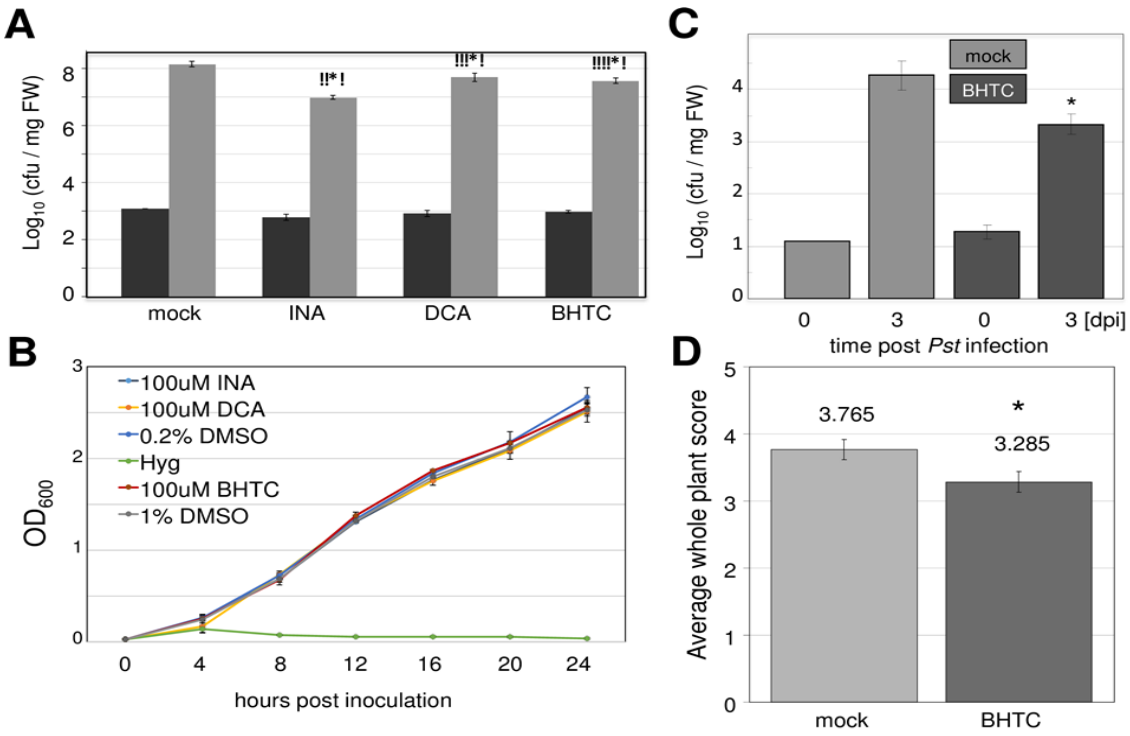


Figure 2.4. BHTC induces defense reactions in multiple plant species against diverse pathogens.

(A) Quantification of *Pst* DC3000 growth on 2 week-old Arabidopsis Col-0 plants by colony forming units (cfu). Col-0 seedlings were pre-treated with 100 µM of indicated chemical or mock solution (solvent only) 24 h prior to dip-inoculation with virulent *Pst* DC3000 (OD_{600}= 0.005). Bacterial titer was evaluated at day 0 (black bars) or day 3 (grey bars). Significant differences were determined using Student's *t*-tests (p<0.05). The shown data represent a typical example of five nearly identical biological replicates. FW = fresh weight.

(B) *Pst* DC3000 grown in liquid culture with 100 µM of the indicated chemicals or 100ug/ml hygromycin (Hyg). The OD_{600} which represents the density of bacteria was measured at indicated times (hours) after inoculation. Error bars represent the standard error of the mean based on at least 3 independent replicates.

(C) Tomato plants cv. Moneymaker root drenched with 50 µM BHTC display lower levels of *Pst* growth in leaves relative to mock-treated (solvent only) plants three days post infection, n = 4, Student's *t*-test p = 0.027.

(D) Cowpea plants sprayed with BHTC exhibit reduced severity of Fot3 pathogenic fungus-induced disease symptoms. Whole plant scores were rated on a scale of zero to five, based on the percentage of the plant that displayed *Fot*3-induced symptoms including: chlorosis, wilting, vascular discoloration and tissue necrosis. 0 = no disease symptoms, 1 = 10%, 2 = 25%, 3 = 50%, 4 = 75%, 5 = 100%, n = 100, p = 0.018. Differences between BTHC- and mock-treated (solvent only) plants were determined by Student's *t*-tests (p<0.05). Data shown is representative of at least three independent experiments. Error bars represent standard errors of the means. cfu = colony forming units, FW = fresh weight. BHTC was applied to the aerial portions of cowpea plants cv CB5 24 h prior to inoculation with *Fot*3 and then biweekly for five weeks. The dosage was increased from 2 mL of 100 µM BHTC to 4 mL of 1 mM BHTC (or the corresponding mock treatment) per plant over time as plant maturity allowed higher tolerance of the solvent DMSO. While the effect of BHTC was only moderate in each case, it was highly reproducible over multiple biological replicates.

BHTC induces hormesis-like responses in Arabidopsis roots

Surprisingly, we found BHTC at concentrations below 1 µM to significantly enhance the root length of Arabidopsis plants grown on BHTC-laced ½MS agar plates, while higher doses of BHTC resulted in reduced root length (Fig. 2.5). In contrast to our *Hpa* defense assays (see above), plants were continuously exposed to BHTC in our root growth assays. Enhancement of root length on low BHTC doses was often detectable within the first three days after germination of the seedlings. However, the timing of this effect varied substantially between different biological replicates. Therefore we measured root lengths at five different time-points (typically at days 3, 5, 7 11 and 14) over a total experimental duration of 14 days and calculated the average change of root length per day over the course of the experiment (Fig. 2.5). Although we observed in some replicates an increase in root length by up to 80%, in most data sets the effect was more moderate averaging to an increased root length of about 20%. The results shown in Figure 2.5 represent a typical biological replicate.

The observation that low doses of BHTC stimulate root elongation while high doses of this compound reduce root length, is reminiscent of the known phenomenon of hormesis, which has been described for a large variety of physical and chemical stimuli in numerous organisms including humans (Calabrese and Baldwin, 2002, Calabrese and Blain, 2011, Mattson and Calabrese, 2010). Hormesis is generally characterized as low-dose stimulation and high-dose inhibition of biological responses.

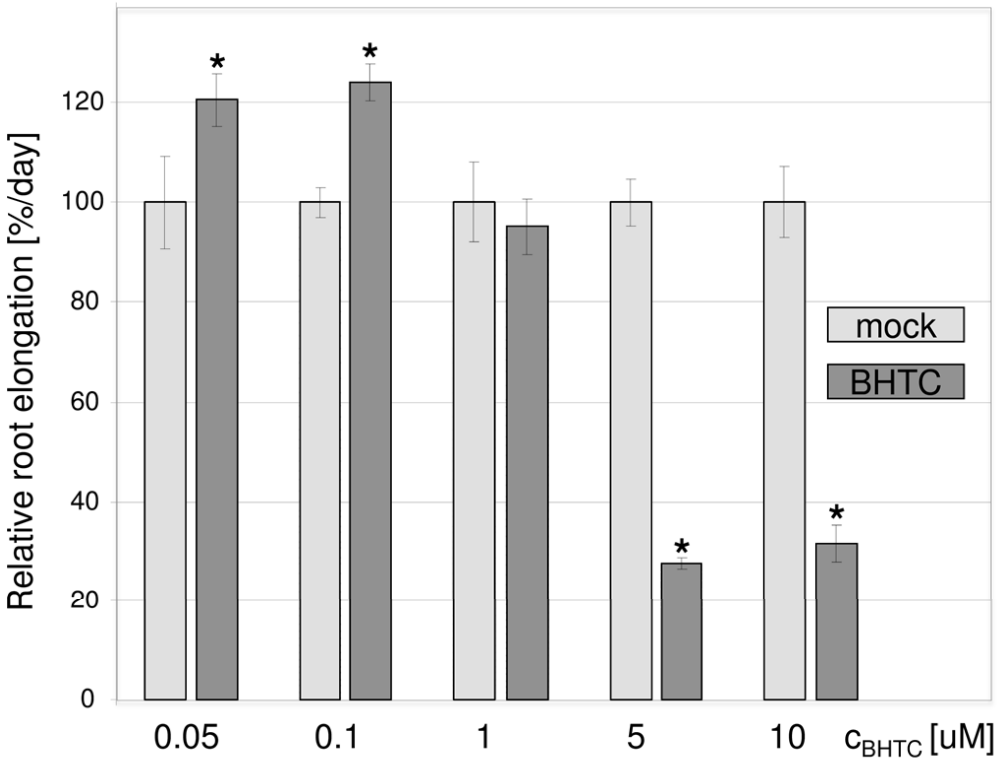


Figure 2.5. Relative root length of Col-0 plants grown on BHTC.
Col-0 seedlings were grown on ½MS medium containing the indicated concentrations of BHTC, or the respective controls (solvent only). Root length was measured at five different time-points, typically at days 3, 5, 7 11 and 14, over a total experimental duration of 14 days and the average relative change of root length per day compared to the respective mock-treated controls is shown. The shown results represent typical examples of at least three biological replicates. Significant differences between BTHC- and control-treated plants were determined by Student's *t*-tests (p<0.05) and are marked by asterisks.

Dose-dependency of BHTC-triggered transcriptome changes

In order to uncover transcriptional patterns associated with defense activation or hormesis-induction, we profiled by mRNA-seq responses triggered in 14 d-old plate-grown Arabidopsis seedlings by continuous exposure to a high-dose (hd) of 5 µM BHTC or a low-dose (ld) of 0.1 µM BHTC. These conditions were chosen because continuous exposure to 5 µM BHTC resulted in strong activation of *CaBP22^{-333}::GUS* reporter gene and suppression of root elongation

in Arabidopsis, while the same kind of application of 0.1 µM BHTC did not induce expression of this pathogen-responsive reporter and stimulated enhanced root elongation (Fig. 2.5 & 2.S1). As controls we used mock treatment (solvent only). For each treatment type, root and shoot tissues were separately analyzed. We performed two independent biological replicates for each experimental condition and sequenced the respective libraries using the Illumina HiSeq2500 platform. Differentially expressed genes (DEGs) were identified by comparing read counts from BHTC-treated samples versus their respective mock controls using a Bonferroni-corrected false discovery rate (FDR)-cut off of 0.05 (Table 2.1, Table 2.S1).

Gene Set	Number of genes in set	Enriched GO terms* (with p values)
hd-BHTC-shoots-up	445	response to stress (p=3.562e-73); response to abiotic or biotic stimulus (p= 1.187e-55); signal transduction (p=6.848e-48); other biological processes (p= 1.133e-38); transport (p= 2.520e-21);
hd-BHTC-shoots-down	54	response to abiotic or biotic stimulus (p=6.084e-04); response to stress (p=4.145e-03); protein metabolism (p=4.965e-03); unknown biological processes (p=9.490e-03; cell organization and biogenesis (p=9.584e-03);
hd-BHTC-roots-up	26	signal transduction (p=6.546e-03); other cellular processes (p= 0.026)
hd-BHTC-roots-down	10	other metabolic processes (p=0.029)
ld-BHTC-shoots-up	34	developmental processes (p=4.062e-05); other cellular processes(p=8.555e-04); other biological processes (p=1.359e-03); response to stress (p=4.527e-03); transport (p=4.699e-03) cell organization and biogenesis p= 0.025);
ld-BHTC-shoots-down	132	electron transport or energy pathways (p=2.718e-80); DNA-dependent transcription (p=2.054e-21); other metabolic processes (p=5.610e-14); other cellular processes (p=1.218e-07);
ld-BHTC-roots-up	1	-
ld-BHTC-roots-down	51	electron transport or energy pathways (p=1.656e-18); other metabolic processes (p=1.901e-06); DNA-dependent transcription, (p=7.534e-05); unknown biological processes(p= 1.549e-04); other cellular processes (p=9.358e-04); developmental processes (p=7.095e-03);

Table 2.1: Set of Arabidopsis genes significantly differentially expressed in response to low- or high-dose BHTC treatment in plate-grown Col-0 seedlings.
* listed are all significantly enriched GO terms regarding the biological function based on the Botany Array Resource classification super viewer (http://bar.utoronto.ca/welcome.htm)

A total of 499 genes exhibited significantly altered transcript levels in shoots after hd-BHTC treatment, with 445 of these DEGs up-regulated (hd-BHTC-shoots-up) and 54 down-regulated (hd-BHTC-shoots-down). In roots the number of DEGs was substantially lower (35 DEGs) with 25 up- (hd-BHTC-roots-up) and 10 down-regulated genes (hd-BHTC-roots-down). The hd-BHTC treatment in shoots resulted in a typical defense-associated transcript profile, including transcript up-regulation of standard defense marker genes, such as *PR1*, *PR5*, *CaBP22* and *LURP1* as well as numerous genes encoding WRKY transcription factors and disease resistance protein family members (Table 2.S1). Highly significantly enriched gene ontology (GO) terms in the hd-BHTC-shoots-up set calculated by the Botany Array Resource classification super viewer [http://bar.utoronto.ca/welcome.htm; (Toufighi et al., 2005)] suggested collective roles of these genes in responses to “stress” and “abiotic/biotic stimuli” as well as “signal transduction” (Table 2.1). Consistent with a role in defense, 1000 bp upstream sequences of the hd-BHTC-shoots-up gene set are highly enriched for known defense-associated promoter motifs. According to the TAIR motif analysis tool (https://www.arabidopsis.org/tools/bulk/motiffinder/index.jsp), the hexameric motifs TTGACT (p=4.25e-19) and TTGACC (p=2.51e-06), that match the WRKY-binding W box element (TTGACC/T) (Eulgem et al., 2000), as well as the TGACGT hexamer (p=6.23e-08) containing the TGA box core motif (TGACG) (Eulgem, 2005) are significantly over-represented in these promoter regions.

Furthermore, many genes of the hd-BHTC-shoots-up set are responsive to pathogen infection with 66% (294 of all 445 genes) being up-regulated in Arabidopsis after infections with the oomycete *Hpa* (Bhattarai et al., 2010), the bacterium *P. syringae* (Thilmony et al., 2006) and/or the powdery mildew fungus *Erysiphae orontii* (Pandey et al., 2010) (Fig. 2.6A). In addition, 65% (290) of all 445 hd-BHTC-shoots-up members are inducible by the SA analogs DCA, INA and/or BTH (Knoth et al., 2009, Wang et al., 2006) (Fig. 2.6B), suggesting that BHTC also mimics some SA functions and acts as a partial agonist of this defense hormone.

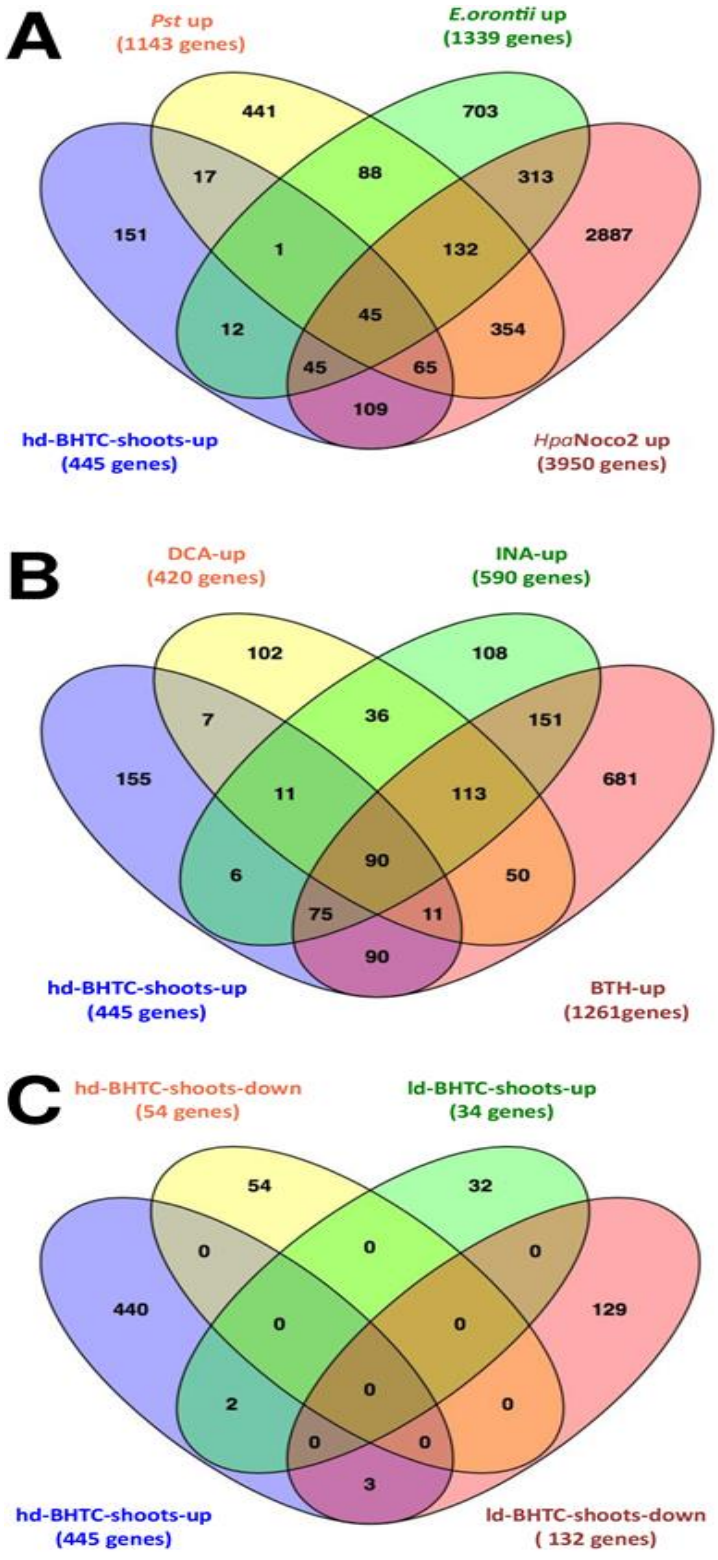


Figure 2.6. **Arabidopsis gene sets responsive to low- and high-dose BHTC treatments differ profoundly.** Four-way Venn diagram analysis highlighting commonalities and differences between the gene set induced by a high dose (hd) of BHTC and various pathogens (**A**), other SA analogs (**B**) and low-dose (ld) BHTC treatment (**C**). High-dose BHTC treatment triggers typical defense-associated transcriptome changes that are qualitatively different from responses triggered by low-dose BHTC treatment. **(A)** Sets of genes up-regulated by *Pst*, *Erysiphae orontii* and *Hpa*, are from (Thilmony et al., 2006), (Pandey et al., 2010) and (Bhattarai et al., 2010), respectively. **(B)** Sets of genes up-regulated by DCA and INA or BTH are from (Knoth et al., 2009) and (Wang et al., 2006), respectively. **(C)** See table 2.S1 for details about gene sets shown in this panel.

The set of hd-BHTC-roots-up genes is substantially smaller (only 25 genes) and features no strongly enriched GO terms. However, like hd-BHTC-shoots-up, this set also contains several canonical defense genes, including *CaBP22* and *WRKY* genes and promoters of this set are enriched for the two W box derivatives TTGACT (p=1.99e-02) and TTGACC (p=1.13e-01) as well as the TGA box core containing hexamer TGACGT (p=1.00e-02). Thus, a collective role of hd-BHTC-roots-up members in defense is likely. Genes down regulated by hd-BHTC treatment in shoots or roots are not strongly enriched for any informative GO terms and common biological roles of any of these two gene sets are unclear (Table 2.1).

Responses triggered by the ld-BHTC treatment in shoots and roots were in stark contrast to those triggered by the high BHTC dose. A set of 166 genes was found to be differentially expressed after ld-BHTC treatment in shoots. This set can be subdivided in 34 genes that exhibit transcriptional up-regulation after 0.1 □M BHTC (ld-BHTC-shoots-up) and 132 genes that are transcriptionally down-regulated by this treatment (ld-BHTC-shoots-down; Table 2.S1). The most strongly enriched GO term of the ld-BHTC-shoots-up set is “developmental processes” (p=4.062e-05). While all other BHTC-responsive gene sets we identified exclusively feature nuclear genes, genes down-regulated by the ld-BHTC treatment in shoots and roots consist of nuclear, chloroplast-resident and mitochondrial genes (Table 2.S1). The set of 132 ld-BHTC-shoots-down genes

can be nearly evenly subdivided into 33 nuclear, 53 chloroplast-resident and 44 mitochondrial genes (Table 2.S1). All three of these subsets are strongly enriched for genes encoding proteins involved in “electron transport or energy pathways”, “DNA-dependent transcription”, “other metabolic processes” or “other cellular processes’ (Table 2.1). Particularly strongly represented are genes involved in photosynthetic or respiratory energy production, such as components of the photosynthetic and respiratory electron transport chains, ATPases, Rubisco or components of the photosynthetic reaction centers (Table 2.S1).

As for hd-BHTC treatment, ld-BHTC treatment resulted in roots in a smaller set of transcriptional changes, with only 1 gene significantly up-regulated (AT3G15450) and 51 genes significantly down-regulated (ld-BHTC-roots-down; Tables 2.1 and 2.S1). The response to ld BHTC treatment qualitatively resembles very much the response triggered by a low dose of this compound in shoots. The set of ld-BHTC-roots-down genes also features nuclear, chloroplast-resident and mitochondrial genes. Furthermore, as in the case of ld-BHTC-shoots-down genes, genes involved in photosynthetic and respiratory energy production are strongly represented among ld-BHTC-roots-down genes and significantly enriched GO terms of this set are “electron transport or energy pathways”, “DNA-dependent transcription”, “other metabolic processes” and other “cellular processes “. Of the 51 ld-BHTC-roots-down genes, 20 (39%) are also present in the ld-BHTC-shoots-down set.

Taken together, two clearly recognizable trends of BHTC-induced transcriptional changes in both shoots and roots are (1) the up-regulation of typical defense genes by the hd-treatment with this compound and (2) a coordinated inter-compartmental response triggered by ld-BHTC treatment manifested in the suppression of photosynthesis- and respiration-related genes in the nucleus, chloroplasts and mitochondria. While it is unclear how the collective differential expression of ld-BHTC-responsive genes may contribute to hormesis mediated by low doses of this compound, it is striking that transcriptional responses triggered by a low dose of BHTC are qualitatively entirely distinct from the responses we observed after treatment with a high BHTC dose (Fig. 2.6C).

BHTC-mediated root hormesis partially depends on the defense regulator WRKY70 as well as the auxin-related signaling components AXR1 and SLR

Auxin is known to trigger hormetic growth effects in roots and the Arabidopsis *axr1-3* auxin-response mutant has been reported previously to exhibit a reduction in enhanced root growth induced by low doses of the auxin indole-3-acetic acid (IAA) (Evans et al., 1994). Therefore we tested several Arabidopsis auxin-related mutants for BHTC-triggered hormetic effects. As expected, continuous exposure to 0.1□M BHTC triggered enhanced elongation of roots in plate grown Col-0, while an intermediate BHTC dose of 1□M had no effect and a high dose of 10□M triggered a severe suppression of root elongation

(Fig. 2.7A). In the *tir1-1*, *msg2-1 axr2-T* and *axr5-1* auxin response mutants this profile was largely unchanged. However, the *axr1-3* and *slr-1* mutants exhibited a significantly altered BHTC-response profile. In both cases the positive growth response to 0.1 □M BHTC was significantly reduced (or even reverted), while the variability of root growth after hd BHTC treatment was extremely enhanced in *axr1-3* plants. We further tested several Arabidopsis defense mutants for BHTC-induced root hormesis. BHTC-induced growth enhancement of roots was unaffected in the tested mutants when this compound was applied at a concentration of 0.1□M (not shown). However, when exposed to 0.05□M BHTC we observed a significant reduction of BHTC-induced root hormesis in the *wrky70-3* mutant compared to Col-0 (Fig. 2.7B). While a similar trend was also observed with the *npr1-3* mutant, this effect was not statistically significant at the 5% level. Strikingly, both the *wrky70-3* and *npr1-3* mutants are also compromised in BHTC-mediated immunity against *Hpa* (Fig. 2.3).

Although *axr1-3* and *slr-1* plants were compromised in the BHTC-induced hormetic response, we did not observe any reduction in BHTC-mediated resistance to *Hpa* in these auxin-response mutants (Fig. 2.S4A). However, we unexpectedly found basal defense to this pathogen to be reduced in *axr1-3* and *slr-1* plants (Fig. 2.S4B).

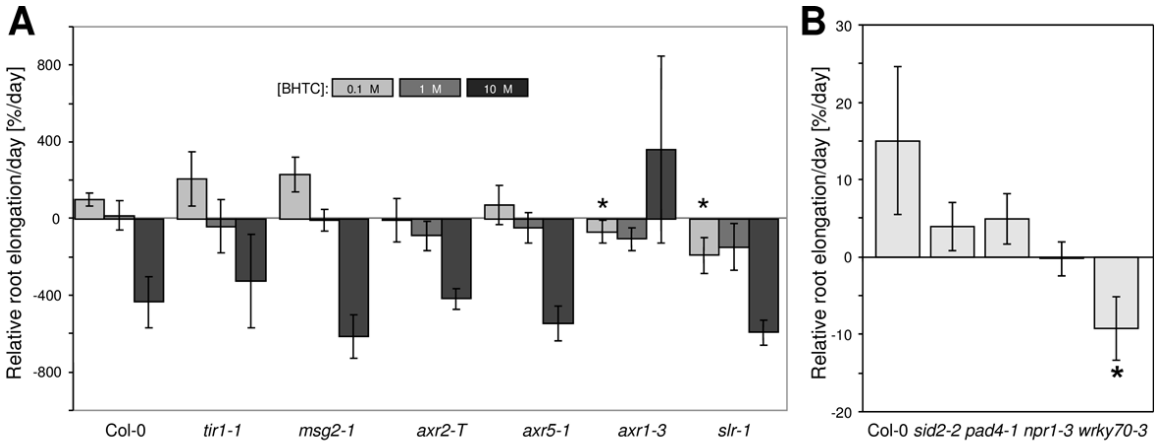


Figure 2.7: The Arabidopsis *axr1-3*, *slr-1* and *wrky70* mutants are compromised in hormetic root elongation by low BHTC doses. (A, B) Relative root elongation per day of Arabidopsis seedlings of the indicated genotypes grown on 0.1□M, 1□M or 10□M BHTC **(A)** or 0.05□M BHTC **(B)**. Relative root elongation was determined as in experiments shown in Figure 2.5. For each BHTC dose significant differences between Col-0 and mutant plants were determined by Student's *t*-tests ($p<0.05$) and are marked by asterisks.

Discussion

Besides the benzoic acid derivative DCA, our chemical screen for inducers of *CaBP22*$^{-333}$*::GUS* in Arabidopsis (Knoth et al., 2009) led to the identification of the 2-phenyl-thiazolidine-4-carboxylic acid (PTC) derivative BHTC as a new synthetic elicitor. To our knowledge, compounds of this class have not been described as plant defense inducers. While plant-based studies on PTCs seem not available, studies in other biological systems have shown some of these compounds to have anticancer, antioxidant, or antimicrobial activities (Alhamadsheh et al., 2006, Ferrandez et al., 1999, Sriharsha et al., 2007, Wlodek et al., 1996). None of these studies, however, examined the effect of PTCs on plant pathogens. The diversity of biological activities of PTCs suggests that these compounds are highly suitable for interactions with a wide range of different cellular targets. Although some PTCs were shown to have antimicrobial activities, BHTC clearly provided disease protection in the tested interactions with *Pst* by inducing plant immune responses and not by having direct biocidal effects against the this pathogen. *Hpa* is a strict biotroph and cannot be grown *in vitro*. Thus, it was not possible to test direct effects of BHTC against this pathogen. However, suppression of *Hpa* growth in Arabidopsis required the plant immune system to be intact. In addition, application of BHTC induced a typical defense-associated transcriptional profile. Hence, BHTC can protect plants against microbial diseases by stimulating natural plant immunity.

Both major moieties of BHTC are necessary for strong elicitor activity, as neither the 4-carboxy-4-thiazolidinyl portion, nor the 5-bromo-2-hydroxy-phenyl portion robustly induced immunity in our assays. While changes of the substituents of the phenyl group resulted in reduced elicitor activity, the PTC-derivatives, HTC, BTC and BMTC that carry at the phenyl group, at least one substituent distinct from the thiazolidine group, still significantly suppressed the formation of *Hpa*Noco2 spores in Arabidopsis. Thus, phenyl-substituted PTCs can be considered a novel class of synthetic elicitors. Of those PTCs tested in our study, BHTC is the most potent and robust plant defense inducer.

All synthetic elicitors identified by our previous chemical screen should induce a common set of defense reactions, which include transcriptional activation of the *LURP* gene cluster, including *CaBP22,* and other responses known to be dependent on SA (Knoth et al., 2009). Nonetheless, we found DCA and BHTC to employ different modes-of action, as their defense-inducing activities differ in the Arabidopsis *npr1-3* mutant. While DCA-mediated immunity is only weakly *NPR1* dependent, no significant levels of disease resistance could be observed in *npr1-3* plants after BHTC application. In contrast to other *NPR1*-dependent synthetic elicitors/plant activators (e.g. INA or BTH), which mediate long-lasting defense induction after a single application, BHTC-treatment resulted only in transient immunity under these conditions. Thus, the mode-of action of BHTC seems to differ from those of INA or BTH as well. A comparison of the transcriptional profiles induced by DCA, INA, BTH or BHTC suggested that all

four compounds act as SA analogs and induce related, yet partly distinct, subsets of transcriptional changes. Thus, with DCA, BHTC and INA or BTH, a set of synthetic elicitors is available that can be used to study distinct aspects of immune responses and regulatory processes associated with the defense hormone SA.

A major strategy of disease control in agriculture and horticulture has been the use of pesticides. Chemical pesticides typically rely on direct antibiotic or biocidal activity, which often leads to undesirable toxic environmental side effects (Gilliom et al., 2007, Kessmann et al., 1994). Synthetic elicitors, however, can protect plants by inducing their natural immune responses, do not rely on toxic effects and are therefore attractive alternatives to conventional pesticides (Bektas and Eulgem, 2014). 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 INA was insufficiently tolerated by some crop plants to warrant practical use as a plant protection compound (Ryals et al., 1996). However, we found DCA and BHTC to be promising in this respect as their defense inducing activity is only transient and weaken within several days after application (Fig. 2.1) (Knoth et al., 2009).

Both DCA and BHTC have similar EC_{50} values regarding their ability to protect Arabidopsis against *Hpa* (6.5 □M and 5.5 □M, respectively). This

suggests that both compounds are equally potent with respect to their uptake, stability *in planta* and/or affinity to their cellular targets (Katzung, 2007). However, we observed maximal inhibition of *Hpa* growth in Arabidopsis to be 100% with DCA, while BHTC can only reduce growth of this pathogen by 73%. Thus, defense reactions induced by DCA appear to be more efficient than those induced by BHTC.

Despite its clear ability to protect Arabidopsis against diseases, the efficiency of BHTC in the tested crop systems seems only weak. While we detected a significant reduction of pathogen growth or disease symptoms in tomato and cowpea, respectively, these effects were quantitatively only marginal and higher BHTC doses compared to Arabidopsis were necessary. Possibly uptake, compound stability and/or target affinity of BHTC may be weaker in these crop species. Testing of additional phenyl-substituted PTC derivatives in tomato or cowpea may lead to the identification of synthetic elicitors better suited for crop protection than BHTC. Nonetheless, BHTC seems to induce defense reactions in multiple plant species that are effective against phylogenetically distinct types of pathogens. The oomycete *Hpa* and the bacterium *Pst* typically infect and reproduce in shoot tissues whereas *Fot*3, a soil-borne fungus, invades plants through the roots, entering the shoot through vascular tissue, causing disease symptoms (Pietro et al., 2003, Slusarenko and Schlaich, 2003, Zeng et al., 2011). Accordingly, synthetic elicitors that promote broad spectrum disease

resistance in crop plants can have a potential application that is far more efficient than the use of pesticides that target one type of pathogen.

Unexpectedly, we found continuous BHTC exposure to trigger hormetic effects in Arabidopsis. While high doses of BHTC activated defense gene expression and strongly reduced root length, low doses of this compound stimulated root elongation. We also found several other synthetic elicitors as well as SA to trigger similar hormetic effects (not shown). However, of those compounds we tested, BHTC is the most efficient one in this respect. Hormesis (*Greek for excite*) is characterized by low-dose stimulation and high-dose inhibition of biological performance parameters, such as growth, metabolic rate or stress tolerance, often resulting in “inverse U"-shaped dose-response curves instead of the sigmoid curves predicted by standard pharmacological threshold models (Calabrese and Baldwin, 2002, Calabrese and Baldwin, 2003). Hormetic phenomena have been described for a wide variety of physical or chemical stimuli in various types of organisms including humans and plants. For example, radioactive radiation, which is a powerful mutagen, metabolic inhibitors, toxic heavy metals or carcinogenic chemicals, such as dioxins, are known to trigger hormetic effects (Calabrese and Baldwin, 2001, Calabrese and Blain, 2005, Kaiser, 2003). At least in some cases, hormesis may constitute an adaptive evolutionary response of organisms to detrimental or otherwise unfavorable biological conditions (Mattson, 2010). Hormetic responses have been proposed to be generally based on compensatory processes following an initial disruption

in homeostasis (Calabrese, 2010). Although such phenomena have been described for a wide variety of organismal types and stimuli, their mechanistic basis has only been established in several non-plant systems (Mattson and Calabrese, 2010; Mattson, 2008; Mattson, 2010; Son et al., 2010) and it is unclear if distinct forms of hormesis share common regulatory processes.

Hormesis seems to be as common among plants as it is among animals (Calabrese and Blain, 2009). In particular, herbicides, natural phytotoxins and radioactivity were found to be potent stimuli of plant hormesis. In the vast majority of cases "growth" or "metabolic rate" were found to be endpoints stimulated by low doses of hormetic agents in plants. Despite the potential significance of hormetic performance enhancement for commercial crop production, the genetic and biochemical basis of hormesis in plants is completely unclear. Surprisingly, no systematic studies on hormesis seem to have been performed using the versatile molecular genetic plant model system Arabidopsis (Calabrese and Blain, 2009). Thus, our results on BHTC-induced root hormesis in Arabidopsis can serve as a starting point for more extended studies on the mechanistic basis underlying this and related phenomena in plants.

Low- and high-dose BHTC treatment elicited profoundly distinct transcriptional profiles. In both shoots and roots, only high levels of BHTC induced typical defense-related transcriptional changes, while low BHTC levels triggered a coordinated inter-compartmental transcriptional response manifested in suppression of photosynthesis- and respiration-related genes in the nucleus,

chloroplasts and mitochondria. In shoots, ld-BHTC treatment also up-regulated transcript levels of a set of 34 genes associated with developmental processes. Inspection of publically available Arabidopsis microarray data in the Botany Array Resource (BAR) (http://bar.utoronto.ca/welcome.htm) showed that nearly all representatives of this ld-BHTC-shoots-up set are specifically up-regulated in various root cell types and only weakly expressed or not expressed in other tissues. In our own mRNA-seq data set this general trend is also very obvious (Table 2.S1). Compared to mock-treated seedlings, all ld-BHTC-shoots-up members exhibit very high transcript levels in mock-treated roots. Thus, a plausible assumption is that these genes play important roles in root development or growth. Possibly, ld-BHTC treatment triggers a transition to root development-specific gene expression patterns in the entire seedling. As transcript levels of ld-BHTC-shoots-up members are already extremely high in untreated roots, but low in untreated shoots, the triggered change may only be clearly detectable in the latter tissues. Consistent with this assumption, we also observed a clear (but not significant) trend of ld-BHTC-shoots-up members to be weakly up-regulated in ld-BHTC-treated roots (average fold change of ld BHTC-treated roots vs. mock-treated roots = 1.25). We observed the opposite trend for these genes in a comparison between hd-BHTC and mock-treated roots (average fold change of hd-BHTC-treated roots vs. mock-treated roots = 0.79) and in a direct comparison 26% of all ld-BHTC-shoots-up exhibited significantly elevated transcript levels in ld-BHTC versus the hd-BHTC treated roots (Table

2.S1). Thus, differential expression of root-specific genes may contribute to the dramatic growth differences we observed between ld- and hd-BTHC treated roots.

It is unclear, however, how the collective down-regulation of ld-BHTC-roots-down genes may contribute to enhanced root growth. In any case, the highly distinct nature of transcriptional responses triggered by low- and high doses of BHTC is striking. Both responses do not differ much in a quantitative manner (e.g. in the amplitude of expression responses of a common set of genes), but are profoundly distinct regarding the identity and predicted roles of the gene sets they affect. This observation may suggest that different BHTC response processes are triggered by dose-sensitive recognition mechanisms. Recently a dose-dependent perception mechanism for SA that involves NPR1 as well as the NPR1-related NPR3 and NPR4 proteins has been proposed in Arabidopsis (Fu and Dong, 2013, Fu et al., 2012). Future studies will have to address if related mechanisms are responsible for dose-dependent perception of BHTC.

Our tests in Arabidopsis defense and auxin-response mutants provided further insight into processes mediating BHTC-triggered root hormesis. The WRKY70 transcription factor seems to be involved in both BHTC-mediated immunity and hormetic root elongation, while components of the auxin response pathway appear to be required for BHTC-mediated root hormesis, but not defense triggered by this compound. The phytohormone auxin is involved in a

wide variety of developmental processes. One well-known function of auxin is the suppression of root elongation when applied at relatively high doses. Interestingly, at low doses auxin can enhance root elongation in Arabidopsis (Evans et al., 1994). Some perception mechanisms of this hormone are well understood and seem to generally involve ARF transcription factors that can be repressed by Aux/IAA proteins. Auxin-responses processes are initiated by auxin-induced ubiquitylation of Aux/IAA proteins by SCF^{AFB} E3 ubiquitin ligase complexes. Several AFB (Auxin signaling F box) proteins, including TIR1, have been identified that mediate specific interactions of auxin-responsive SCF complexes with their respective AUX/IAA targets (Dharmasiri et al., 2005a, Dharmasiri et al., 2005b, Kepinski and Leyser, 2005). The accumulation of auxin beyond certain threshold levels can trigger SCF^{AFB}–mediated ubiquitylation of defined Aux/IAA members followed by the targeted proteasome-dependent degradation of these transcriptional repressors. This results in the de-repression of certain ARFs that induce transcription of auxin-response genes upon binding to auxin-responsive promoter elements (Aux-REs) in their target genes.

While most auxin-signaling mutants we tested did not show any clear reduction of BHTC-mediated hormesis, the *axr1-3* and *slr-1* mutants were clearly compromised in this response. *AXR1* encodes an E1 enzyme subunit that plays a central role in the perception of auxin by transferring the ubiquitin-related peptide RUB to SCF^{AFB} complexes and, thereby, activating them (Leyser et al., 1993, Quint and Gray, 2006). Mutants of *AXR1* are known to comprehensively

block multiple aspects of auxin responses (Gray and Estelle, 2000). Described phenotypes of *axr1* mutants include reduced auxin sensitivity in roots as well as several abnormalities or defects in leaf, inflorescence and flower morphology (Estelle and Somerville, 1987). Most importantly, the *axr1-3* mutant has been shown to exhibit a reduction in auxin-mediated root hormesis (Evans et al., 1994). Thus, an AXR1-dependent mechanism may be common to auxin and BHTC-mediated root hormesis.

Besides *axr1-3*, a second known Arabidopsis auxin response mutant, *slr-1*, was compromised in hormetic root enlargement by BHTC. This mutant bears a dominant negative mutation leading to a version of the Aux/IAA member IAA14 with an increased half-life (Fukaki et al., 2002). Several dominant negative Aux/IAA mutants are known to affect a subset of auxin responses (Liscum and Reed, 2002). The *slr* mutation is known to completely block lateral root formation, as well as to inhibit root hair formation and the gravitropic responses of roots and hypocotyls (Fukaki et al., 2002). However, dominant negative Aux/IAA mutations are known to have pleiotropic effects that do not always accurately reflect authentic roles of the respective gene (Liscum and Reed, 2002). While our results link auxin-related signaling processes to hormetic responses triggered by a synthetic plant defense elicitor, mechanistic details of this link are still enigmatic and will have to be resolved in future studies. Results from our current study can serve as a basis for more detailed analyses on connections between defense

signaling and root development as well as fundamental processes generally underlying hormetic phenomena in plants.

Materials and Methods

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

Arabidopsis *(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 Arabidopsis mutants *wrky70-3* (Knoth et al., 2007), *pad4-1* (Glazebrook et al., 1997), *wrky72-2* (Bhattarai et al., 2010), *sid2-2* (Dewdney et al., 2000), *npr1-3* (Cao et al., 1994b, Cao et al., 1997), *axr1-3* (Lincoln et al., 1990) and *slr-1* (Fukaki et al., 2002) have been described. *Hyaloperonospora arabidopsidis* (*Hpa*) was grown and propagated as described previously (McDowell et al., 2000). Two- or three-week old Arabidopsis plants were spray-infected with *Hpa* spore suspensions at 2 x 10^4 spores ml^{-1} for *Hpa*Noco2 with Preval sprayers (http://www.prevalspraygun.com). Plants were scored for *Hpa* growth 7 days post-infection (dpi) by counting spores per 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.

Pathogen infection experiments with tomato and cowpea

Tomato (*Solanum lycopersicum*) cv. Moneymaker (Everwilde Farms Inc.) seeds were sown on autoclaved vermiculite. Plants were fertilized with Miracle-Gro Tomato Plant Food (18-18-21; Scott's Miracle-Gro Products, Inc.) biweekly and maintained at 25°C under 200 µmol m^{-2} $s^{-}1$ light intensity for a 12-h-light photoperiod for four weeks. Each pot received 30 mL 50 µM BHTC poured over the vermiculite as a root drench 24 h prior to pathogen inoculation and two, 24 and 48 h post-inoculation. *Pseudomonas syringae* pv. *tomato* strain DC3000 (*Pst*) was cultured on King's B medium with 50 mg mL^{-1} rifampicin (Sigma Aldrich) at 28°C. Aerial portions of tomato plants were submerged in a 10mM $MgCl_2$ solution containing *Pst* (OD_{600} 0.005) and 0.02% Silwet-77 (Lehle Seeds) for 30 seconds. A single leaf was removed one h post-inoculation at day 0 and all remaining leaves were used for the 3 dpi time point. Leaves were weighed, ground in 10mM $MgCl_2$, diluted and plated. Colonies were counted 40-48 h after plating. Cowpea (*Vigna unguiculata)* cv. California Blackeye 5 (CB5) seedlings grown in vermiculite were inoculated at 8 days after germination with *Fusarium oxysporum* f. sp. *tracheiphilum* race 3 (*Fot*3) using a root clip and dip inoculation method. CB5 roots were rinsed free of vermiculite in water, cut to a length of five cm, submerged in a 10^4 spores mL^{-1} *Fot*3 suspension for 30 seconds and then replanted individually into pots containing UC Mix 3 soil. Pots were randomized on benches and plants were fertilized with Miracle-Gro (14-14-14; Scott's

Miracle-Gro Products, Inc.) biweekly and watered every other day. Aerial parts of plants were sprayed with either BHTC or a mock treatment containing the corresponding amount of DMSO solvent. Relatively mature plants could tolerate higher concentrations of DMSO better than younger plants, so the chemical concentrations increased over time and the amount sprayed increased for thorough coverage as plant size increased; 24h prior to inoculation = 100 µM BHTC 2mL per plant, week one post inoculation = 100 µM BHTC 2ml/plant, week two post inoculation = 200 µM BHTC 3mL per plant, week three post inoculation = 500 µM BHTC 4mL per plant, week four post inoculation = 750 µM BHTC 4ml/plant, and week five post inoculation = 1 mM BHTC 4mL per plant. Plants were evaluated five weeks post-inoculation for severity of disease symptoms (leaf chlorosis/wilting and vascular stem discoloration) relative to non-inoculated plants (ten BHTC-treated, ten mock-treated, ten untreated) using a zero to five rating scale as previously described (Pottorff et al., 2012). One hundred DMSO-treated and 100 BHTC-treated plants were scored individually, averaged and statistical significance was determined using the Student's *t* test ($p < 0.05$). *Fot*3 was grown and inoculum prepared as previously described (Pottorff et al., 2012). Cowpea-*Fot*3 experiments were conducted in a greenhouse with day temperatures up to 35°C and night temperatures down to 16°C.

Analysis of GUS Activity and Treatment of Homozygous *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 et al., 2009).

Synthetic Elicitors

BHTC, HTC, BTC, PTC, BMTC, CMP389 were all ordered from Sigma TimTec. T4CA, 2BP, and DCA were ordered from Sigma-Aldrich. BHTC can be easily synthesized following a protocol described previously (Khan et al., 2006; Song et al., 2009). A preparation of BHTC we synthesized using this protocol (s-BHTC) produced a nuclear magnetic resonance (NMR) spectrum identical to that obtained with BHTC purchased from Sigma TimTec (p-BHTC; data not shown) and the efficacy of s-BHTC and p-BHTC in reducing *Hpa* spore development in Arabidopsis was nearly identical (not shown).

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 per 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 per pot of chemicals at the

indicated concentrations and times prior to pathogen challenge. Disease symptoms were analyzed as described above.

Arabidopsis Root Growth Assays

Col-0 seeds were surface-sterilized in a 75% ethanol then 0.02% Triton X, 10% Bleach and water solution, for 10 and 15 min respectively. Seeds were then rinsed with sterile water and plated on solid media laced with: ½ MS (Murashige and Skoog), 1.5% agar, 3% sucrose and defined concentrations of synthetic elicitors or the equivalent concentration of DMSO (control) in square Petri plates. 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 (Schneider et al., 2012).

mRNA-seq analysis with plate-grown Arabidopsis seedlings

Col-0 seeds were surface-sterilized in 75% ethanol and a 0.02% Triton X, 10% Bleach solution, for 10 and 15 min respectively. Seeds were then rinsed with sterile water and plated on solid media laced with ½ MS, 1.5% agar, 3% sucrose and 0.1 or 5 µM of BHTC or solvent only (0.1% DMSO). Seeds were stratified for two days at 4°C and then placed on plates which were vertically positioned under fluorescent lights. After 14 days, seedling tissue was separated into shoot and root parts using a blade. To prevent any tissue contamination,

seedlings were cut into three parts, and root-shoot intersection areas were discarded. Total RNA was isolated from Shoot and root separately using TRIZOL (Invitrogen). RNA was processed and libraries were prepared with the NEBNext Ultra RNA library prep kit by following manufacturer's instruction. For each treatment type, root and shoot tissues were separately analyzed. We performed two independent biological replicates for each experimental condition and sequenced the respective libraries using the Illumina HiSeq2500 platform. Sequence reads were analyzed using TopHat for alignment of reads to the TAIR10 Arabidopsis genome annotation. Differentially expressed genes (DEGs) were identified by comparing read counts from BHTC-treated samples versus their respective mock controls by EdgeR using a Bonferroni-corrected false discovery rate (FDR)-cut off of 0.05. All mRNA-seq data generated for this study were deposited as format files in the NCBI sequence read archive (http://www.ncbi.nlm.nih.gov/sra/) data under the following accession number xxxxxx.

Acknowledgements

Dr. Melinda Rodriguez-Salus performed the biological experiments for Fig. 2.1, Fig. 2.2, Supplementary Figures 2.2 and 2.3. She also drafted results and materials & methods sections related to these figures. Mercedes Schroeder performed the biological experiments for figures 2.4C & 2.4D and provide draft results and materials & methods sections related to these figures. All remaining biological experiments were performed by Yasemin Bektas, the results and materials & methods related to these figures and tables were written by Yasemin Bektas and edited by Thomas Eulgem. I further thank Gregory Barding and Dr. Cynthia Larive (both University of California at Riverside) for helpful discussions and advice as well as Natalie Williams and Adilene Gomez (UCR) for excellent technical assistance. This work was supported by NSF-EAGER-IOS-1313814 grant to TE, a pre-doctoral fellowship Turkish Republic Ministry of National Education to YB as well as pre-doctoral fellowships to MRS, MS and CK from the NSF-funded ChemGen IGERT program (DGE-0504249).

References

Ahmad, S., Van Hulten, M., Martin, J., Pieterse, C.M., Van Wees, S.C., and Ton, J. (2011) Genetic dissection of basal defence responsiveness in accessions of Arabidopsis thaliana. *Plant Cell Environ* 34, 1191-1206.

Alhamadsheh, M.M., Hudson, R.A., and Viranga Tillekeratne, L.M. (2006) Design, total synthesis, and evaluation of novel open-chain epothilone analogues. *Organic letters* 8, 685-688.

Bektas, Y. and Eulgem, T. (2014) Synthetic plant defense elicitors. *Frontiers in plant science* 5, 804.

Bhattarai, K.K., Atamian, H.S., Kaloshian, I., and Eulgem, T. (2010) WRKY72-type transcription factors have a conserved role in basal plant immunity and contribute to gene-for-gene resistance mediated by the tomato R protein Mi-1 *The Plant Journal* 63, 229-240.

Bowling, S.A., Clarke, J.D., Liu, Y., Klessig, D.F., and Dong, X. (1997) The cpr5 mutant of Arabidopsis expresses both NPR1-dependent and NPR1-independent resistance. *Plant Cell* 9, 1573-1584.

Calabrese, E. (2010) Hormesis: Once Marginalized, Evidence Now Supports Hormesis as the Most Fundamental Dose Response. *in "Hormesis - A Revolution in Biology, Toxicology and Medicine"*.

Calabrese, E.J. and Baldwin, L.A. (2001) The frequency of U-shaped dose responses in the toxicological literature. *Toxicol Sci* 62, 330-338.

Calabrese, E.J. and Baldwin, L.A. (2002) Defining hormesis. *Hum Exp Toxicol* 21, 91-97.

Calabrese, E.J. and Baldwin, L.A. (2003) Hormesis: the dose-response revolution. *Annu Rev Pharmacol Toxicol* 43, 175-197.

Calabrese, E.J. and Blain, R. (2005) The occurrence of hormetic dose responses in the toxicological literature, the hormesis database: an overview. *Toxicol Appl Pharmacol* 202, 289-301.

Calabrese, E.J. and Blain, R.B. (2009) Hormesis and plant biology. *Environ Pollut* 157, 42-48.

Calabrese, E.J. and Blain, R.B. (2011) The hormesis database: the occurrence of hormetic dose responses in the toxicological literature. *Regul Toxicol Pharmacol* 61, 73-81.

Cao, H., Bowling, S.A., Gordon, A.S., and Dong, X. (1994a) Characterization of an Arabidopsis Mutant That Is Nonresponsive to Inducers of Systemic Acquired Resistance. *Plant Cell* 6, 1583-1592.

Cao, H., Bowling, S.A., Gordon, S., and Dong, X. (1994b) Characterization of an Arabidopsis mutant that is non-responsive to inducers of systemic acquired resistance. *Plant Cell* 6, 1583-1592.

Cao, H., Glazebrook, J., Clark, J.D., Volko, S., and Dong, X. (1997) The Arabidopsis *NPR1* gene that controls systemic acquired resistance encodes a novel protein containing ankyrin repeats. *Cell* 88, 57-64.

Casida, J.E. (2009) Pest toxicology: the primary mechanisms of pesticide action. *Chem Res Toxicol* 22, 609-619.

Chisholm, S.T., Coaker, G., Day, B., and Staskawicz, B.J. (2006) Host-microbe interactions: shaping the evolution of the plant immune response. *Cell* 124, 803-814.

Dewdney, J., Reuber, T.L., Wildermuth, M.C., Devoto, A., Cui, J., Stutius, L.M., Ausubel, F.M. (2000) Three unique mutants of Arabidopsis identify eds loci required for limiting growth of a biotrophic fungal pathogen. *Plant J.* 24, 205-208.

Dharmasiri, N., Dharmasiri, S., and Estelle, M. (2005a) The F-box protein TIR1 is an auxin receptor. *Nature* 435, 441-445.

Dharmasiri, N., Dharmasiri, S., Weijers, D., Lechner, E., Yamada, M., Hobbie, L., Estelle, M. (2005b) Plant development is regulated by a family of auxin receptor F box proteins. *Developmental cell* 9, 109-119.

Dong, X. (2004) NPR1, all things considered. *Curr Opin Plant Biol* 7, 547-552.

Estelle, M. and Somerville, C. (1987) Auxin resistant mutants of arabidopsis thaliana with an altered morphology. *molecular & general genetics* 206, 200-206.

Eulgem, T. (2005) Regulation of the Arabidopsis defense transcriptome. *Trends Plant Sci* 10, 71-78.

Eulgem, T., Rushton, P.J., Robatzek, S., and Somssich, I.E. (2000) The WRKY superfamily of plant transcription factors. *Trends in Plant Science* 5, 199-206.

Evans, M., Ishikawa, I., and Estelle, M. (1994) Responses of Arabidopsis roots to auxin studies with high temporal resolution: Comparison of wild type and auxin-response mutants. *Planta* 194, 215-222.

Ferrandez, M.D., Correa, R., Del Rio, M., and De la Fuente, M. (1999) Effects in vitro of several antioxidants on the natural killer function of aging mice. *Experimental gerontology* 34, 675-685.

Fu, Z.Q. and Dong, X. (2013) Systemic acquired resistance: turning local infection into global defense. *Annual review of plant biology* 64, 839-863.

Fu, Z.Q., Yan, S., Saleh, A., Wang, W., Ruble, J., Oka, N., . . . Dong, X. (2012) NPR3 and NPR4 are receptors for the immune signal salicylic acid in plants. *Nature* 486, 228-232.

Fukaki, H., Tameda, S., Masuda, H., and Tasaka, M. (2002) Lateral root formation is blocked by a gain-of-function mutation in the SOLITARY-ROOT/IAA14 gene of Arabidopsis. *Plant J* 29, 153-168.

Gilliom, R., Barbash, J., Crawford, G., Hamilton, P., Martin, J., Nakagaki, N., Wolock, D. (2007) The Quality of our nation's waters: Pesticides in the nation's streams and ground water, 1992-2001. *US Geological Survey* Chapter 1.

Glazebrook, J. (2001) Genes controlling expression of defense responses in Arabidopsis--2001 status. *Curr Opin Plant Biol* 4, 301-308.

Glazebrook, J., Chen, W., Estes, B., Chang, H.S., Nawrath, C., Metraux, J.P., Katagiri, F. (2003) Topology of the network integrating salicylate and jasmonate signal transduction derived from global expression phenotyping. *Plant J* 34, 217-228.

Glazebrook, J., Zook, M., Mert, F., Kagan, I., Rogers, E.E., Crute, I.R., . . . Ausubel, F.M. (1997) Phytoalexin-deficient mutants of *Arabidopsis* reveal that *PAD4* encodes a regulatory factor and that four *PAD* genes contribute to downy mildew resistance. *Genetics* 146, 381-392.

Gorlach, J., Volrath, S., Knauf-Beiter, G., Hengy, G., Beckhove, U., Kogel, K.H., Ryals, J. (1996) Benzothiadiazole, a novel class of inducers of systemic acquired resistance, activates gene expression and disease resistance in wheat. *Plant Cell* 8, 629-643.

Gray, W.M. and Estelle, I. (2000) Function of the ubiquitin-proteasome pathway in auxin response. *Trends Biochem Sci* 25, 133-138.

Hein, I., Gilroy, E.M., Armstrong, M.R., and Birch, P.R. (2009) The zig-zag-zig in oomycete-plant interactions. *Mol Plant Pathol* 10, 547-562.

Jones, J.D. and Dangl, J.L. (2006) The plant immune system. *Nature* 444, 323-329.

Kaiser, J. (2003) Hormesis. Sipping from a poisoned chalice. *Science* 302, 376-379.

Katzung, B. (2007) Basic and Clinical Pharmacology. *McGraw-Hill, Boston*; 10th edition.

Kepinski, S. and Leyser, O. (2005) The Arabidopsis F-box protein TIR1 is an auxin receptor. *Nature* 435, 446-451.

Kessmann, H., Staub, T., Hofmann, C., Maetzke, T., Herzog, J., Ward, E., .Ryals, J. (1994) Induction of systemic acquired resistance in plants by chemicals. *Annu. Rev. Phytopathol* 32, 439-459.

Knoth, C., Ringler, J., Dangl, J.L., and Eulgem, T. (2007) Arabidopsis WRKY70 is required for full RPP4-mediated disease resistance and basal defense against Hyaloperonospora parasitica. *Molecular Plant Microbe Interactions* 20, 120-128.

Knoth, C., Salus, M.S., Girke, T., and Eulgem, T. (2009) The synthetic elicitor 3,5-dichloroanthranilic acid induces NPR1-dependent and NPR1-independent mechanisms of disease resistance in Arabidopsis. *Plant Physiol* 150, 333-347.

Lawton, K., Friedrich, L., Hunt., M., Weymann, K., Delaney, T., Kessman, H., Ryals, J. (1996) Benzothiadiazole induces disease resistance in *Arabidopsis* by activation of the systemic acquired resistance signal transduction pathway. *Plant J.* 10, 71-82.

Leyser, H.M.O., Lincoln, C.A., Timpte, C., Lammer, D., Turner, J., and Estelle, M. (1993) Arabidopsis auxin-resistance gene AXR1 encodes a protein related to ubiquitin-activating enzyme E1. *Nature* 364, 161-164.

Lincoln, C., Britton, J.H., and Estelle, M. (1990) Growth and development of the axr1 mutants of Arabidopsis. *Plant Cell* 2, 1071-1080.

Liscum, E. and Reed, J.W. (2002) Genetics of Aux/IAA and ARF action in plant growth and development. *Plant Mol Biol* 49, 387-400.

Mattson, M. and Calabrese, E. (2010) Hormesis: What It Is and Why It Matters. *in "Hormesis - A Revolution in Biology, Toxicology and Medicine"*, 1 - 14.

Mattson, M.P. (2008) Hormesis defined. *Ageing Res Rev* 7, 1-7.

Mattson, M.P. (2010) The Fundamental Role of Hormesis in Evolution. *in "Hormesis A Revolution in Biology, Toxicology and Medicine"*, 57-68.

McDowell, J.M., Cuzick, A., Can, C., Beynon, J., Dangl, J.L., and Holub, E.B. (2000) Downy mildew (*Peronospora parasitica*) resistance genes in Arabidopsis vary in functional requirements for *NDR1*, *EDS1*, *NPR1*, and Salicylic Acid accumulation. *Plant J.* 22, 523-530.

Métraux, J.P., Ahl Goy, P., Staub, T., Speich, J., Steinemann, A., Ryals, J., and Ward, E. (1991) Induced resistance in cucumber in response to 2,6-dichloroisonicotinic acid and pathogens. In *Advances in Molecular Genetics of Plant-Microbe Interactions* (Hennecke, H. and Verma, D.P.S., eds), pp. 432-439, Kluwer.

Nimchuk, Z., Eulgem, T., Holt, I.B., and Dangl, J.L. (2003) Recognition and response in the plant immune system. *Annu Rev Genet* 37, 579-609.

Pandey, S.P., Roccaro, M., Schon, M., Logemann, E., and Somssich, I.E. (2010) Transcriptional reprogramming regulated by WRKY18 and WRKY40 facilitates powdery mildew infection of Arabidopsis. *Plant J* 64, 912-923.

Pietro, A.D., Madrid, M.P., Caracuel, Z., Delgado-Jarana, J., and Roncero, M.I. (2003) Fusarium oxysporum: exploring the molecular arsenal of a vascular wilt fungus. *Mol Plant Pathol* 4, 315-325.

Pottorff, M., Wanamaker, S., Ma, Y.Q., Ehlers, J.D., Roberts, P.A., and Close, T.J. (2012) Genetic and physical mapping of candidate genes for resistance to Fusarium oxysporum f.sp. tracheiphilum race 3 in cowpea [Vigna unguiculata (L.) Walp]. *PLoS One* 7, e41600.

Quint, M. and Gray, W.M. (2006) Auxin signaling. *Curr Opin Plant Biol* 9, 448-453.

Ryals, J.L., Neuenschwander, U.H., Willits, M.C., Molina, A., Steiner, H.-Y., and Hunt, M.D. (1996) Systemic acquired resistance. *Plant Cell* 8, 1809-1819.

Sato, M., Tsuda, K., Wang, L., Coller, J., Watanabe, Y., Glazebrook, J., and Katagiri, F. (2010) Network modeling reveals prevalent negative regulatory relationships between signaling sectors in Arabidopsis immune signaling. *PLoS Pathog* 6, e1001011.

Schneider, C.A., Rasband, W.S., and Eliceiri, K.W. (2012) NIH Image to ImageJ: 25 years of image analysis. *Nature methods* 9, 671-675.

Schreiber, K.J. and Desveaux, D. (2008) Message in a Bottle: Chemical Biology of Induced Disease Resistance in Plants. *The Plant Pathology Journal* 24, 245-268.

Slusarenko, A.J. and Schlaich, N.L. (2003) Downy mildew of Arabidopsis thaliana caused by Hyaloperonospora parasitica (formerly Peronospora parasitica). *Molecular Plant Pathology* 4, 159-170.

Son, T., Cutler, R., and Mattson, M.P. (2010) Transcriptional Mediators of Cellular Hormesis. *in "Hormesis - A Revolution in Biology, Toxicology A Revolution in Biology, Toxicology and Medicine"*, 69-94.

Sriharsha, S.N., Pai, K.S., Suhas, Shashikanth, S., Chandra, N., and Prabhu, K.R. (2007) Synthesis, docking and anti-tumor activity of beta-L-1,3-thiazolidine pyrimidine nucleoside analogues. *Medicinal chemistry* 3, 425-432.

Tao, Y., Xie, Z., Chen, W., Glazebrook, J., Chang, H.S., Han, B., . . . Katagiri, F. (2003) Quantitative nature of Arabidopsis responses during compatible and incompatible interactions with the bacterial pathogen Pseudomonas syringae. *Plant Cell* 15, 317-330.

Thilmony, R., Underwood, W., and He, S.Y. (2006) Genome-wide transcriptional analysis of the Arabidopsis thaliana interaction with the plant pathogen Pseudomonas syringae pv. tomato DC3000 and the human pathogen Escherichia coli O157:H7. *Plant J* 46, 34-53.

Toufighi, K., Brady, S.M., Austin, R., Ly, E., and Provart, N.J. (2005) The Botany Array Resource: e-Northerns, Expression Angling, and promoter analyses. *Plant J* 43, 153-163.

Tsuda, K., Sato, M., Stoddard, T., Glazebrook, J., and Katagiri, F. (2009) Network properties of robust immunity in plants. *PLoS Genet* 5, e1000772.

Uknes, S., Mauch-Mani, B., Moyer, M., Potter, S., Williams, S., Dincher, S., Ryals, J. (1992) Acquired resistance in Arabidopsis. *The Plant Cell* 4, 645-656.

Wang, D., Amornsiripanitch, N., and Dong, X. (2006) A Genomic Approach to Identify Regulatory Nodes in the Transcriptional Network of Systemic Acquited Resistance in Plants. *PloS Pathogens* in press.

Ward, E.R., Uknes, S.J., Williams, S.C., Dincher, S.S., Wiederhold, D.L., Alexander, D.C., . . . Ryals, J.A. (1991) Coordinate gene activity in response to agents that induce systemic acquired resistance. *Plant Cell* 3, 1085-1094.

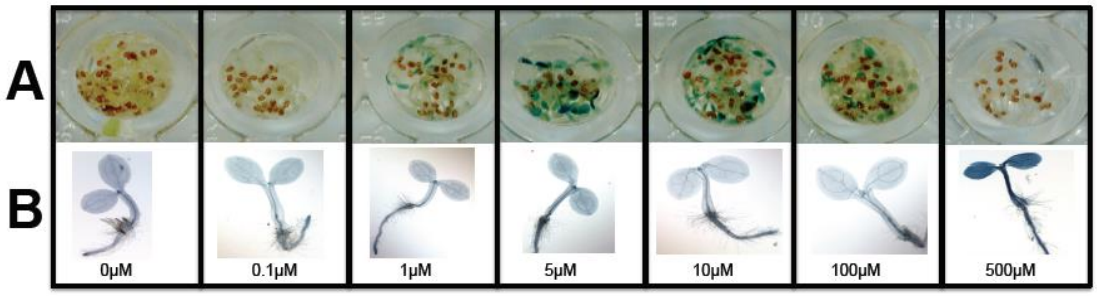
Wlodek, L., Wrobel, M., and Czubak, J. (1996) Selective effect of 2-(polyhydroxyalkyl)-thiazolidine-4-carboxylic acids on nonprotein sulfhydryl groups in tumor bearing mice. *General pharmacology* 27, 1373-1376.

Zeng, W., Brutus, A., Kremer, J.M., Withers, J.C., Gao, X., Jones, A.D., and He, S.Y. (2011) A genetic screen reveals Arabidopsis stomatal and/or apoplastic defenses against Pseudomonas syringae pv. tomato DC3000. *PLoS Pathog* 7, e1002291.

Zipfel, C. (2014) Plant pattern-recognition receptors. *Trends in immunology* 35, 345-351.

Supplementary Figures:

Figure 2.S1: Analysis of BHTC activity under saturation treatment conditions.



(**A)** Wells of "96-weel plates" containing 7d-old liquid-grown *CaBP22*$^{-333}$*:GUS* seedlings after X-Gluc histochemical staining comparing reporter responses after 24 h incubation at indicated compound concentrations. Blue/green color of cotyledons indicates induction of the *GUS* gene expression. Chlorophyll was completely removed during the ethanol-destaining process prior to GUS staining.
(B) Trypan blue staining of *CaBP22*$^{-333}$*:GUS* seedlings incubated for 24 h in liquid medium containing BHTC at the indicated concentrations. Dark blue/black color of the cotyledons indicates cell death (toxicity). The seed coats of seedlings always stain blue/black and can been seen in some images. All histochemical staining analyses were performed at least three times with similar results.

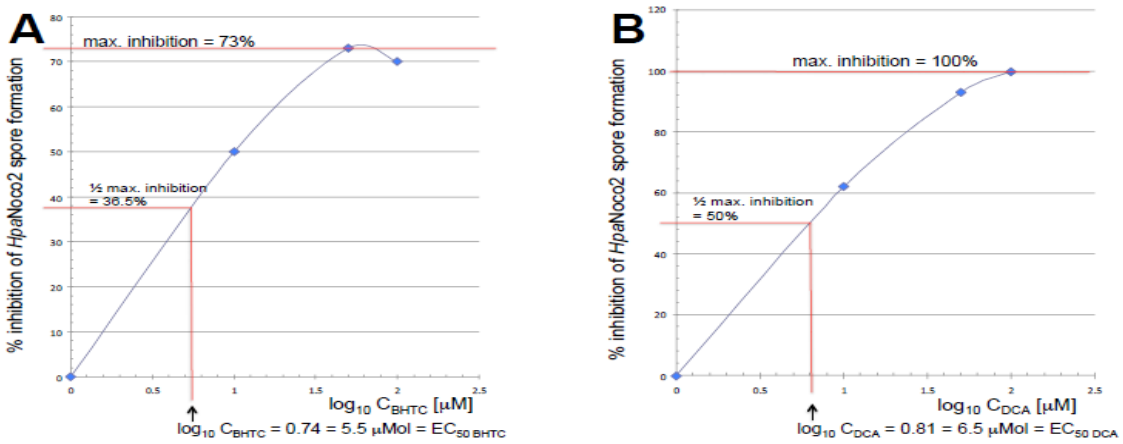


Figure 2.S2: Quantitative characteristics of BHTC and DCA.
Dose-dependency of BHTC- **(A)** of DCA- **(B)** mediated protection of Arabidopsis Col-0 against *Hpa*Noco2. While DCA can inhibit *Hpa* spore formation by 100%, the level of maximal inhibition mediated by BHTC is 73%. The EC_{50} values (concentration of a bioactive compound at which half-maximal biological activity is observed) for BHTC and DCA derived from the shown data are 5.5 □M and 6.5 □M, respectively.

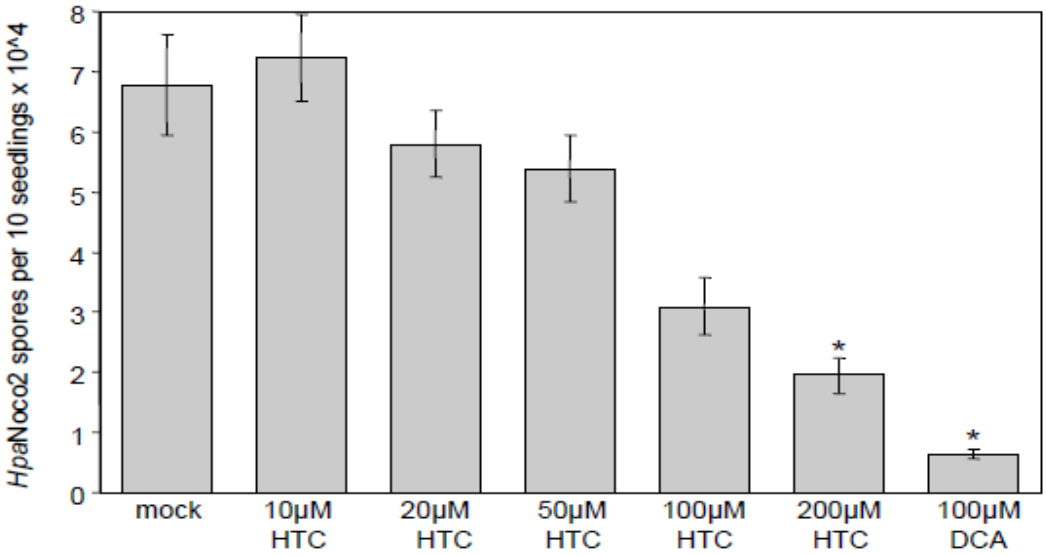


Figure 2.S3: The BHTC derivative HTC is a weak defense inducer.
Three-week-old soil-grown Col-0 seedlings were sprayed with HTC, DCA, or equivalent Mock (DMSO) at the indicated concentrations 24 h prior to spray infection with 2 x 10^4 *Hpa*Noco2 spores mL^{-1} (2 mL per pot). Spores were counted 7 dpi. Mean and SE values were calculated from a minimum of three biological replicates and the average of the three is shown above. The Student's *t*-test (*p*<0.05) was used to determine significant differences relative to the Mock.

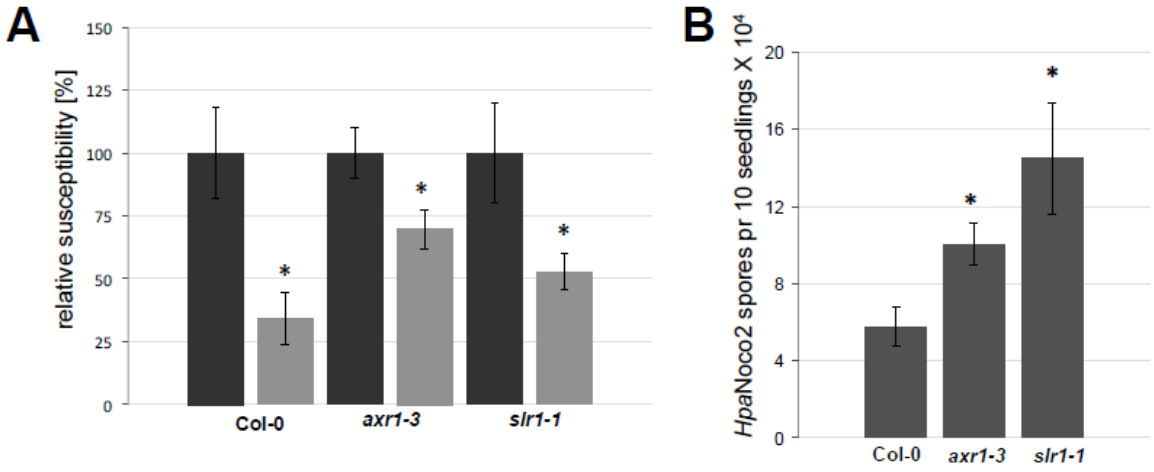


Figure 2.S4: The Arabidopsis *axr1-3* and *slr-1* mutants are not compromised in BHTC-mediated immunity against *Hpa*Noco2.
(A) Relative levels of *Hpa*Noco2 susceptibility of Arabidopsis seedlings of the indicated genotypes in mock-treated (solvent only, black bars) or 100mM BHTC pre-treated seedlings.
(B) Absolute levels of *Hpa*Noco2 susceptibility in mock-treated Arabidopsis seedlings of the indicated genotypes.
(A, B) See legend of Fig. 2.3 for details.

Table 2.S1: List of differentially expressed BHTC-responsive genes identified by mRNA-seq in this study. Supplementary data submitted.

CHAPTER 3: The Synthetic Elicitor DPMP (2,4-dichloro-6-{(E)-[(3-methoxyphenyl)imino]methyl}phenol) Induces Disease Resistance and Hormesis-Like Responses *in Arabidopsis thaliana*

Abstract

Synthetic elicitors are drug-like compounds that are structurally distinct from natural defense elicitors. Exogenous application of these compounds induces plant defense responses. By high-throughput screening we previously identified 114 synthetic elicitor candidates that activate expression of the pathogen-responsive *CaBP22^{-333}::GUS* reporter gene in *Arabidopsis thaliana* (Arabidopsis). Here we report on the characterization of one of these compounds, 4-dichloro-6-{(E)-[(3-methoxyphenyl)imino]methyl}phenol (DPMP). DPMP strongly triggers disease resistance of Arabidopsis against bacterial and oomycete pathogens. Interestingly, DPMP has two separate independently defense-inducing moieties. Both are interfering with defense signaling dependent on NPR1. We further found that low doses of DPMP enhance root growth in Arabidopsis, while, high doses of this compound inhibited root growth, besides inducing defense. This effect is an example of the known pharmacological phenomenon of dose-response of hormesis. By mRNA-seq analysis we identified substantial differences of transcriptional profiles triggered by low and high doses of DPMP. Only high levels of DPMP include typical defense-related transcriptional changes.

Introduction

Plant innate immunity is based a complex set of integrated defense mechanisms against microbial diseases (Glazebrook et al. 2003; Sato et al. 2010; Tsuda et al. 2009). Plants can recognize microbe-associated molecular patterns (MAMPs), which are highly conserved molecular structures of pathogens, by pattern recognition receptors (PRRs) on the surface of plant cell. These interactions activate pattern-triggered immunity (PTI) (Nürnberger and Lipka 2005; Chisholm et al. 2006; Gómez-Gómez and Boller 2002; Zipfel 2014; Segonzac and Zipfel 2011; Zipfel et al. 2004; Abramovitch and Martin 2004; Ahmad et al. 2011). To attenuate or block PTI, pathogens typically utilize effector molecules that enable them to use a given plant species as a host, resulting in compatible interactions or a condition termed effector-triggered susceptibility (ETS). During this type of interaction plants can still exhibit a weakened immune response, called basal defense, which limits pathogen spread, but is insufficient for preventing diseases (Ahmad et al. 2011; Glazebrook 2001). As a countermeasure to ETS, plants often can recognize the presence or activity of effector proteins by highly specific plant resistance (R) proteins and induce effector-triggered immunity (ETI). This leads to incompatible interactions leaving the pathogen avirulent and the plant resistant (Chisholm et al. 2006; Jones and Dangl 2006).

PTI, basal defense and ETI seem to involve a common set of defense signals, reactive oxygen species (ROIs), Ca^{2+}, salicylic acid (SA), ethylene (ET) and jasmonic acid (JA) (Nimchuk et al. 2003). The massive release of ROIs at pathogen infection sites is one of the earliest observable features of a plant's defense program. Induced changes of ion fluxes typically precede this oxidative burst (McDowell and Dangl 2000). The oxidative burst conditions a programmed form of localized cell death at infection sites, termed hypersensitive response (HR). HR can limit invasion of biotrophic pathogens, as these require host tissues to remain intact (Dangl et al. 1996). These early responses are coordinated by various components of the SA signaling pathway (Nimchuk et al. 2003). In addition, crosstalk between the SA, JA and ET hormone pathways are important for the fine tuning of plant defense responses (Durrant and Dong 2004).

Inducible immune responses are tightly associated with extensive transcriptional- and metabolic–reprogramming controlled by a complex regulatory network (Sato et al. 2010; Tsuda et al. 2009; Glazebrook et al. 2003). This network can be subdivided into various defined sectors that can interact with each other (Sato et al. 2010; Tsuda et al. 2009). For example, distinct defense signaling sectors dependent on early MAMP-activated MAP kinases (MAPKs) or the defense hormones SA or JA, have been described for Arabidopsis.

Synthetic elicitors (aka plant activators) are small molecules, which activate plant immune responses and can protect plants from diseases without the need to be directly toxic to pathogens. With this, they may offer alternative

disease control strategies that can be environmentally friendly (Bektas and Eulgem 2015). Synthetic elicitors may trigger defense reactions by directly interacting with immune receptors, such as PRRs or R-proteins, or by interfering with other defense signaling components. They can permit the study of biological functions of functionally redundant proteins, which are difficult to examine by traditional genetically methods.

One of the first class of synthetic elicitors, low molecular weight polyacrylic acid derivatives, were identified in 1974 and were shown to activate disease resistance in tobacco against tobacco mosaic virus (TMV) or tobacco necrosis virus (TNV) (Gianinazzi and Kassanis 1974; Kassanis and White 1975). Subsequently a large number of synthetic compounds were found to exhibit defense elicitor activity in plants. Most of them can be broadly classified as functional SA analogs, imprimatins, sulfonamides, adipic acid derivatives or jasmonic acid analogs (Bektas and Eulgem 2015). While some of them were used in basic research, others have been effectively used in crop protection.

The frequently used SA analogs 2,6-dichloro-isonicotinic acid (INA) and benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester (BTH) were discovered by the Switzerland-based pharmaceutical corporation Ciba-Geigy (now Syngenta) in early 1990ies (Metraux et al. 1991; Ward et al. 1991; Uknes et al. 1992). Interactions of these two compounds with plant defense system have been well characterized and they have been abundantly used as defense triggers in basic and applied studies on plant immunity. Both BTH and INA trigger

defense-associated effects similar to SA, but are less phytotoxic and more efficient than this natural defense hormone (Bektas and Eulgem 2015; Görlach et al. 1996; Lawton et al. 1996; Ward et al. 1991). In addition BTH, which has been marketed by Syngenta under the name BION® or Actigard®, some of other SA analogs (Probenazole, Tiadinil, Isotianil) have been successfully used in agriculture to protect plants against disease resistance (Bektas & Eulgem, 2015).

By high-throughput chemical screening our lab has previously identified 114 drug-like organic compounds that induce expression of the pathogen-responsive *CaBP22*$^{-333}$*::GUS* reporter gene in transgenic Arabidopsis (Knoth et al. 2009; Knoth and Eulgem 2014). One of them, 3-5-dichloroanthranilic acid (DCA) induces fast and transient defense responses against the pathogenic oomycete *Hyaloperonospora arabidopsidis* (*Hpa*) and the bacterial pathogen *Pseudomonas syringae* (Knoth et al. 2009). Unlike the SA analogs INA and BTH, the defense-inducing activity of DCA is not fully blocked in *npr1 Arabidopsis* mutants. DCA-triggered immune responses are largely independent from NPR1 and this compound partially targets a defense-mechanism dependent on the WRKY70 transcription factor. Thus, the mode-of-action of DCA seems distinct from that of INA and BTH. Another synthetic elicitor that was characterized by our lab is 2-(5-bromo-2-hydroxy-phenyl)-thiazolidine-4- carboxylic acid (BHTC). Similar to DCA, it induces plant defense quickly and transiently, but its mode-of-action is different from that of DCA, since it strongly depends on NPR1.

We surprisingly found that, while high doses of BHTC inhibit root growth, low doses of this synthetic elicitor have the opposite effect and enhance root elongation (Rodriguez-Salus, Bektas et al. 2015, submitted). This effect can be reminiscent of the phenomenon of hormesis, which has been described as dose-response relationships with low-dose stimulation and high-dose inhibition of biological performance (Calabrese 2015; Calabrese and Baldwin 2001). This phenomenon is often resulting in “inverse U"-shaped dose-response curves and has been described for various biological systems including humans and plants (Calabrese 2008; Calabrese and Blain 2005; Mattson et al. 2010). Our previous results showed that low doses of BHTC stimulate root elongation, while high doses of BHTC, which induce plant defense responses, inhibit root growth. A mRNAseq analysis we performed revealed that transcriptional profiles associated with low- and high-dose application of BTHC are highly distinct from each other. Furthermore, experiments with Arabidopsis defense mutants revealed that the WRKY70 transcription factor links plant defense responses to BHTC-induced hormesis.

Here, we report on another new synthetic elicitor, 2,4-dichloro-6-{(E)-[(3-methoxyphenyl)imino]methyl}phenol (DPMP), which is a member of a large group of related compounds identified as candidate synthetic elicitors in by our previous high throughput screen. DPMP is the most potent synthetic elicitor that we have identified so far, since it induces plant defense responses at very low concentrations (1 µM). It’s activity is distinct from that of DCA and similar to

BHTC, since its ability to induce immunity against *Hpa* is completely blocked in *npr1* mutant plants. Interestingly, we found that two distinct moieties of this compound are inducing defense responses *against Hpa and Pst*. An mRNA-seq analysis of DPMP-induced transcriptional responses has further reveal that, although DPMP acts as a partial agonist of SA and mimics some SA functions, it also induces expression of 376 genes are uniquely targeted by this compound.

Results

DPMP elicits *CaBP22^{-333}::GUS* expression

Candidate synthetic elicitors identified by our high throughout screen can be categorized into several structural classes. One structural class, phenyl-imino-methyl-phenol derivatives (PMPs) and PMP-related compounds, are represented by more than 30 members in our set of compound candidates. PMPs share a phenyl-imino-methyl-phenol skeleton. The PMP, 2,4-dichloro-6-{(E)-[(3-methoxyphenyl)imino]methyl}phenol (DPMP) particularly strongly induced *CaBP22^{-333}::GUS* expression. In one week-old liquid-grown *CaBP22^{-333}::GUS* seedlings DPMP activated reporter gene expression at a concentration as low as 1 µM (Fig. 3.1A). We further tested for possible synthetic elicitor-induced phytotoxicity by trypan blue staining of Arabidopsis *CaBP22^{-333}::GUS* seedlings 24 h after incubation with various concentrations of DPMP (Fig. 3.1B). Seedlings treated with a concentration of 500 µM DPMP stained dark blue, indicating extensive cell death, while no cell death was observed at lower concentrations (1 µM, 5 µM and 10 µM), which induces *CaBP22^{-333}::GUS* expression. Thus, DPMP-mediated phytotoxicity is not responsible for expression of this defense-associated reporter gene.

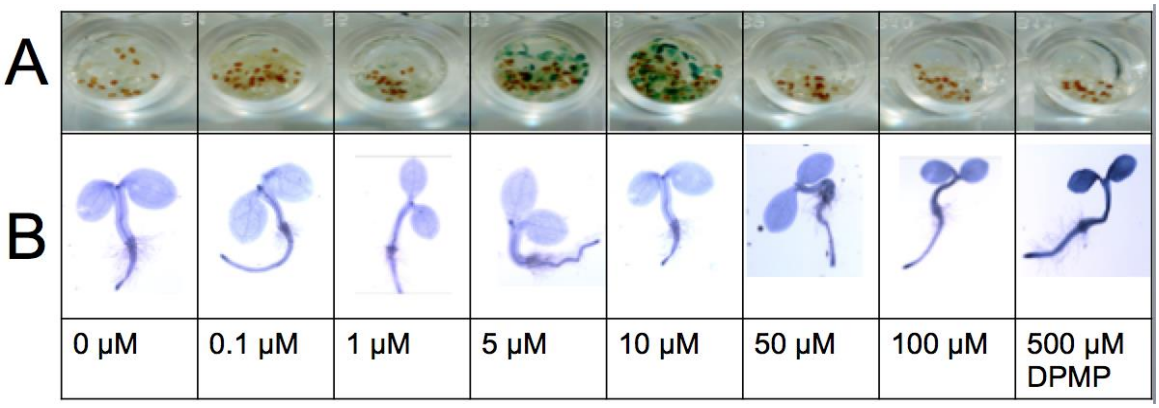


Figure 3.1: Activity of DPMP in *CaBP22^{-333}::GUS* Arabidopsis plants.
(**A)** After 24 h incubation with the indicated DPMP concentrations, wells of "96-well plates" containing 7d-old liquid-grown *CaBP22^{-333}::GUS* seedlings were processed by X-Gluc histochemical staining. Blue/green color of cotyledons treated with 1 to 10 µM DPMP indicates induction of *GUS* gene expression. Chlorophyll was completely removed during the ethanol-destaining process after GUS staining.
(B) Trypan blue staining of *CaBP22^{-333}::GUS* seedlings incubated for 24 h in liquid medium containing DPMP at the indicated concentrations. Dark blue/black color of the cotyledons indicates cell death (toxicity). The seed coats of seedlings always stain blue/black and can been seen in some images. Extensive cell death is only detectable after treatment with 500 µM DPMP.
(A, B) All histochemical staining analyses were performed at least three times with similar results. Shown are typical examples.

DPMP induces rapidly and transiently disease resistance of Arabidopsis to *Hpa*

We further examined whether DPMP induces in soil-grown plants disease resistance against the pathogenic oomycete *Hpa.* Three-week-old seedlings of the Arabidopsis ecotype Columbia (Col-0) wild type (WT) were pretreated with different concentrations of DPMP by a single foliar spray application 24 h prior to infection with the virulent *Hpa* isolate Noco2 (*Hpa*Noco2). *Hpa* spores were counted 7 days post infection (dpi). Plants sprayed with concentrations as low as 1 µM of DPMP showed a significant reduction in spore production compared to mock-pretreated plants (Fig. 3.2A) and at 10 µM DPMP, this effect already reached its maximal level of *Hpa*Noco2 immunity. Therefore, all further experiments were performed with a DPMP concentration of 10 µM. Compared to

our previously characterized synthetic elicitors DCA and BHTC (Knoth et al, 2009; Rodriguez-Salus, Bektas et al. 2015, submitted; see also chapter 2), DPMP displayed maximal effects suppression of spore formation at 10 times lower concentrations. DPMP is the most efficient plant defense-inducing compound we have identified so far.

Based on the dose-response data shown in Fig. 3.2A, we estimated the median effective concentration (EC_{50}) of DPMP regarding its ability to protect Arabidopsis from *Hpa*Noco2 as 0.75 µM (Fig. 3.2B). As expected, the EC_{50} value of DPMP is much lower than that of DCA and BHTC (Rodriguez-Salus, Bektas et al. 2015, submitted). Their EC_{50} are 6.5 µM and 5.5 µM, respectively. These results suggest that DPMP has a high efficiency regarding uptake and/or ability to interact with its target(s).

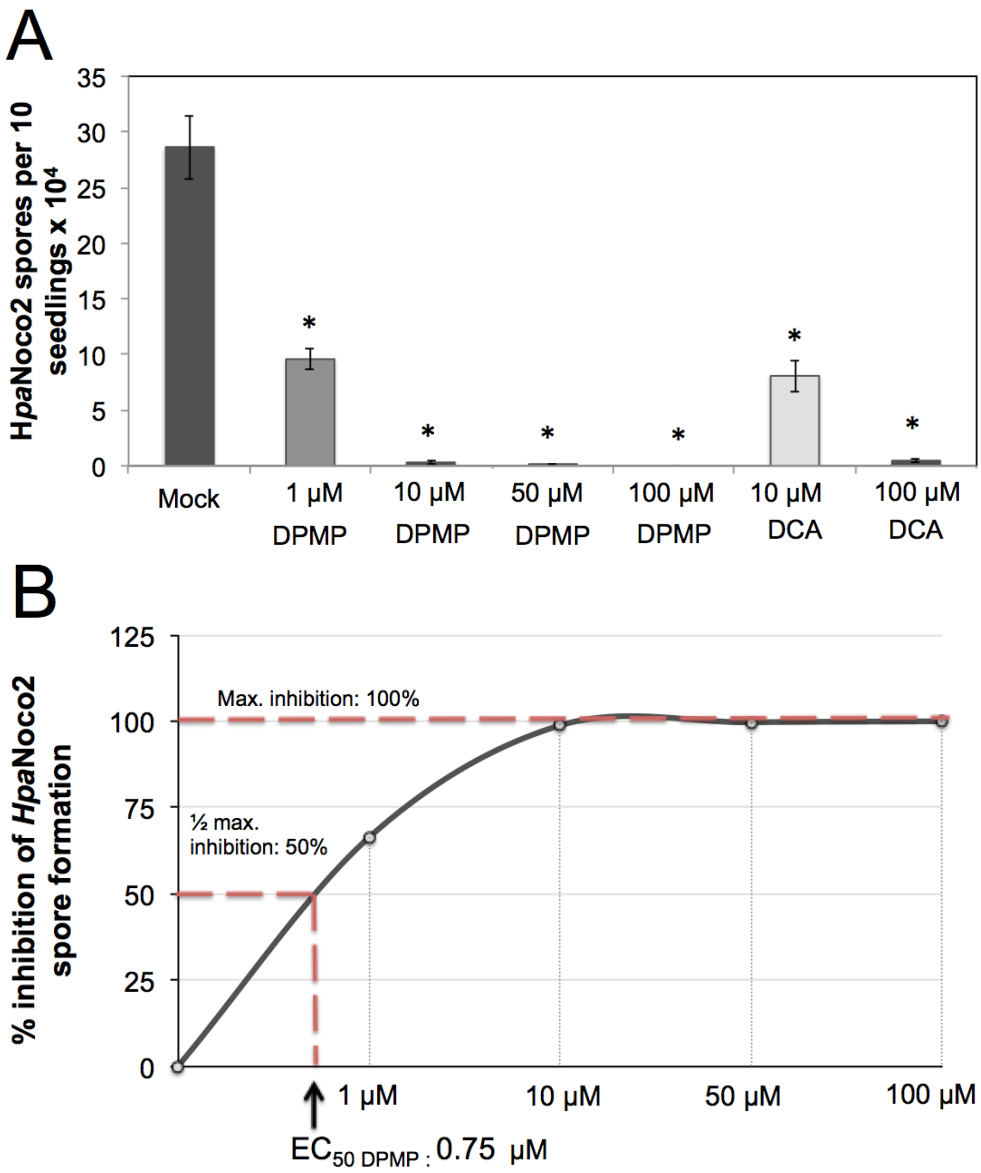


Figure 3.2: Dose-response curve and quantitative characteristics of DPMP.
(A) Dose response curve for DPMP-elicited immunity against *Hpa*. Three-week-old seedlings were sprayed with DPMP or DCA at the indicated concentrations or their respective mock solutions (solvent only) 24 h prior to *Hpa*Noco2 (3 x 10^4 spores mL^{-1}) spray infection. *Hpa* spores were counted 7 days post infection. Error bars represent the standard error of the mean (SE) based on at least 3 independent replicates. Spore numbers that are significantly reduced after synthetic elicitor treatments compared to mock-treatments based on Student *t*-test ($p < 0.05$) are marked by asterisks.
(B) *Hpa*Noco2 growth inhibition assay and estimated EC_{50} value. Based on the data shown in panel A. SE values were all less than 0.9 % and are not visible in the graph. The assay was repeated three times with similar results. The student *t*-test ($p < 0.05$) used to determine significant differences of DPMP treated samples compared to mock.

We further analyzed the kinetics of DPMP-induced defense induction and compared it with the kinetic behavior of other synthetic elicitors. Col-0 plants were pretreated with 100 µM of BHTC, DCA or INA or 10 µM of DPMP at various time points ranging from 1 hour to 6 days prior to pathogen challenge (Fig. 3.3). At these concentrations each of the tested compounds exhibits maximal activity. Mock treatment itself reduced spore growth when time points between *Hpa*Noco2 infection and chemical treatment were less than one day apart. This effect might be due to residual liquid coating of plants before being treated with pathogen. The synthetic elicitor-induced defense reaction is fast. At 1-hour post treatment (hpt), all of the tested chemicals suppressed *Hpa* spore production (Fig. 3.3). At 1-day post treatment (dpt), 10 µM DPMP exhibited similar strength of defense induction as 100 µM DCA and INA. Of all of four tested treatments, BHTC showed the weakest activity in inducing disease resistance.

Between 3 and 6 dpt levels of DPMP and DCA-mediated immunity began to decline while that of INA-mediated immunity remained constant and that of BHTC-mediated immunity already dropped between 1 and 3 dpt. At 6 dpt DPMP did not trigger any detectable immunity against the pathogen. Consistent with previous reports, DCA and BHTC induce plant immunity transiently (Knoth et al., 2009; Rodriguez-Salus, Bektas et al. 2015, submitted). Distinctively, the activity of INA is known to be long-lasting (Bowling 1997; Gorlach 1996; Metraux, 1991). Taken together our results clearly showed that the defense-inducing activity of

DPMP against *Hpa*Noco2 is stronger than that of other tested synthetic elicitors. Furthermore, its activity is rapid and transient, like those of DCA and BHTC.

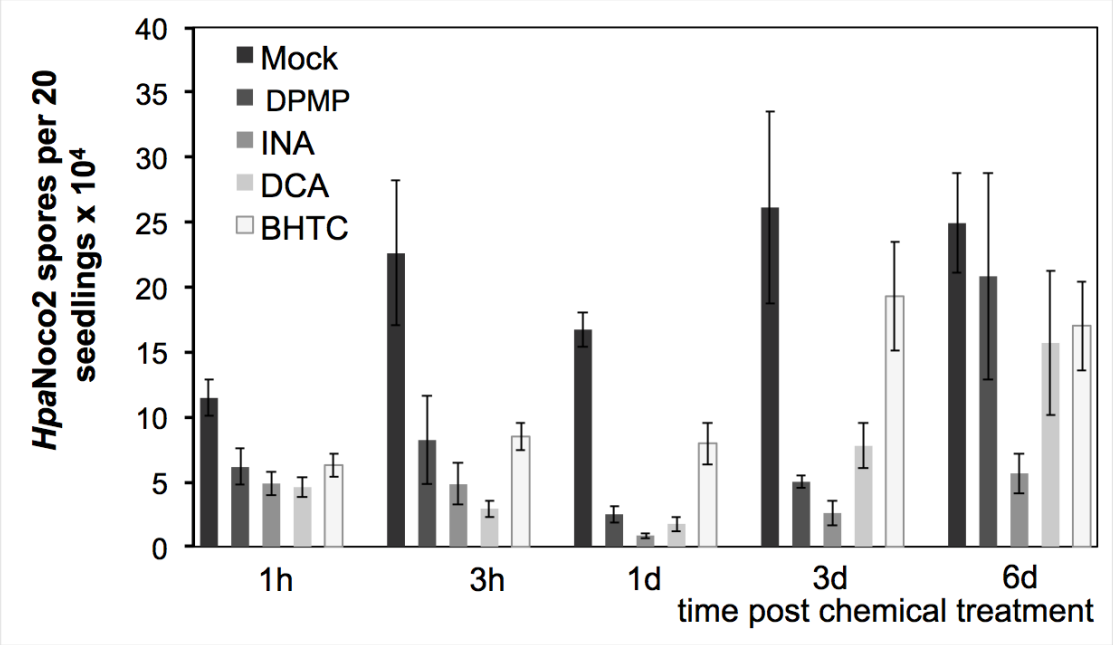


Figure 3.3: Kinetic analysis of DPMP-induced disease resistance against *Hpa*.
Two-week-old Col-0 seedlings were sprayed with 10 µM DPMP or 100 µM INA, DCA or BHTC or mock solution (0.2% DMSO) at the indicated times prior to *Hpa*Noco2 (3 x 10^4 spores mL^{-1}) spray-infection. *Hpa* spores were counted 7 days post infection. Mean and SE values were calculated from a minimum of three biological replicates and the average of those is shown above. The Student *t*-test ($p < 0.05$) showed significant differences for all of the synthetic elicitor treatments relative to the mock-treated control, except 6 dpt. At 6 days, only INA mediates significant immunity against *Hpa* relative to mock treatment.

DPMP provides disease protection against the bacterial plant pathogen *Pseudomonas syringae*

We further tested the ability of DPMP to induce resistance to the bacterial plant pathogen *Pseudomonas syringae* pathovar *tomato* strain DC3000 (*Pst*). Arabidopsis plants were pretreated with synthetic elicitors at concentrations

which they trigger strong immunity 24 h prior to dip-inoculation with *Pst*. Like with INA and DCA, DPMP-pretreated plants showed a significant reduction in bacterial growth (Fig. 3.4A). To test for a potential direct toxic activity of DPMP against bacteria, we grew *Pst* in liquid medium containing DPMP, other synthetic elicitors or the antibiotic hygromycin. None of the tested synthetic elicitors reduced bacterial growth, but hygromycin completely eliminated growth of *Pst* (Fig. 3.4B). Taken together, DPMP can induce plant immunity against *Pst* without being directly toxic to this pathogen.

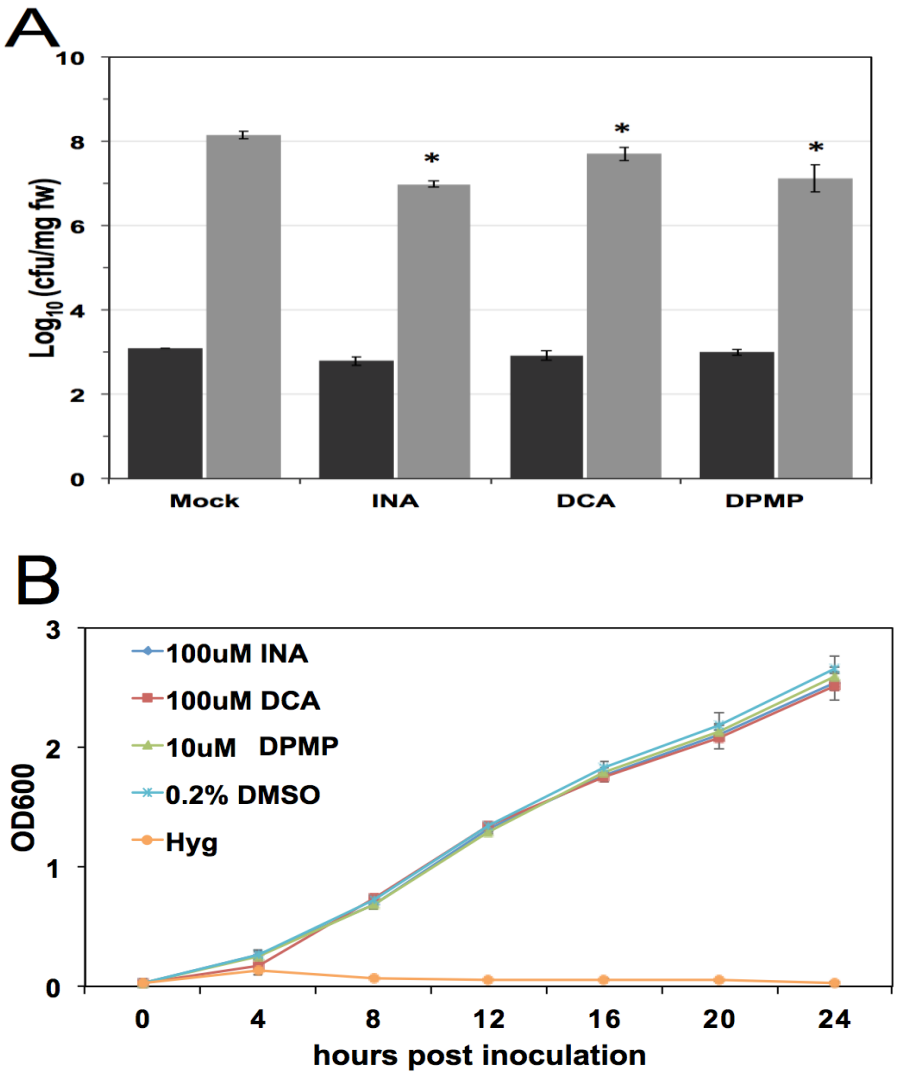


Figure 3.4: DPMP induces disease resistance against *Pst*.
(A) Quantification of *Pst* DC3000 growth on Arabidopsis Col-0 plants by the number of colony forming units (cfu). Two-week-old Col-0 seedlings were pre-treated with 10 µM DPMP, 100 µM INA, DCA or mock solution (0.2% DMSO) 24 h prior to dip-inoculation with virulent *Pst* DC3000 (OD_{600}= 0.005). Bacterial titers in the infected tissues were determined at day 0 (black bars) and day 3 (gray bars). Significant differences were identified using Student's *t*-test ($p< 0.05$). The shown data represent a typical example of five nearly identical biological replicates.
(B) *Pst* DC3000 grown in liquid culture with 10 µM DPMP, 100 µM INA, DCA, mock solution (0.2% DMSO) or 100 mg mL^{-1} hygromycin (HYG). OD600, which represents the density of bacteria was measured at indicated times after inoculation. Error bars represent the standard error of the mean based on at least 3 independent replicates.

DPMP interacts with targets operating downstream or independently from SA biosynthesis and is fully dependent on NPR1

To determine the mode of action of this new synthetic elicitor, we analyzed the defense-inducing activity of DPMP in the *ndr1-1*, *pad4-1*, *sid2-1*, *npr1-3* and *wrky70-3* Arabidopsis defense mutants as well as the transgenic *nahG* line. While *ndr1*, *sid2* and *pad4* mutant plants are known to be compromised in defense-associated SA biosynthesis and the transgenic *nahG* line does not accumulate significant levels of this defense hormone (Nimchuck et al., 2003), *npr1* mutants are deficient in the perception of SA (Glazebrook et al. 1997; Wildermuth et al. 2001; Cao et al. 1997; Cao et al. 1994) (Fig. 3.5A). WRKY70 is a transcription factor that partially operates in defense signaling downstream from NPR1 and is partially NPR1-independent (Li et al., 2004, 2006; Knoth et al, 2007, 2009). Col-0 and mutants plants were treated with *Hpa*Noco2 24 h after single foliar-spray applications with 10 µM DPMP. *Hpa* spores were counted 7 dpi. DPMP induced strong resistance against *Hpa* in Col-0 wild type plants and the *sid2-2*, *pad4-1* and *ndr1* mutants. DPMP-mediated immunity was slightly, but significantly reduced in *wrky70* mutants (Fig. 3.5B). However, in *npr1*-3 plants the defense inducing activity of DPMP was fully abolished; similar to INA (Fig. 3.5C). Our lab previously reported that DCA is partially dependent on *NPR1* and *WRKY70* (Knoth et al. 2009). DPMP is weakly dependent on *WRKY70* and completely dependent on *NPR1*, which are discriminates this compound from DCA. Surprisingly, in *nahG* transgenic plants, no significant protection was

observed against *Hpa* after the application of DPMP. The *nahG* transgenic plants express a SA hydroxylase, an enzyme that degrades SA to catechol. It is possible that, this enzyme is also degrading DPMP (Fig. 3.5A).

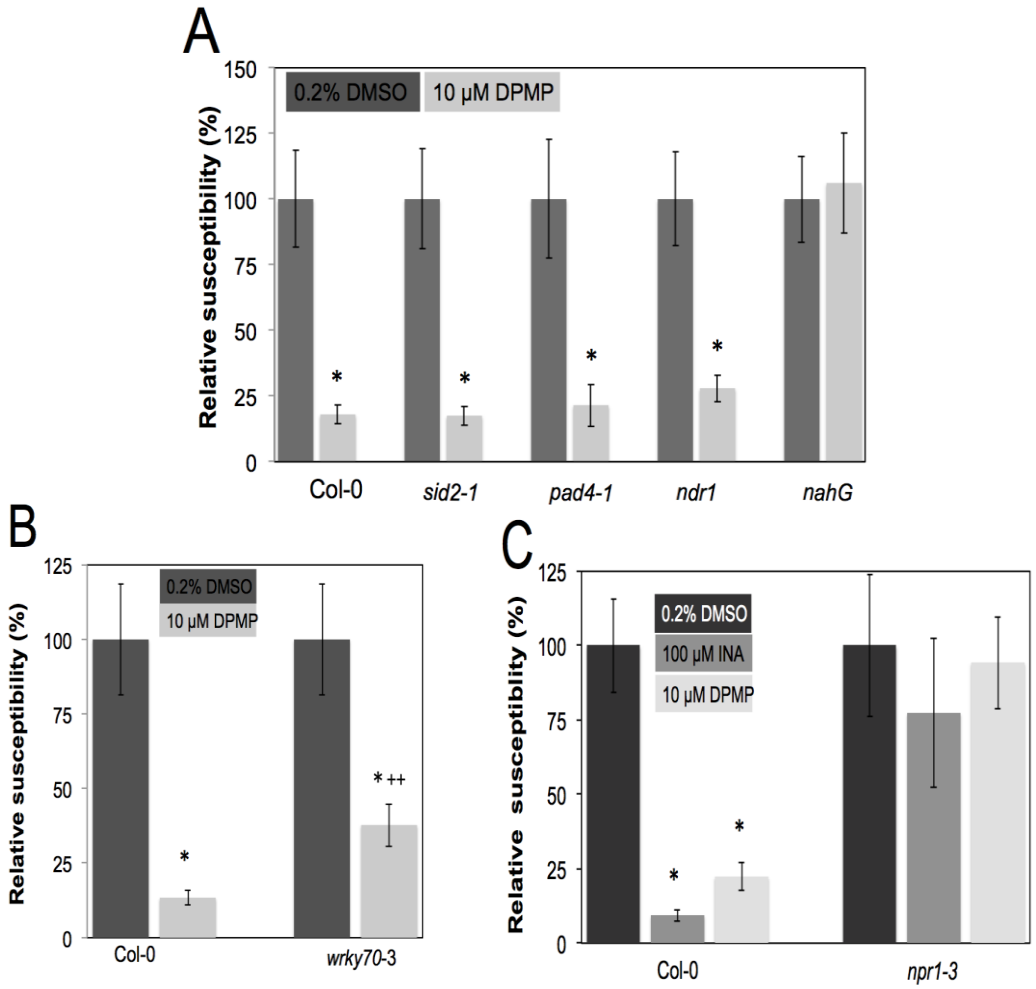


Figure 3.5: Analysis of DPMP activity in known defense mutants. (A,B) Analysis of DPMP activity in Arabidopsis Col-0 plants and Col-0 defense mutants. Three-week-old seedlings were sprayed with 10 µM DPMP or mock solution (0.2% DMSO) 24 h prior to *Hpa*Noco2 (3 x 10^4 spores mL^{-1}) spray infection. *Hpa* spores were counted 7dpi. Shown are relative numbers of spores per seedling. Error bars represent the standard error of the mean based on at least four independent replicates. Spore numbers that are significantly reduced after synthetic elicitor treatments compared to mock-treatments based on Student *t*-test ($p < 0.05$) are marked by asterisks. Relative susceptibility that are significantly increased on wrky70 mutants compared to Col-0 after synthetic elicitor treatments based on Student *t*-test ($p < 0.05$) are marked by double plus sign (++) **(C)** Analysis of DPMP and INA activity in Arabidopsis Col-0 and the *npr1-3* mutant. Two-week-old seedlings were sprayed with 10 µM DPMP, 100 µM INA or mock solution (0.2% DMSO) 24 h prior to *Hpa*Noco2 (3 x 10^4 spores mL^{-1}) spray infection. *Hpa* spores were counted 7 days post infection. Shown are relative numbers of spores per seedling compared to values obtained with the respective mock-treated controls. Error bars represent the standard error of the mean based on at least three independent replicates. Spore numbers that are significantly reduced after synthetic elicitor treatments compared to mock-treatments based on Student *t*-test ($p < 0.05$) are marked by asterisks.

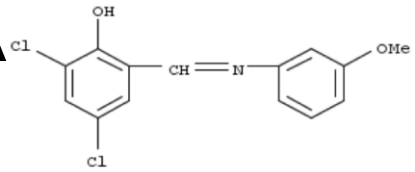
Structure activity analysis of other PMPs

DPMP is only one member of 33 PMP-related compounds identified by our high throughput synthetic elicitor screen. While not all of them share a common methyl-imino-phenol skeleton, they all bear an imino group linked to a phenyl moiety. We tested ten of these additional 33 PMP-related compounds and compared their defense-inducing activities to DPMP (Fig. 3.6A & 3.6B). Arabidopsis Col-0 plants were pretreated with a concentration of 10 µM or 25 µM of the respective chemicals 24 h prior to *Hpa*Noco2 infection. The tested chemicals exhibited three major activity trends: strong, moderate or weak compared DPMP, which we considered a strong defense inducer. PMP-related compounds that induced significant levels of immunity against *Hpa* at 10 µM and 25 µM were categorized as “strong”. If they only induced significant protection against *Hpa* at 25 µM, we categorize them as “moderate”. If they did not exhibit any significant defense induction at the tested concentrations, we classified them as “weak” inducers, as they were still able to induce *CaBP22-*^{333}*::GUS* expression.

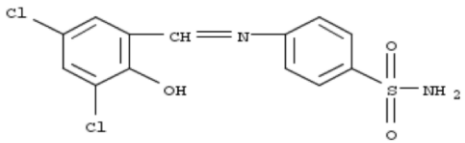
DPMP, 4-[[(3,5-dichloro-2-hydroxyphenyl)methylene]amino] Benzenesulfonamide (CMP974) and 4-tert-butyl-2-[(5-chloro-2-hydroxybenzylidene)amino]phenol (CMP993), provided strong protection against *Hpa* infection. They suppressed *Hpa* spore growth at concentrations of both 10 µM and 25 µM and reached levels of protection of up to 73%, 80% and 50% respectively.

Benzoic acid, 3-fluoro-, 2-[(5-chloro-2-hydroxyphenyl)methylene]hydrazide (CMP508), N'-benzylidene-2-hydroxybenzohydrazide (CMP318), Benzenamine, N-[(5-bromo-2-thienyl)methylene]-2-methyl-3-nitro-, [N(E)]- (CMP686) exhibited moderate defense induction and reduced susceptibility only at the 25 µM concentration to 75% to 50%. Although the five remaining PMPs did not trigger defense induction at concentrations of 10 µM or 25 µM, and considered “weak” inducers, they might induce plant defense at higher concentrations. The two strongest compounds are true PMPs, while the class of moderate defense inducers also includes with CMP318 and CMP686 compounds that deviate from this core structure.

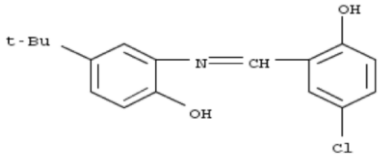
A



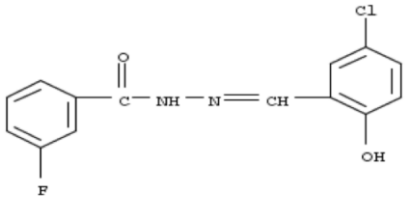
Phenol, 2,4-dichloro-6-[[(3-methoxyphenyl)imino]methyl]- **(DPMP)**



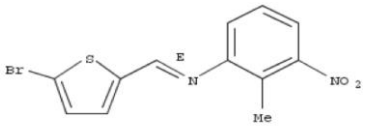
Benzenesulfonamide, 4-[[(3,5-dichloro-2 hydroxyphenyl)methylene]amino]- **(CMP974)**



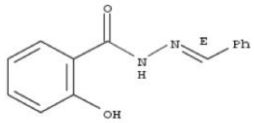
4-tert-butyl-2-[(5-chloro-2-hydroxybenzylidene)amino]phenol **(CMP993)**



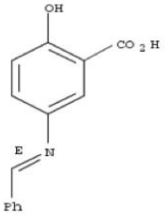
Benzoic acid, 3-fluoro-, 2-[(5-chloro-2-hydroxyphenyl)methylene]hydrazide **(CMP508)**



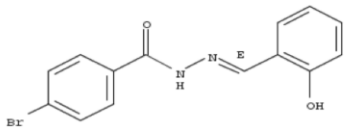
Benzenamine, N-[(5-bromo-2-thienyl)methylene]-2-methyl-3-nitro-, [N(E)] **(CMP686)**



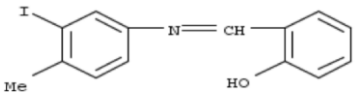
N'-benzylidene-2-hydroxybenzohydrazide **(CMP318)**



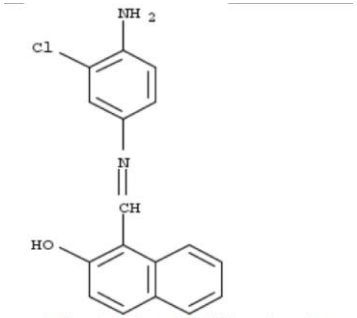
5-(benzylideneamino)-2-hydroxybenzoic acid **(CMP762)**



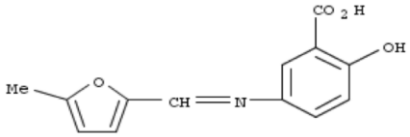
4-bromo-N'-(2-hydroxybenzylidene)benzohydrazide **(CMP673)**



2-{[(3-iodo-4-methylphenyl)imino]methyl}phenol **(CMP782)**



2-Naphthalenol, 1-[[(4-amino-3 chlorophenyl)imino]methyl]- **(CMP447)**



2-hydroxy-5-{[(5-methyl-2-furyl)methylene]amino}benzoic acid **(CMP24)**

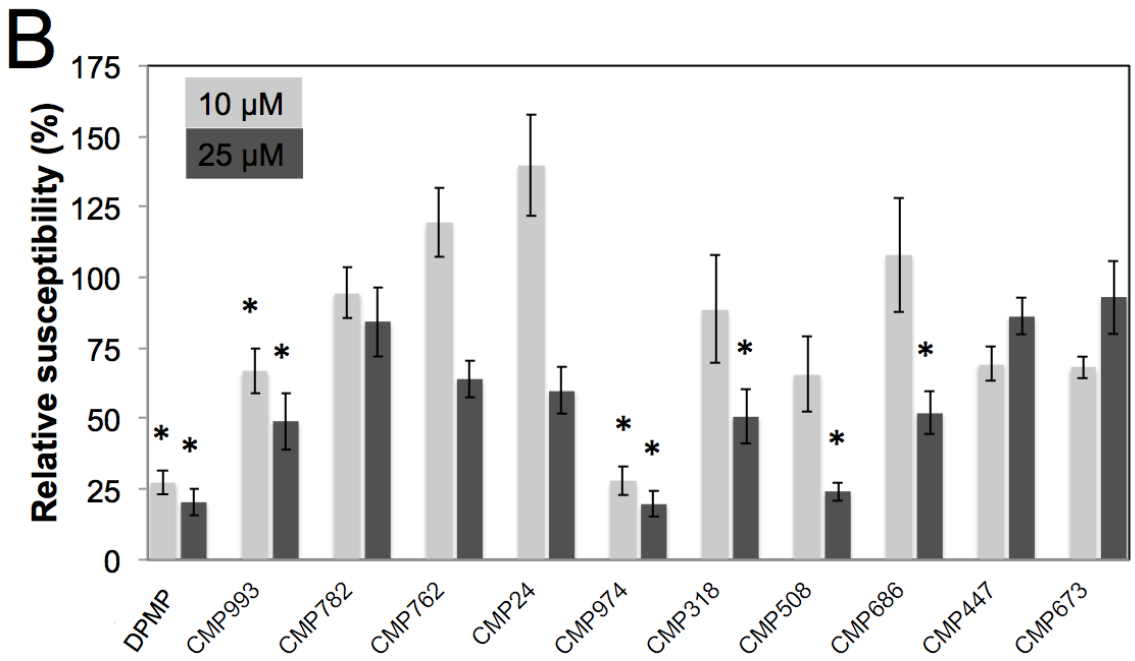


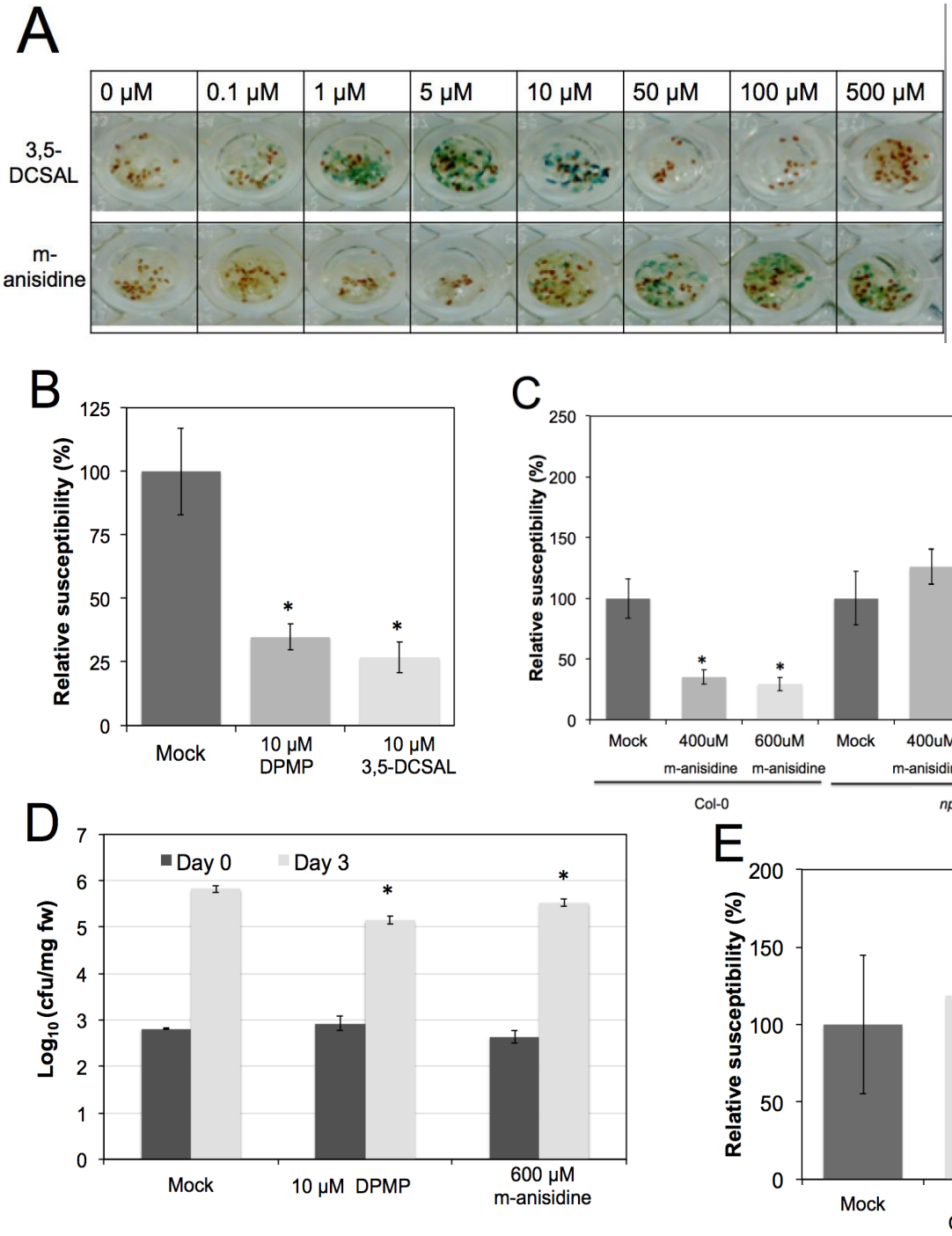
Figure 3.6: Structure activity analysis of PMP-related compounds.
(**A)** Chemical structures of compounds analyzed.
(B) Relative susceptibility of Arabidopsis against *Hpa*Noco2. Two-week-old seedlings were sprayed with 10 µM or 25 µM of the indicated compounds or their respective mock controls (1 % DMSO or 2.5 % DMSO respectively) 24 h prior to *Hpa*Noco2 (3 x 10^4 spores mL^{-1}) spray infection. *Hpa* spores were counted 7 days post infection. Shown are relative numbers of spores per seedling compared to values obtained with the respective mock-treated controls. Error bars represent the standard error of the mean based on at least 3 independent replicates. Spore numbers that are significantly reduced after synthetic elicitor treatments compared to mock-treatments based on Student *t*-test ($p < 0.05$) are marked by asterisks.

Biologically active moieties of DPMP

The imine group of PMPs may be subject to hydrolysis in an aqueous environment (Layer 1963). Thus, moieties released upon their hydrolysis may be the actual biologically active compounds of some PMPs. In order to determine moieties of the DPMP molecule that are critical for its defense-inducing activity, we analyzed its possible hydrolysis products.

3,5-dichlorosalicylaldehyde (3,5-DCSAL) and m-anisidine are possibly released by hydrolysis of DPMP (Fig. 3.7F). We first tested the ability of these two compounds to induce GUS expression in transgenic *CaBP22*$^{-333}$*::GUS* Arabidopsis plants (Fig. 3.7A). The putative hydrolysis product 3,5-DCSAL induced expression of this pathogen responsive reporter gene at the same concentration as DPMP (1 µM – 10 µM). Surprisingly, m-anisidine also induced GUS expression in this line, albeit at much higher concentrations (50 µM - 500 µM). To confirm these results, we tested different concentrations of 3,5-DCSAL and m-anisidine and compared their bioactivity to DPMP. At a concentration of 10 µM 3,5-DCSAL exhibits a similar strength of defense activation as 10 µM DPMP against *Hpa* (Fig. 3.7B). However, m-anisidine trigger defense induction at concentrations of 400 µM and 600 µM against *Hpa* and, as that of DPMP, its activity is completely dependent on *NPR1* (Fig. 3.7C). In addition to *Hpa*, m-anisidine induced plant immunity against *Pst* (Fig. 3.7D). In order to test whether the PMPs bridge skeleton itself induce immunity against *Hpa*Noco2, we analyzed N-[(E)-2-Thienylmethylidene]-2-Propanamine (CMP500), a molecule of relative simple structure that contains an imine bridge. CMP500 did not induce detectable resistance of Arabidopsis Col-0 against *Hpa*Noco2 at the concentrations we tested (Fig. 3.7E & 3.7F). Taken together these results show that, although the PMP imine bridge structure itself seems not trigger immunity, two moieties of DPMP that are potentially released upon hydrolysis of this molecule actively induce immunity. Furthermore, as 3,5-DCSAL is of similar efficiency as DPMP,

this moiety itself may be mainly responsible for DPMP-triggered immunity at lower DPMP concentrations.


A
0 µM
0.1 µM
1 µM
5 µM
10 µM
50 µM
100 µM
500 µM
3,5-DCSAL
m-anisidine
B
Relative susceptibility (%)
125
100
75
50
25
0
Mock
10 µM DPMP
10 µM 3,5-DCSAL
C
Relative susceptibility (%)
250
200
150
100
50
0
Mock
400uM m-anisidine
600uM m-anisidine
Col-0
npr1-3
D
Log10 (cfu/mg fw)
Day 0
Day 3
Mock
10 µM DPMP
600 µM m-anisidine
E
Relative susceptibility (%)
Mock
600 µM CMP500

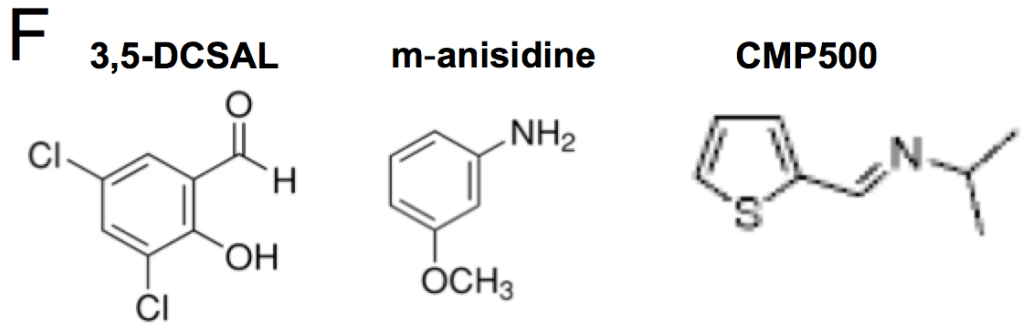


Figure 3.7: Two separate putative hydrolysis products of DPMP induce disease resistance. **(A)** After 24 h incubation at indicated DPMP concentrations, wells of "96-well plates" that contain 7d-old liquid-grown *CaBP22^{-333}::GUS* seedlings were processed by X-Gluc histochemical staining. Blue/green color of cotyledons indicates induction of the *GUS* gene expression. Chlorophyll was completely removed during the ethanol-destaining process after GUS staining. **(B)** Two-week-old seedlings were sprayed with 10 µM DPMP, 10 µM 3,5-DCSAL or mock control (1 % DMSO) 24 h prior to *Hpa*Noco2 (3 x 10^4 spores mL^{-1}) spray infection. *Hpa* spores were counted 7 days post infection. Shown are relative numbers of spores per seedling compared to values obtained with the respective mock-treated controls. Error bars represent the standard error of the mean based on at least 5 independent replicates. Spore numbers that are significantly reduced after synthetic elicitor treatments compared to mock-treatments based on Student *t*-test ($p< 0.05$) are marked by asterisks. **(C)** Two-week-old seedlings were sprayed with 400 µM m-anisidine, 600 µM m-anisidine or Mock control (H_2O) 24 h prior to *Hpa*Noco2 (3 x 10^4 spores mL^{-1}) spray infection on Col-0 or *npr1-3* mutants. *Hpa* Spores were counted 7 days post infection. Shown are relative numbers of spores per seedling compared to values obtained with the respective mock-treated controls. Error bars represent the standard error of the mean based on at least 3 independent replicates. Spore numbers that are significantly reduced after synthetic elicitor treatments compared to mock-treatments based on Student *t*-test ($p< 0.05$) are marked by asterisks. **(D)** The m-anisidine moiety of DPMP induces disease resistance against *Pst*. Quantification of Pst DC3000 growth on Arabidopsis Col-0 plants by colony forming units (cfu). Two-week-old Col-0 seedlings were pre-treated with 10 µM DPMP, 600 µM m-anisidine or Mock solution (0.2% DMSO) 24 h prior to dip-inoculation with virulent *Pst* DC3000 (OD_{600}= 0.005). Bacterial titer was evaluated at day 0 (black bars) and day 3 (gray bars). Significant differences were tested using Student *t*-test ($p< 0.05$). The shown data represent a typical example of two identical biological replicates. **(E)** Two-week-old seedlings were sprayed with 600 µM CMP500 or mock control (H_2O) 24 h prior to *Hpa*Noco2 (3 x 10^4 spores mL^{-1}) spray infection. *Hpa* Spores were counted 7 dpi. Shown are relative numbers of spores per seedling compared to values obtained with the respective mock-treated controls. Error bars represent the standard error of the mean based on at least 3 independent replicates. Spore numbers that are significantly reduced after synthetic elicitor treatments compared to mock-treatments based on Student *t*-test ($p< 0.05$) are marked by asterisks. **F)** Chemical structures of 3,5 DCSAL, m-anisidine and CMP500.

DPMP induces hormesis-like responses similar to BHTC in Arabidopsis

Similar to the synthetic elicitor BHTC (chapter 2; Rodriguez-Salus, Bektas et al. 2015, submitted) DPMP significantly enhanced root length of plate-grown Arabidopsis plants at concentrations below 1 µM, while high doses of DPMP reduced Arabidopsis root growth. For these assays plants were grown on various concentrations of DPMP-containing $^1/_2$ MS agar plates for 14 days and root length of the plants were measured at five different time-points (days at 3,5,7,11,14). Enhancement of root growth on low DPMP doses reached up to 80% in some biological replicates. However, in most data sets, results were moderate and increased root length of about 20-30 % was observed.

Besides BHTC and DPMP, we had tested several additional putative hormetic agents under a variety of different conditions. These included the herbicide glyphosate, which had previously been reported to induce hormetic effects in a range of plant species (Velini et al. 2008), as well as other chemical defense inducers such as BTH, INA and SA. While we found DPMP, BHTC, BTH, INA and SA to induce hormetic growth effects in Arabidopsis roots, glyphosate, seemed not to have such activity in Arabidopsis. Results obtained with DPMP are shown in Fig. 3.8. At concentrations 0.01 µM and 0.1 µM DPMP significantly enhanced the root length of Arabidopsis plants. Above 1 µM, this enhancement was abolished and inhibition of root growth was observed. DPMP and BHTC both exhibited similar hormetic effects at low concentrations, while hormetic effects of SA, BTH and INA were weaker than with these two synthetic

elicitors. We further established that the optimum concentration low-dose stimulation is 0.1 µM and for high-dose inhibition is 3 µM (Fig. 3.8B). These two doses were applied in our mRNA-seq experiments (see below).

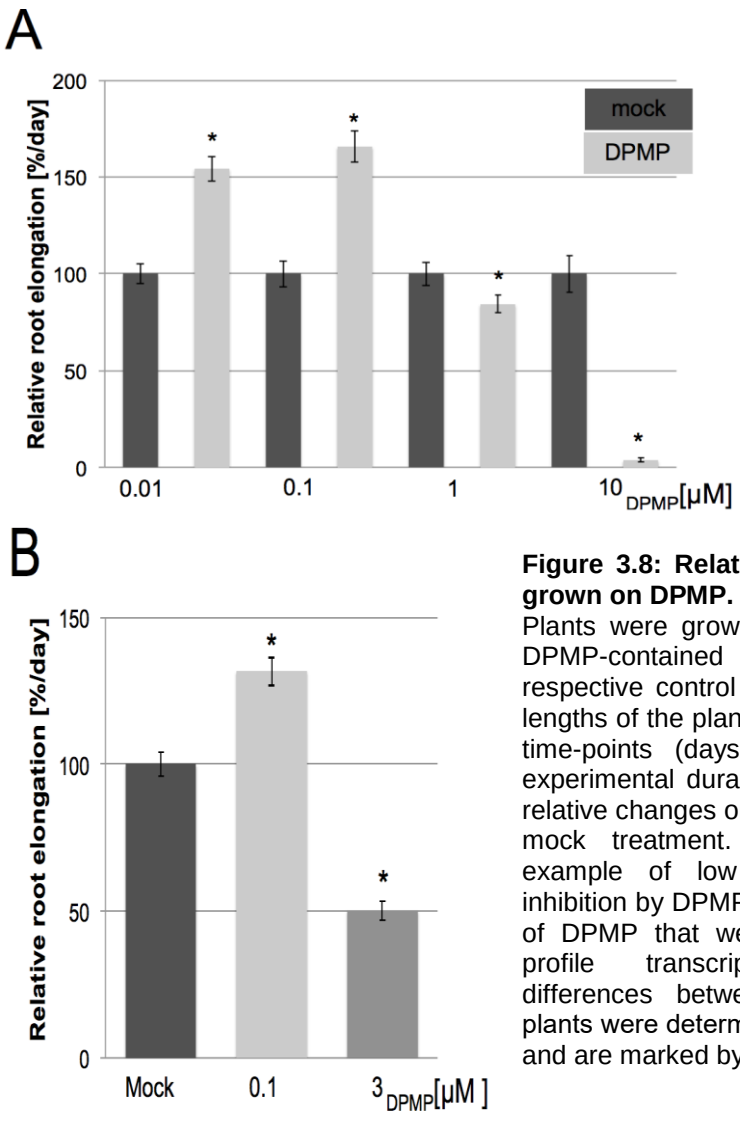


Figure 3.8: Relative root length of Col-0 plants grown on DPMP.
Plants were grown on indicated concentrations of DPMP-contained $^1/_2$ MS agar plates or the respective control (solvent only) for 14 days. Root lengths of the plants were measured at five different time-points (days at 3,5,7,11,14), over a total experimental duration of 14 days and the average relative changes on root length per day compared to mock treatment. **(A).** A representative typical example of low dose stimulation, high dose inhibition by DPMP. **(B).** An optimum concentrations of DPMP that were used on mRNA analysis to profile transcriptome changes. Significant differences between DPMP and control-treated plants were determined by Student's t-tests ($p<0.05$) and are marked by asterix.

Transcriptome changes triggered by high- and low-s of DPMP in Arabidopsis

To profile global transcriptional patterns associated with defense activation or hormesis induction, we performed mRNA-seq analysis. After Arabidopsis plants were grown for 14 days on ½ MS agar plates containing either 3 µM DPMP (high-dose (hd)) or 0.1 µM DPMP (low-dose (ld)) or mock (solvent only), shoot and root tissues were analyzed separately. These conditions were chosen because continuous exposure to 3 µM DPMP triggers strong activation of the *CaBP22*$^{-333}$*::GUS* reporter gene activation, whereas 0.1 µM of this compound did not induce expression of this reporter gene. However, the latter treatment induced robust hormetic growth enhancement in Arabidopsis roots. We performed two independent biological replicates for each experimental condition and sequenced the respective libraries using the Illumina HiSeq2500 platform. Differentially expressed genes (DEGs) after ld or hd DPMP treatment were identified by comparing read counts to those observed in the respective mock-treated control samples using a Bonferroni-corrected false discovery rate (FDR)-cut off of 0.05.

In shoots, treatment with hd-DPMP significantly altered transcript levels of 1364 genes, 1061 of which were transcriptionally up-regulated (hd-DPMP-shoots-up) and 303 of which were transcriptionally down-regulated (hd-DPMP-shoots-down) relative to the mock controls. The hd-DPMP treatment in shoot tissue resulted in a typical defense-associated transcriptional profile. Standard

defense marker genes, such as *PR1*, *PR5*, *CaBP22* and *LURP1*, as well as numerous WRKY transcription factors genes were transcriptionally up-regulated. Enriched gene ontology (GO) terms were calculated by the Botany Array Resource classification super viewer [http://bar.utoronto.ca/welcome.htm; (Toufighi et al. 2005)] and suggested that collective roles of hd-DPMP-shoots-up genes are in “response to stress” and “abiotic/biotic stimuli” as well as “signal transduction” (Table 3.1). Furthermore, the analysis of promoter motifs of these genes using the TAIR motif analysis tool (https://www.arabidopsis.org/tools/bulk/motiffinder/index.jsp) revealed that these hd-DPMP-shoots-up gene set are highly enriched for known defense-associated promoter motifs such as the hexameric motifs TTGACT (p=2.92E-38) that match the WRKY-binding W box element (TTGACC/T) (Eulgem et al. 2000). Also, a TGA box core motif (TGACG) was represented in the significantly enriched hexamer TTGACG (p=2.41E-05). Interestingly, hd-DPMP-shoot-down genes feature, along with “response to stress” and “abiotic/biotic stimuli”, the additional enriched GO term “electron transport or energy pathways”. Defense responses are associated with increased demands for energy and plant respiration is highly stimulated during plant defense responses (Bolton 2009). On the contrary, studies on photosynthesis and plant defense have shown that photosynthetic metabolism is repressed locally during plant defense (Berger et al. 2007; Bolton 2009; Scholes and Rolfe 1996). It has been suggested that this might be due to the need to produce free ROS resources for defense response or to protect the

photosynthetic apparatus from the defense-associated oxidative burst (Bolton 2009). Alternatively this may be a consequence of oxidative damage (Bolton 2009).

To profile transcriptional patterns associated with defense activation, we grew plants on 3 µM DPMP and harvested their issues 14 days later. The constant drain of metabolic resources due high-dose DPMP responses may have activated stress response mechanisms that reduce the rate of photosynthesis. This would be consistent with the fact that most of the down-regulates genes under these conditions are related to “electron transport or energy pathways” and associated with chloroplast or photosynthesis genes, such as the small subunit of RUBISCO and chlorophyll a-b binding protein. These results suggest that the role of hd-DPMP-shoot-down members is also indirectly related to defense responses.

Gene Set	Number of genes in set	Enriched GO terms* (with p values)

hd-DPMP-shoots-up	1031	response to stress (p=5.238e-120);
		response to abiotic or biotic stimulus (p=2.239e-92);
		signal transduction (p=3.030e-91);
		other biological processes (p= 7.769e-46);
		transport (p= 4.875e-40)

hd-DPMP-shoots-down	333	electron transport or energy pathways (p= 1.540e-36);
		other metabolic processes (p= 1.037e-11);
		transcription,DNA-dependent (p= 3.319e-08);
		response to stress (p= 9.890e-03);
		abiotic or biotic stimulus (p= 7.598e-03);
		other cellular processes (p=8.862e-07);
		transport (p= 0.016)

hd-DPMP roots-up	207	response to stress (p= 1.269e-12);
		response to abiotic or biotic stimulus (p= 1.147e-11);
		signal transduction (p= 1.415e-05);
		transport (p= 2.139e-03);
		other metabolic processes (p= 9.875e-03)

hd-DPMP roots-down	351	other metabolic processes (p= 1.989e-03);
		response to stress (p= 7.419e-03);
		abiotic or biotic stimulus (p= 7.598e-03);
		other cellular processes (p=8.862e-07);
		transport (p= 4.095e-03)

ld-DPMP-shoots-up	0	NA
ld-DPMP-shoots-down	0	NA

ld-DPMP-roots-up	31	other cellular processes (p= 0.012);
		other metabolic processes (p= 0.028);
		response to stress (p= 0.048)

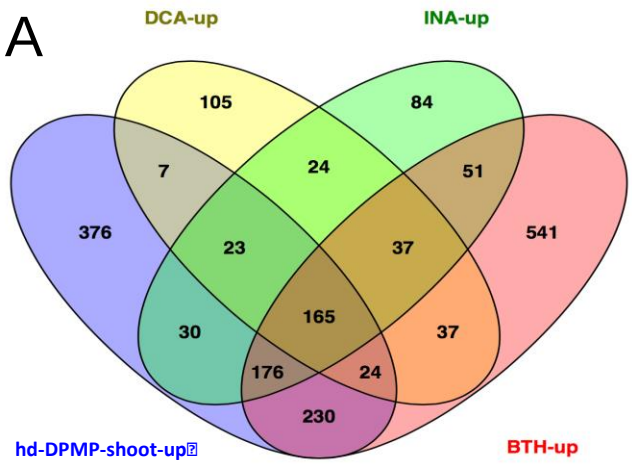
ld-DPMP-roots-down	2	NA

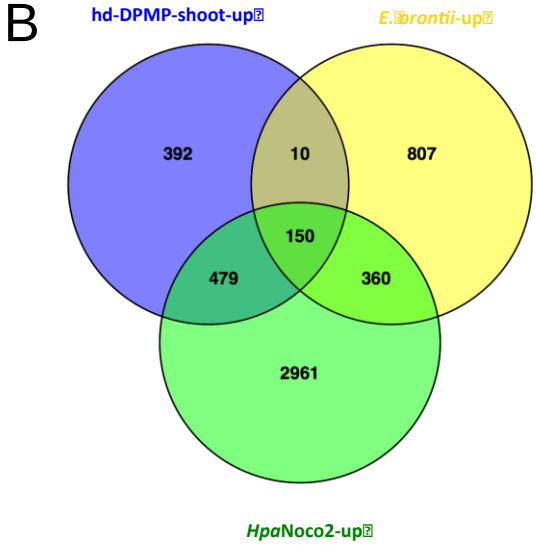
Table 3.1: Set of Arabidopsis genes significantly differentially expressed in response to low- or high-dose DPMP treatment in plate-grown Col-0 seedlings.

Comparison of set of genes that were induced by pathogens or known elicitors revealed that 63% of all hd-DPMP-shoot-up gene members are also inducible by the SA analogs DCA, INA and/or BTH (Knoth et al. 2009; Wang et al. 2006). This may indicate that, like DCA, INA and BTH, DPMP also acts as a partial agonist of SA and mimics some SA functions (Fig. 3.9A). Additionally, 62% of all hd-DPMP-shoot-up gene members are also responsive to infections with the oomycete *Hpa* and/or the powdery mildew fungus *Erysiphae orontii* (Fig. 3.9B). Transcriptional reponses triggered by these virulent pathogens are associated with basal defense (Bhattarai et al. 2010; (Pandey et al. 2010).

In roots, the number of DEGs was lower (558 DEGs), with 207 up-regulated (hd-DPMP-roots-up) and 351 down-regulated genes (hd-DPMP-roots-down) (Fig. 3.9C). However, the set of hd-DPMP-roots-up genes shares similar putative collective roles with hd-DPMP-shoots-up members. As in the case of hd-DPMP-treated shoots, enriched gene ontology (GO) terms showed that this set also contains genes with likely collective roles in “response to stress” and “abiotic/biotic stimuli” as well as “signal transduction” (Table 3.1). Moreover, this set also contains established defense marker genes like *LURP1*, *CaBP22* and *WRKY* genes. Genes down responsive to the hd-DPMP treatment in root tissues further contain genes related to “response to stress” and “abiotic/biotic stimuli”. These results indicate that the role of hd-DPMP-root members is also likely related to defense responses.

The number of DEGs responsive to the ld-DPMP treatments was substantially smaller than that responsive to the hd-DPMP treatments. A small set of 33 genes was found to be differentially expressed after ld-DPMP treatments in roots. Furthermore, ld-DPMP-root transcriptome changes are qualitatively different from responses induced by hd-DPMP treatment in roots (Fig. 3.9D). In this set of transcriptional changes, 31 genes were transcriptionally up-regulated and 2 genes were down-regulated by this treatment. Unfortunately, for the ld-DPMP-shoot treatment, we did not observe any significant DEGs in the comparisons between 0.1 µM DPMP versus mock in shoot. In this treatment, possibly many transcript level changes associated with the tested condition were relatively weak and having only two biological replicates may not have provided enough statistical power to detect such subtle changes as significant.


A
DCA-up
INA-up
105
84
7
24
51
376
23
37
541
165
30
37
176
24
230
hd-DPMP-shoot-up
BTH-up


B
hd-DPMP-shoot-up
E. brontii-up
392
10
807
150
479
360
2961
HpaNoco2-up

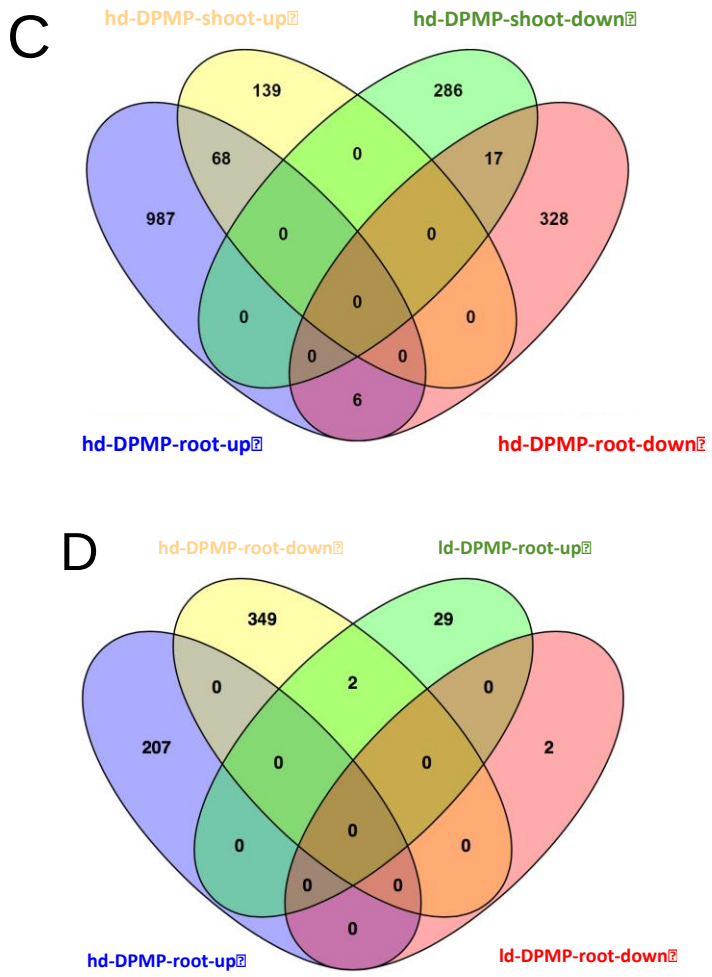


Figure 3.9: DPMP-triggered transcriptome changes different on low- and high-dose DPMP treatments. Venn diagram analysis highlights differences and similarities between the gene sets that were up-regulated from high-dose of DPMP and SA analogs **(A)**, set of genes up-regulated by *Erysiphae orontii (Pandey et al. 2010)* and *Hpa* (Bhattarai et al. 2010) **(B)**, comparison of high-dose of DPMP treatments in shoot and root **(C)** comparison of high- and low-dose of DPMP treatments show qualitatively different responses **(D)**.

Discussion

In this study, we identified and characterized the phenyl-imino-phenyl (PMP) derivative DPMP as a particularly potent novel synthetic elicitor after screening of total of 60.000 diverse organic compounds (Knoth et al. 2009). DPMP strongly induced *CaBP22*$^{-333}$*::GUS* reporter gene expression. Furthermore, it induced disease resistance against two phylogenetically distinct pathogens (*Hpa*Noco2 and *Pst*). Its defense-inducing activity is transient and strong, as it activates immune reactions at concentrations 10-fold lower than previously identified SA analogs without being direct toxic to pathogens. DPMP is the most potent novel compound that was identified in our synthetic elicitor screening with an unusually low estimated EC_{50} value of 0.75 µM. Low active concentrations are often correlated with high target affinity, high target specify and low levels of undesired side effects (Burdine and Kodadek 2004). Interestingly, DPMP consists of two separate moieties, each of which independently induces plant immune responses. Both of these moieties are linked by a labile imino bridge, which is likely subject to hydrolysis in the aqueous environment this compound encounters in plant tissues.

Our mode-of-action analysis of DPMP using Arabidopsis defense mutants revealed that DPMP acts downstream from SA biosynthesis and SA accumulation or acts independently from these defense-related processes. However, the defense-inducing activity of DPMP was completely blocked in the *npr1-3* mutant and partially reduced in the *wrky70-3* mutant. Based on these

results, we propose that DPMP activates the NPR1-dependent branch of the defense-signaling network. It seems further to interfere with WRKY70-dependent defense signaling processes. Although, DPMP activity is completely dependent on NPR1 similar to that of SA and the well-characterized SA analogs INA and BTH,(Kunz et al. 1997; Lawton et al. 1996; Friedrich et al. 1996; Metraux et al. 1991; Ward et al. 1991), its active concentration is 10-fold lower than that of INA and its activity is transient unlike that of INA and BTH, which induce sustained disease resistance in plants.

Furthermore, DPMP is functionally distinct from our previously characterized synthetic elicitors DCA and BHTC, Unlike DCA it is completely dependent on NPR1 and unlike BHTC, it is still active in *wrky70-3* plants. Based on its low EC_{50} value, the affinity of DPMP for its target protein(s) might be higher than that of other synthetic elicitors, such as INA, DCA or BHTC. Differences in the genetic requirements for synthetic elicitor activity further suggest that DPMP interacts either with different targets than DCA, BHTC or INA, or that it interferes with common targets of these synthetic elicitors in a distinct manner. Combined, these results made DPMP an interesting new bioactive compound for further studies.

The structure-activity analysis uncovered 3,5-DCSAL and m-anisidine to be two independently bioactive moieties of DPMP. Most likely both moieties get released *in planta* from DPMP by hydrolysis of its labile imino bridge. As it is as potent and efficient as DPMP itself, 3,5-DCSAL is likely mainly responsible for

the bioactivity of DPMP. To our knowledge neither 3,5-DCSAL nor m-anisidine have been previously reported to induce plant immune responses.

It is further possible that 3,5-DCSAL gets converted to 3,5-dichloro salicylic acid (3,5-DCSA) *in planta*. 3,5-DCSA is one of the structurally related active analogs of SA and it has been tested along with other SA derivatives in studies on plant defense induction (Silverman et al. 2005; Conrath et al. 1995). It was shown that 3,5-DCSA, 4-chloro salicylic acid (4-CSA), and 5-chloro salicylic acid (5-CSA), functionally mimic SA in plants, induce *PR1* gene expression and enhance disease resistance to TMV infection in tobacco (Conrath et al. 1995). Also, 3,5-DCSA primed Arabidopsis plants for enhanced induction of defense similar to SA, BTH and SA-derivatives; 4-CSA, and 5-CSA (Kohler et al. 2002; Katz et al. 1998; Thulke and Conrath 1998; Conrath 2011). Wu et al. reported that NPR1 could directly binds to SA in an equilibrium dialysis assay with the Kd value about 140 nM. In addition to SA, they showed that 4-CSA, 5-CSA and 3,5-DCSA bind to NPR1 with similar or slightly higher affinity than SA but not inactive analogs of SA (catechol, methyl-salicylate, 4-hydroxy benzoic acid and 3-hydroxy benzoic acid) (Wu et al. 2012). This observation is also consistent with our finding that the activity of DPMP is blocked in *npr1-3* mutants. Since release of 3,5-DCSAL may be mainly responsible for the defense-inducing activity of DPMP, this molecule may act after oxidation to 3,5-DCSAL by binding to NPR1.

The imprimatins C1 and C2 and their potential break-down products 4-chlorobenzoic acid (4-CBA) and 3,4-dichlorobenzoic acid (3,4-DCBA) were

identified as enhancers of pathogen-induced cell death in *Arabidopsis* suspension culture as well as inducers of disease resistance against both avirulent and virulent bacteria in Arabidopsis (Noutoshi et al. 2012). It was also shown that 3,5-dichlorobenzoic acid (3,5-DCBA) exhibits stronger activity than 3,4-DCBA and 3,4-DCBA is stronger than 4-CBA (Noutoshi et al. 2012; Knoth et al. 2009). These results indicate that the activities of these compounds are depending on the number and the position of chlorine substituents at their benzene rings. At the 3-, 4- and 5-positions chlorines seem important for defense activation. 3-5 DCBA shares a common dichlorinated benzoic acid core structure with INA and DCA. The exchange of a carbon atom by a nitrogen atom at position 4 of the ring converts 3,5-DCBA to INA, while the addition of an amino group to a position 2 of the ring converts it to DCA. Compared to 3,5-DCBA, DCA and INA more efficiently induced *CaBP22-333::GUS* expression and defense activation against *Hpa* in our assays.

To our knowledge, we showed here for the first time that DPMP and its one of its likely degradation products 3,5-DCSAL induce basal defense against virulent *Hpa*Noco2 as well as *Pst*. DPMP and 3,5-DCSAL share a structure of a dichloronated aromatic six-member ring with a carbonyl group, in addition to a hydroxyl group to a position 2, similar to SA. This substituted benzene ring core structure represents the most efficient synthetic elicitor class (regarding efficiency against *Hpa* and *Pst* in Arabidopsis) we have tested so far. Although Wu and co-workers (Wu et al. 2012) found SA and SA derivatives to show similar levels of

NPR1 affinity in their *in vitro* biding studies, our results suggest that subtle structural differences of these compounds affect their *in vivo* activity and, thus, the strength of the disease resistance they mediate against pathogens. Such structural differences may alter affinities of these molecules for target proteins under *in vivo* conditions. Alternatively, these changes might affect efficiency of metabolic conversions they encounter *in planta.* The unusually high level of synthetic elicitor activity we observed for 3,5-DCSAL (compared to SA and benzoic acid derivatives, such as 3,5-DCBA, INA or DCA) may be due to a higher affinity to its target protein(s) or additional hypothetical co-receptors. Alternatively, this compound may be taken up more efficiently or may be metabolically more stable.

Surprisingly, in addition to the 3,5-DCSAL moiety, a second moiety, m-anisidine also has defense-inducing activity. At concentrations of 400 µM and 600 µM, m-anisidine mediated immunity against *Hpa* and *Pst* (Fig. 3.7C & 3.7D). To our knowledge, m-anisidine has not been described as plant defense inducer before. It also activates plant defense responses dependent on NPR1 similar to 3,5-DCSAL. 3,5-DCSAL and m-anisidine are structurally unrelated and they may either interact with different cellular targets or interact in different ways with the same or related receptors. If they interact with distinct targets, they may have the ability to induce immunity in interact synergistic fashion by activating parallel defense mechanisms. Future studies with these two compounds can reveal their role in immune responses.

DPMP is only one PMP representative identified in our original high throughput synthetic elicitor screen. Tests for *Hpa* resistance of ten additional PMP-related compounds we had identified as inducers of *CaB22*$^{-333}$*::GUS* expression in Arabidopsis demonstrated that DPMP and the related compound CMP974 are the most efficient defense inducers of this set of compounds. Both contain a 3,5-DCSAL moiety and most likely this sub-structure is responsible for their common defense inducing activity. In addition to these compounds, four additional PMP-related compounds protected Arabidopsis against *Hpa*. However, these compounds are less efficient defense inducers than DPMP and CMP974. Two of them, CMP993 and CMP508, contain moieties that are similar to 3,5-DCSAL. They both contain an aromatic six-member ring with a carbonyl group and a chlorine at position 5. The lack of additional chlorine may be the reason for the weaker defense induction compared to 3,5-DCSAL. The remaining two moderately active PMP-related defense inducers, CMP318 and CMP686, do not contain moieties similar to 3,5-DCSAL or m-anisidine. The five additional PMP-related compounds we tested, did not trigger defense against *Hpa* at the tested concentrations and they seem less active compared to others. However, they may induce efficient plant defense responses at higher concentrations. Taken together PMPs and PMP-related compounds are a large and structurally diverse class of synthetic elicitors that have the potential to be highly efficient. It is interesting that DPMP bears two independently active moieties. Further studies with these compounds along with other so far untested PMP derivatives may

reveal whether this is characteristic for all PMPs or not. Additional studies with PMP-related compounds are needed to define structural features that are possibly commonly required for the synthetic elicitor activities of these molecules.

Interestingly, we found that, like BHTC, DPMP triggered hormetic effects and significantly enhanced root length of plate-grown Arabidopsis plants when applied at concentrations below 1 µM, while high doses of DPMP reduced Arabidopsis root growth and induce defense gene expression. As in the case of BHTC, low and high-dose DPMP treatments triggered distinct transcriptional profiles. In both shoots and roots, only high levels of DPMP induced typical defense-related transcriptional changes. The high dose application of DPMP resulted in a typical defense-associated transcriptional profile. Standard defense marker genes, such as *PR1*, *PR5*, *CaBP22* and *LURP1*, as well as numerous WRKY transcription factors genes were transcriptionally up-regulated. Furthermore, many genes of the hd-DPMP-shoot-up set are also induced during natural immune responses in Arabidopsis after infection with the oomycete *Hpa* and/or the powdery mildew fungus *Erysiphae orontii*. The conserved promoter motifs of these genes are enriched with W box element as well as a TGA box derivative. Moreover several hd-DPMP-shoot-up gene members are overlap with genes induced by other SA analogs; DCA, INA and/or BTH. These results suggest, and further support, that DPMP is a SA mimic.

Unfortunately, our low-dose DPMP treatments did not reveal clear information regarding putative biological roles of the respective response genes.

In ld-DPMP-treated roots only a small number of genes were differentially expressed (Table 3.S1). The analysis of the ld-DPMP-shoot responses did not result in the identification of any significant DEGs in the comparisons between 0.1 µM DPMP versus mock in shoot. Possibly many transcript level changes associated with the tested condition were relatively weak and having 2 biological replicates did not provide enough statistical power to detect these subtle changes as significant. Further additional biological replicates may reveal a clear profile of low-dose DPMP induced transcriptional changes.

As described in chapter 2, hormetic effects of BHTC were tested in a set of defense and auxin signaling-mutants. These data suggested that the WRKY70 transcription factor contributes to in BHTC-induced immunity along with hormetic root elongation. Although, most of the tested auxin-signaling mutants did not exhibit clear effects on BHTC-mediated hormesis, the *axr1-3* and *slr-1* mutants were compromised in this response. Further studies with DPMP, in addition to BHTC may reveal common and distinct roles of these two synthetic elicitors in hormesis and may also uncover links between plant immunity and hormetic growth effects.

Materials and Methods

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

Arabidopsis *(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 Arabidopsis mutants *wrky70-3* (Knoth et al. 2007), *pad4-1* (Glazebrook et al. 1997), *ndr1-1 (Century et al. 1997), sid2-2* (Dewdney et al. 2000), *npr1-3 (Cao et al. 1994; Cao et al. 1997),* nahG (Delaney et al. 1994) have been described. *Hyaloperonospora arabidopsidis* (*Hpa*) was grown and propagated as described previously (McDowell et al. 2000). Two- or three-week old Arabidopsis plants were spray-infected with *Hpa*Noco2 spore suspensions at 3x 10^4 spores ml^{-1} 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 or 20 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. Arabidopsis plants were dip inoculated with *Pseudomonas syringae* pv tomato DC3000 with an indicated inoculum concentration (optical density at 600 nm). For these experiments, infections and scoring were performed as described previously (Tornero and Dangl 2001). Plants were also visually scored for disease symptoms 2 days after inoculation.

Analysis of GUS Activity and Treatment of Homozygous *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 et al. 2009).

Synthetic Elicitors

DPMP, 3-5 DCSAL and m-anisidine, CMP500 and CMP974 were all ordered from Sigma-Aldrich (https://www.sigmaaldrich.com). CMP993, CMP762, CMP24, CMP508, CMP686, CMP447, CMP673 were ordered from Interchim (https://www.interchim.com). CMP782 and CMP318 were purchased from Ryan Scientific (https://www.ryansci.com).

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 at the indicated times and concentrations with Preval sprayers. Final DMSO concentrations never exceeded 2.5%. 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.

Arabidopsis Root Growth Assays

Col-0 seeds were surface-sterilized in a 75% ethanol then 0.02% Triton X, 10% Bleach and water solution, for 10 and 15 min respectively. Seeds were then rinsed with sterile water and plated on solid media laced with: ½ MS (Murashige and Skoog), 1.5% agar, 3% sucrose and defined concentrations of 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 (Schneider et al. 2012).

mRNA-seq analysis with plate-grown Arabidopsis seedlings

Col-0 seeds were surface-sterilized in 75% ethanol and a 0.02% Triton X, 10% Bleach solution, for 10 and 15 min respectively. Seeds were then rinsed with sterile water and plated on solid media laced with ½ MS (Murashige and Skoog), 1.5% agar, 3% sucrose and 0.1 or 3 µM of BHTC or solvent only (0.1% DMSO). Seeds were stratified for two days at 4°C and then placed on plates which were vertically positioned under fluorescent lights. After 14 days, plant tissue was separated into shoot and root parts using blade. To prevent any tissue contamination, seedlings were cut into three parts, and root-shoot intersection areas were discarded. Total RNA was isolated from Shoot and root separately by using TRIZOL (Invitrogen, http://www.invitrogen.com). RNA was processed and

libraries were prepared with the NEBNext Ultra RNA library prep kit by following manufacturer's instruction (New England Biolabs, http://www.neb.com). For each treatment type root and shoot tissues were separately analyzed. We performed two independent biological replicates for each experimental condition and sequenced the respective libraries using the Illumina HiSeq2500 platform. Sequence reads were analyzed using TopHat for alignment of reads to the TAIR10 Arabidopsis genome annotation. Differentially expressed genes (DEGs) were identified by comparing read counts from BHTC-treated samples versus their respective mock controls by EdgeR using a Bonferroni-corrected false discovery rate (FDR)-cut off of 0.05.

Acknowledgments

Dr. Melinda Rodriguez-Salus performed the biological experiments for Figure 3.2, All remaining biological experiments were performed by Yasemin Bektas. This work was supported by NSF-EAGER-IOS-1313814 grant to TE, a pre-doctoral fellowship Turkish Republic Ministry of National Education to YB.

References

Abramovitch RB, Martin GB (2004) Strategies used by bacterial pathogens to suppress plant defenses. Current opinion in plant biology 7 (4):356-364. doi:10.1016/j.pbi.2004.05.002

Ahmad S, Van Hulten M, Martin J, Pieterse CM, Van Wees S, Ton J (2011) Genetic dissection of basal defence responsiveness in accessions of Arabidopsis thaliana. Plant, cell & environment 34 (7):1191-1206

Bektas Y, Eulgem T (2015) Synthetic plant defense elicitors. Frontiers in plant science 5

Berger S, Sinha AK, Roitsch T (2007) Plant physiology meets phytopathology: plant primary metabolism and plant–pathogen interactions. Journal of experimental botany 58 (15-16):4019-4026

Bhattarai KK, Atamian HS, Kaloshian I, Eulgem T (2010) WRKY72-type transcription factors contribute to basal immunity in tomato and Arabidopsis as well as gene-for-gene resistance mediated by the tomato R gene Mi-1. The Plant journal : for cell and molecular biology 63 (2):229-240. doi:10.1111/j.1365-313X.2010.04232.x

Bolton MD (2009) Primary metabolism and plant defense- fuel for the fire. MPMI 22:487-497

Burdine L, Kodadek T (2004) Target Identification in Chemical Genetics: The (Often) Missing Link. Chemistry & Biology 11 (5):593-597. doi:http://dx.doi.org/10.1016/j.chembiol.2004.05.001

Calabrese EJ (2008) Hormesis: why it is important to toxicology and toxicologists. Environmental toxicology and chemistry / SETAC 27 (7):1451-1474. doi:10.1897/07-541

Calabrese EJ (2015) Hormesis within a mechanistic context. Homeopathy: the journal of the Faculty of Homeopathy 104 (2):90-96. doi:10.1016/j.homp.2015.01.002

Calabrese EJ, Baldwin La (2001) Hormesis: a generalizable and unifying hypothesis. Critical reviews in toxicology 31 (4-5):353-424. doi:10.1080/20014091111730

Calabrese EJ, Blain R (2005) The occurrence of hormetic dose responses in the toxicological literature, the hormesis database: an overview. Toxicology

and applied pharmacology 202 (3):289-301. doi:10.1016/j.taap.2004.06.023

Cao H, Bowling SA, Gordon AS, Dong X (1994) Characterization of an Arabidopsis Mutant That Is Nonresponsive to Inducers of Systemic Acquired Resistance. The Plant Cell 6:1583-1592

Cao H, Glazebrook J, Clarke JD, Volko S, Dong X (1997) The Arabidopsis NPR1 gene that controls systemic acquired resistance encodes a novel protein containing ankyrin repeats. Cell 88 (1):57-63

Century KS, Shapiro AD, Repetti PP, Dahlbeck D, holub E, Staskawicz BJ (1997) NDR1, a pathogen-induced component required for Arabidopsis disease resistance. Science 278: 1963-1965. science 278:1963-1965

Chisholm ST, Coaker G, Day B, Staskawicz BJ (2006) Host-microbe interactions: shaping the evolution of the plant immune response. Cell 124 (4):803-814. doi:10.1016/j.cell.2006.02.008

Conrath U (2011) Molecular aspects of defence priming. Trends in plant science 16 (10):524-531. doi:10.1016/j.tplants.2011.06.004

Conrath U, Chen Z, Ricigliano JR, Klessig DF (1995) Two inducers of plant defense responses, 2,6-dichloroisonicotinec acid and salicylic acid, inhibit catalase activity in tobacco. Proc Natl Acad Sci U S A 92 (16):7143-7147

Dangl JL, Dietrich Ra, Richberg MH (1996) Death Don't Have No Mercy: Cell Death Programs in Plant-Microbe Interactions. The Plant cell 8 (10):1793-1807. doi:10.1105/tpc.8.10.1793

Delaney TP, Uknes S, Vernooij B, Friedrich L, Weymann K, Negrotto D, Gaffney T, Gut-Rella M, Kessmann H, Ward E, Ryals J (1994) A central role of salicylic Acid in plant disease resistance. Science (New York, NY) 266 (5188):1247-1250. doi:10.1126/science.266.5188.1247

Dewdney J, Reuber TL, Wildermuth MC, Devoto A, Cui J, Stutius LM, Drummond EP, Ausubel FM (2000) Three unique mutants of Arabidopsis identify eds loci required for limiting growth of a biotrophic fungal pathogen. Plant J 24:205-208

Durrant WE, Dong X (2004) Systemic acquired resistance. Annu Rev Phytopathol 42:185-209. doi:10.1146/annurev.phyto.42.040803.140421

Eulgem T, Rushton PJ, Robatzek S, Somssich IE (2000) The WRKY superfamily of plant transcription factors. Trends in plant science 5 (5):199-206

Friedrich L, Lawton K, Ruess W, Masner P, Specker N, Rella MG, Meier B, Dincher S, Staub T, Uknes S, Métraux J-P, Kessmann H, Ryals J (1996) A benzothiadiazole derivative induces systemic acquired resistance in tobacco. The Plant Journal 10 (1):61-70. doi:10.1046/j.1365-313X.1996.10010061.x

Fu ZQ, Yan S, Saleh A, Wang W, Ruble J, Oka N, Mohan R, Spoel SH, Tada Y, Zheng N, Dong X (2012) NPR3 and NPR4 are receptors for the immune signal salicylic acid in plants. Nature 486 (7402):228-232. doi:10.1038/nature11162

Gianinazzi S, Kassanis B (1974) Virus Resistance Induced in Plants by Polyacrylic Acid. Journal of General Virology 23:1-9

Glazebrook J (2001) Genes controlling expression of defense responses in Arabidopsis--2001 status. Current opinion in plant biology 4 (4):301-308

Glazebrook J, Chen W, Estes B, Chang HS, Nawrath C, Metraux JP, Zhu T, Katagiri F (2003) Topology of the network integrating salicylate and jasmonate signal transduction derived from global expression phenotyping. Plant J 34 (2):217-228

Glazebrook J, Zook M, Mert F, Kagan I, Rogers EE, Crute IR, Holub EB, Ausubel FM (1997) Phytoalexin-deficient mutants of *Arabidopsis* reveal that *PAD4* encodes a regulatory factor and that four *PAD* genes contribute to downy mildew resistance. Genetics 146:381-392

Gómez-Gómez L, Boller T (2002) Flagellin perception: a paradigm for innate immunity. Trends in plant science 7 (6):251-256

Görlach J, Volrath S, Knauf-Beiter G, Hengy G, Beckhove U, Kogel K-H, Oostendorp M, Staub T, Ward E, Kessmann H, Ryals J (1996) Benzothiadiazole, a novel class of inducers of systemic acquired resistance, activates gene expression and disease resistance in wheat. Plant Cell 8 (4):629-643. doi:10.1105/tpc.8.4.629

Jones JDG, Dangl JL (2006) The plant immune system. Nature 444 (7117):323-329. doi:10.1038/nature05286

Kassanis B, White RF (1975) Polyacrylic acid -induced resistance to tobacco mosaic virus in tobacco cv. Xanthi. Annals of Applied Biology 79:215-220

Katz VA, Thulke OU, Conrath U (1998) A benzothiadiazole primes parsley cells for augmented elicitation of defense responses. Plant Physiol 117 (4):1333-1339

Knoth C, Eulgem T (2014) High-Throughput Screening of Small-Molecule Libraries for Inducers of Plant Defense Responses. In: Hicks GR, Robert S (eds) Plant Chemical Genomics, vol 1056. Methods in Molecular Biology. Humana Press, pp 45-49. doi:10.1007/978-1-62703-592-7_5

Knoth C, Ringler J, Dangl JL, Eulgem T (2007) Arabidopsis WRKY70 is required for full RPP4-mediated disease resistance and basal defense against Hyaloperonospora parasitica. Molecular plant-microbe interactions : MPMI 20 (2):120-128. doi:10.1094/MPMI-20-2-0120

Knoth C, Salus MS, Girke T, Eulgem T (2009) The synthetic elicitor 3,5-dichloroanthranilic acid induces NPR1-dependent and NPR1-independent mechanisms of disease resistance in Arabidopsis. Plant physiology 150 (1):333-347. doi:10.1104/pp.108.133678

Kohler A, Schwindling S, Conrath U (2002) Benzothiadiazole-Induced Priming for Potentiated Responses to Pathogen Infection, Wounding, and Infiltration of Water into Leaves Requires the NPR1 / NIM1 Gene in Arabidopsis. 128 (March):1046-1056. doi:10.1104/pp.010744.1046

Kunz W, Schurter R, Maetzke T (1997) The Chemistry of Benzothiadiazole Plant Activators. Pesticide Science 50 (4):275-282. doi:10.1002/(SICI)1096-9063(199708)50:4<275::AID-PS593>3.0.CO;2-7

Lawton Ka, Friedrich L, Hunt M, Weymann K, Delaney T, Kessmann H, Staub T, Ryals J (1996) Benzothiadiazole induces disease resistance in Arabidopsis by activation of the systemic acquired resistance signal transduction pathway. The Plant journal : for cell and molecular biology 10 (1):71-82

Layer RW (1963) The Chemistry of Imines. Chemical Reviews 63 (5):489-510. doi:10.1021/cr60225a003

Mattson MP, Calabrese EJ, Son TG, Cutler RG, Camandola S, Chadwick W, Maudsley S, Stranahan AM, Martin B, Ji S, White CM (2010) Hormesis A revolution in biology, toxicology and medicine. springer, New York, NY

McDowell JM, Cuzick A, Can C, Beynon J, Dangl JL, Holub EB (2000) Downy mildew (*Peronospora parasitica*) resistance genes in Arabidopsis vary in functional requirements for *NDR1*, *EDS1*, *NPR1*, and Salicylic Acid accumulation. Plant J 22:523-530

McDowell JM, Dangl JL (2000) Signal transduction in the plant immune response. Trends in biochemical sciences 25 (2):79-82

Metraux JP, Ahlgoy P, Staub T, Speich J, Steinemann A, Ryals J, Ward E (1991) Induced Systemic Resistance in Cucumber in Response to 2,6-Dichloro-Isonicotinic Acid and Pathogens. In: Hennecke H, Verma D (eds) Advances in Molecular Genetics of Plant-Microbe Interactions Vol. 1, vol 10. Current Plant Science and Biotechnology in Agriculture. Springer Netherlands, pp 432-439. doi:10.1007/978-94-015-7934-6_66

Nimchuk Z, Eulgem T, Holt BF, Dangl JL (2003) Recognition and response in the plant immune system. Annual review of genetics 37:579-609. doi:10.1146/annurev.genet.37.110801.142628

Noutoshi Y, Jikumaru Y, Kamiya Y, Shirasu K (2012) ImprimatinC1, a novel plant immune-priming compound, functions as a partial agonist of salicylic acid. Scientific reports 2:705. doi:10.1038/srep00705

Nürnberger T, Lipka V (2005) Non-host resistance in plants: new insights into an old phenomenon. Mol Plant Pathol 6 (3):335-345. doi:10.1111/j.1364-3703.2005.00279.x

Pandey SP, Roccaro M, Schön M, Logemann E, Somssich IE (2010) Transcriptional reprogramming regulated by WRKY18 and WRKY40 facilitates powdery mildew infection of Arabidopsis. The Plant journal : for cell and molecular biology 64 (6):912-923. doi:10.1111/j.1365-313X.2010.04387.x

Sato M, Tsuda K, Wang L, Coller J, Watanabe Y, Glazebrook J, Katagiri F (2010) Network modeling reveals prevalent negative regulatory relationships between signaling sectors in Arabidopsis immune signaling. PLoS pathogens 6 (7):e1001011-e1001011. doi:10.1371/journal.ppat.1001011

Schneider CA, Rasband WS, Eliceiri KW (2012) NIH Image to ImageJ: 25 years of image analysis. Nature methods 9 (7):671-675

Scholes JD, Rolfe SA (1996) Photosynthesis in localised regions of oat leaves infected with crown rust (Puccinia coronata): quantitative imaging of chlorophyll fluorescence. Planta 199 (4):573-582

Segonzac C, Zipfel C (2011) Activation of plant pattern-recognition receptors by bacteria. Current opinion in microbiology 14 (1):54-61. doi:10.1016/j.mib.2010.12.005

Silverman FP, Petracek PD, Heiman DF, Fledderman CM, Warrior P (2005) Salicylate activity. 3. structure relationship to systemic acquired resistance. Journal of agricultural and food chemistry 53:9775-9780

Thulke O, Conrath U (1998) Salicylic acid has a dual role in the activation of defence-related genes in parsley. Plant J 14 (1):35-42. doi:10.1046/j.1365-313X.1998.00093.x

Tornero P, Dangl JL (2001) A high throughput method for quantifying growth of phytopathogenic bacteria in Arabidopsis thaliana. Plant Journal 28:475-481

Toufighi K, Brady SM, Austin R, Ly E, Provart NJ (2005) The Botany Array Resource: e-Northerns, Expression Angling, and promoter analyses. Plant J 43 (1):153-163

Tsuda K, Sato M, Stoddard T, Glazebrook J, Katagiri F (2009) Network properties of robust immunity in plants. PLoS Genet 5 (12):e1000772. doi:10.1371/journal.pgen.1000772

Uknes S, Mauch-Mani B, Moyer M, Potter S, Williams S, Dincher S, Chandler D, Slusarenko a, Ward E, Ryals J (1992) Acquired resistance in Arabidopsis. The Plant cell 4 (6):645-656. doi:10.1105/tpc.4.6.645

Velini ED, Alves E, Godoy MC, Meschede DK, Souza RT, Duke SO (2008) Glyphosate applied at low doses can stimulate plant growth. 496 (January):489-496. doi:10.1002/ps

Wang D, Amornsiripanitch N, Dong X (2006) A genomic approach to identify regulatory nodes in the transcriptional network of systemic acquired resistance in plants. PLoS pathogens 2 (11):e123-e123. doi:10.1371/journal.ppat.0020123

Ward ER, Uknes SJ, Williams SC, Dincher SS, Wiederhold DL, Alexander DC, Ahl-Goy P, Metraux JP, Ryals Ja (1991) Coordinate Gene Activity in Response to Agents That Induce Systemic Acquired Resistance. The Plant cell 3 (10):1085-1094. doi:10.1105/tpc.3.10.1085

Wildermuth MC, Dewdney J, Wu G, Ausubel FM (2001) Isochorismate synthase is required to synthesize salicylic acid for plant defence. Nature 414 (6863):562-565. doi:10.1038/35107108

Wu Y, Zhang D, Chu JY, Boyle P, Wang Y, Brindle ID, De Luca V, Després C (2012) The Arabidopsis NPR1 protein is a receptor for the plant defense hormone salicylic acid. Cell reports 1 (6):639-647. doi:10.1016/j.celrep.2012.05.008

Zipfel C (2014) Plant pattern-recognition receptors. Trends in Immunology 35 (7):345-351. doi:10.1016/j.it.2014.05.004

Zipfel C, Robatzek S, Navarro L, Oakeley EJ, Jones JD, Felix G, Boller T (2004) Bacterial disease resistance in Arabidopsis through flagellin perception. Nature 428 (6984):764-767. doi:10.1038/nature02485

Supplementary Figures

Table 3.S1: List of differentially expressed DPMP-responsive genes identified by mRNA-seq in this study. Supplementary data submitted.

General Conclusion

Plants defend themselves against pathogens by preformed and inducible defense measures. Inducible plant immune responses are triggered by receptor-mediated recognition of pathogen-derived molecules and involve extensive reprogramming of the host transcriptome, proteome and metabolome. So far the plant immune system has been mainly studied by forward and reverse genetic approaches combined by biochemical and transcriptomic studies. Besides several types of immune receptors, signal transducers, transcriptional regulators and defense-executing proteins as critical components of the plant immune system, such studies have uncovered that the regulation of inducible plant immune responses is governed by a complex regulatory network.

The use chemical genetics combined with synthetic elicitors, as specific inducers of defined defense mechanisms, provides an attractive alternative to forward and reverse genetic studies, as this may reveal novel components and regulatory circuits of plant defense network that cannot be accessed through genetic classical analyses. Previously, our lab initiated a chemical genomics-based approach to identify and characterize new types of synthetic elicitors. By high-throughput chemical screening we identified 114 synthetic elicitors that activate expression of the pathogen-responsive *CaBP22-^{333}::GUS* reporter gene in transgenic Arabidopsis. One example of a synthetic elicitor identified by this screen is DCA (Knoth et all., 2009). Although most of these elicitors are structurally related to SA, some of them appear entirely novel. Here first, I

discussed various types of synthetic elicitors that have been used for studies on the plant immune system and crop protection. Furthermore, I described and characterized new types of synthetic elicitors identified in the screen performed by Knoth et al., 2009.

Synthetic elicitors can be used as a tool to dissect plant defense mechanisms. However, they have also been successfully applied to crop protection. In chapter one, I illustrated that the vast majority of known synthetic elicitors belong to the large group of functional SA analogs. They mimic roles of SA in defense induction and their structures are related to this messenger molecule. The dominance of SA-related synthetic elicitors may be due to a bias in the elicitor screening strategies, which have commonly utilized known SA-triggered immune responses as indicators of successful defense induction. Alternatively, it is possible that the SA-dependent sector of the defense network is particularly enriched for drugable targets and that there is a particularly large and diverse set of SA receptor present in plants. In addition to SA-analogs, imprimatins, sulfonamides, adipic acid derivatives and jasmonic acid analogs were classified as synthetic elicitors. While additional screens for synthetic elicitors that are more potent and possibly distinct from those that are known are desirable, the defense-inducing compounds are known at this point already have great potential for research and application. Systematic comparisons of their functional characteristics in a single plant system such as *Arabidopsis* may uncover new features of the plant immune system and may allow to rank them

regarding their efficiencies, potencies, kinetic characteristics and range of pathogens they are effective against.

The characterization of BHTC was initiated by former PhD student Melinda Rodriguez-Salus. She had shown that BHTC quickly and transiently induces disease resistance against *Hpa* in Arabidopsis, and has a distinct mode-of-action from DCA. She also showed that it induces root growth elongation at lower concentrations. I further continue to work with BHTC and have shown that in addition to *Hpa*, it also induces disease resistance of Arabidopsis against *Pst* without being toxic to this pathogen. Experiments with known Arabidopsis defense mutants demonstrated that BHTC interfere with signaling processes downstream or independently from SA and requires both NPR1 and WRKY70 for full defense induction.

The observed dose-dependent effects of BHTC on Arabidopsis root growth are reminiscent of the phenomenon of hormesis. Hormesis is a process that is characterized by low-dose stimulation and high-dose inhibition of biological responses. While the application of BHTC at low doses (lower than 1 µM) triggers root growth enhancement, high doses of this compound (10 µM) suppress root elongation. Although, former student PhD student Melinda Rodriguez-Salus first observed this phenomenon, I further characterized hormetic effects of BHTC and initiated systematic studies to uncover the mechanistic basis of this phenomenon in Arabidopsis. In order to analyze

transcriptional patterns associated with hormesis-induction at low-doses and defense activation at high-doses application of BHTC, I profiled mRNAseq transcriptional responses of 14-d old plate-grown Arabidopsis seedlings. Strikingly, low- and high-dose BHTC treatments triggered highly distinct transcriptional profiles. In both shoots and roots, only the high dose of BHTC induces typical defense-related transcriptional changes, while the low BHTC dose triggered a coordinated inter-compartmental transcriptional response manifested in suppression of photosynthesis and respiration-related genes in the nucleus, chloroplast and mitochondria. To provide further insight into processes of BHTC-mediated hormesis, I tested Arabidopsis defense- and auxin-response mutants with low and high doses of this synthetic elicitor. This implicated the WRKY70 transcription factor in both BHTC-mediated immunity and hormetic root elongation. Additionally, of all of the tested auxin-response mutants *axr1-3* and *slr-1* seem to be required for BHTC-mediated root hormesis. However, these mutants are not required for defense induction that is triggered by this compound. These results link auxin-related signaling process to the hormetic response, but the mechanistic details of this link are still enigmatic and needs be resolved in future studies. My results can be used as a basis to understand connection between defense responses and root development as well as fundamental processes generally underlying hormetic phenomena in plants.

In addition to BHTC, also I have worked on the characterization of DPMP, a member of the large group of PMP derivatives identified by our previous high

throughput synthetic elicitor screen. Similar to BHTC and DCA, DPMP also induces quickly and transiently disease resistance against *Hpa* in Arabidopsis. It also induces disease resistance of Arabidopsis against *Pst* without being toxic to this pathogen. The applied concentrations and the EC_{50} values of DPMP are at least 10 fold lower than that of INA, DCA or BHTC. Based on these characteristics DPMP is a particularly potent synthetic elicitor. The analysis DPMP activity in known Arabidopsis defense mutants demonstrated that this compound interferes with signaling processes downstream or independently from SA (similar to DCA and BHTC). However, unlike DCA, the defense-inducing activity of DPMP is fully-dependent on NPR1. Unlike BHTC, DPMP activity is not fully dependent on WRKY70.

The structure-activity analysis of DPMP showed that two separate moieties of DPMP, 3,5-DCSAL and m-anisidine, which may be released from DPMP by hydrolysis of its imino bridge, can independently induce immune responses in Arabidopsis. The efficiency of 3,5-DCSAL is similar to DPMP, thus this moiety may be mainly responsible for the bioactivity of DPMP. To what extent m-anisidine contributes to the defense-inducing activity of DPMP has to be addressed in future studies. Particularly attractive is the idea that a molecule like DMPM can deliver two independent bioactive moieties with synergistic activities. This is unlikely the case for DPMP, as 3,5-DCSAL seems to be as active as DPMP and the efficiency of m-anisidine is relatively low. However, new molecules can be designed that carry two independent bioactive moieties linked

by a labile molecular bridge that may have more promising properties than DPMP.

The mRNA seq analyses showed that DMPM is likely a functional analog of the defense hormone SA. It induces transcriptional responses that overlap to a large extent with those triggered by other known SA analogs. Of all SA analogs we tested (DCA, INA and BHTC), DPMP is seems to be the most potent one, as it induces strong immunity against *Hpa* at a very low dose. It is possible that the structural differences between DPMP and other SA-analogs affect affinities of these molecules for common target proteins. Alternatively, these differences may affect the selectivity of these compounds to different targets. In addition, these changes might affect uptake and/or metabolic stability of these compounds. Further comparative studies are needed to resolve effects of subtle structural differences between different SA analogs on their performance as plant defense inducers.

PMP and PMP-related compounds are a large group of new synthetic elicitors group. They contain 33 potential synthetic elicitors identified in our high throughput screen. Along with the 10 PMP representatives I characterized, analysis of the remaining PMP candidates identified by our screen will lead to a pool of interesting new molecules that should provide ample information useful for the design of highly potent and efficient novel synthetic elicitors.

The effect of low-dose stimulation and high-dose inhibition of root

elongation was also observed with DPMP. As in the case of BHTC, a high dose of DPMP induced typical defense-related transcriptional changes in both roots and shoots. However, mRNA-seq analysis of low-dose DPMP responses did not reveal useful insights. Only transcript levels of a small number of genes were affected by this type of treatment. This is possibly because the two biological replicates I performed, do not provide sufficient statistical power to uncover relatively weak transcriptional changes. The analysis of additional biological replicates may reveal a clear profile for low-dose DPMP transcriptional changes. Furthermore, the characterization of low-dose responses of DPMP along with BHTC in an extended set of Arabidopsis defense mutants and auxin-response mutants may reveal additional information about whether hormetic effects triggered by these two synthetic elicitors are similar or distinct from each other.

Our lab identified several distinct types of synthetic elicitors. My work further characterized the mode-of action of two of them, BHTC and DPMP regarding to plant defense responses and hormesis. While their targets in plant defense are still yet to be defined, it is clear that these compounds are powerful tools to dissect plant defense responses. I have identified 10 additional PMP-related compounds as synthetic elicitors, which show potential for future research as well. The studies with BHTC and DPMP showed that they have some similar and distinct aspects with each other. Genetic screens can be developed to identify mutants that show altered responses to BHTC and DPMP. The discovered mutants with altered sensitivity to these compounds may point to

novel features of the defense network and allow to identify the target receptor/s of our synthetic elicitors. Once their biological targets are known, these compounds might be used as molecular probes to assist hierarchical analysis of defense-related processes they trigger. In addition to working with Arabidopsis, continued studies with other plants might be of great potential for their use to study of homologous processes across species barriers. As I pointed out in chapter 1, synthetic elicitors have already been shown to valuable tools in plant defense pathway studies. Furthermore, there is a great potential to develop novel pesticide alternatives that are not harmful to the environment and protect plant against pathogen by not being directly toxic to pathogens (and other life forms), but inducing plant immunity.