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From Model System to Crops: Translational Approaches to Design New Crop Disease Protection Strategies Based on Findings in *Arabidopsis thaliana*

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

Doctor of Philosophy

in

Plant Biology

by

Mercedes Mae Schroeder

September 2018

Dissertation Committee:
Dr. Thomas Eulgem, Chairperson
Dr. Isgouhi Kaloshian
Dr. Philip Roberts

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The Dissertation of Mercedes Mae Schroeder is approved:

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Portions of the information in this dissertation are reprints, although in different text and figure format, of the material as it appears in two publications: 1) The synthetic elicitor BHTC (2-(5-bromo-2-hydroxy-phenyl)-thiazolidine-4-carboxylic acid) links plant immunity to hormesis. Plant Physiol. 170, 444-58. 2016

and 2) The synthetic elicitor DPMP (2,4-dichloro-6-{(E)-[(3-methoxyphenyl)imino]methyl}phenol) triggers strong immunity in *Arabidopsis thaliana* and tomato. Sci Rep. 6, doi: 10.1038/srep29554. 2016. The corresponding author, Thomas Eulgem, listed in these publications, directed and supervised the research which forms the basis for portions of Chapter I of this dissertation.

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Dedication

It is with heart-felt gratitude that I dedicate this dissertation to my mom, Professor Susan Schroeder. Thank you, Mom, for your endless support. You are the person that I love, like, respect and admire most in all the world. I could not have done this without you.

ABSTRACT OF THE DISSERTATION

From Model System to Crops: Translational Approaches to Design New Crop Disease Protection Strategies Based on Findings in *Arabidopsis thaliana*

by

Mercedes Mae Schroeder

Doctor of Philosophy, Graduate Program in Plant Biology
University of California, Riverside, September, 2018
Dr. Thomas Eulgem, Chairperson

Plants are subject to attack by microbial pathogens. This research utilized four distinct plant-pathosystems combined with methods of phytopathology, molecular genetics and chemical biology to study mechanisms of plant immunity against microbial phytopathogens. Chemical biology is the use of small molecule compounds to study biological processes. A previous chemical screen identified 114 compounds, called synthetic elicitors, that activate innate immune responses in the model plant *Arabidopsis thaliana* (Arabidopsis). Here, experiments conducted with three synthetic elicitors were found to provide disease protection

in one or more of the following plant-pathogen interactions: *Solanum lycopersicum* (tomato) and *Pseudomonas syringae* pathovar *tomato* (*Pst*), *Vigna unguiculata* (cowpea) and *Fusarium oxysporum* formae speciales *tracheiphilum* race 3 (*Fot*3) or Arabidopsis and *Hyaloperonospora arabidopsidis* (*Hpa*). The results of these studies demonstrated the potential power of synthetic elicitors as both tools for molecular/chemical biology research and for direct protection of agricultural crop plants. In addition to synthetic elicitor-applied research, innovative agar plate-based infection assays were developed to study defense responses in Arabidopsis against *Macrophomina phaseolina* (*Mp*). Dubbed as a "global destroyer of crops", the necrotrophic, soil-borne fungus *Mp* infects more than 500 plant species including many economically important crops. Plant defenses against this powerful pathogen are poorly understood. The novel model phyto-pathosystem was used to quantify *Mp* biomass growth in roots and to analyze shoot disease severity in different Arabidopsis genotypes, quantitatively assessing host factors affecting the outcome of *Mp* infections. By comparative profiling of host transcripts in roots of the wild type Arabidopsis accession Col-0, with and without *Mp* infection, expression changes were uncovered in hundreds of genes potentially contributing to *Mp* defense mechanisms or putative effector targeting schemes. The results established the *Mp*-Arabidopsis interaction as a useful model pathosystem, which allowed for application of the vast genomics-related resources of this versatile model plant to the systematic investigation of previously understudied host defenses against a major broad host-spectrum plant pathogen.

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General Introduction

Plant immunity research

Plants have evolved a complex defense system that includes a wide range of protective mechanisms, from preformed physical barriers (for example, spiny structures, antimicrobial compounds or thick/waxy cuticles) to intricate, genetically-regulated, inducible responses to pathogens (de Wit, 2002). Detailed knowledge of plant immunity broadens the understanding of organismal interactions in the natural world and proposes to benefit humankind by helping to provide a basic necessity, food. Plant defense research is a requisite weapon to fight disease-caused crop loss that could be devastating to future generations of the ever-expanding human population.

Multiple approaches are taken to study plant immunity. For example, ecological, physiological and evolutionary studies often provide a macroscopic view of plants relative to their environment, whereas molecular biology and biochemistry typically analyze molecular mechanisms in and/or surrounding plant cells. Within each discipline, is a broad variety of research areas. Molecular biology alone encompasses studies of transcriptional reprogramming, vesicle trafficking, hormone effects/crosstalk, individual gene function, receptor activity, signaling, post translational regulation, small RNA operation, chromatin modification and more. The research described here uses primarily molecular and chemical

techniques to study plant defense responses to microbial phyto-pathogens. These will be discussed in detail in each chapter. It is important, however, to introduce one method that is less commonly used in plant defense research, but was applied to a large portion of this work, i.e. chemical biology.

Chemical biology (also called chemical genetics) is the use of small molecule compounds to study protein functions or biological processes (Stockwell, 2000; Smukste and Stockwell, 2005; O'Connor *et al*., 2011). The chemicals need to be cell-permeable yet of sufficient complexity to specifically bind to proteins in biological organisms. Chemical biology strategies have been successfully applied to various areas of plant science research, including disease resistance and biocidal pesticide generation (Serrano *et al*., 2007; Schreiber *et al*., 2008; Walsh, 2007). Using a high-throughput screening procedure, the lab of Professor Thomas Eulgem, identified 114 candidate compounds that induce defense reactions in the model plant *Arabidopsis thaliana* (Arabidopsis) (Knoth *et al.*, 2009). These compounds can be referred to as synthetic elicitors because they are synthetic drug-like molecules that interact with regulators of plant immunity and activate disease resistance (Knoth *et al.*, 2009). The work described here has added to the lab's previous chemical biology studies in Arabidopsis and applied them to crop plants. The most robust synthetic elicitors were tested in the lab and greenhouse and compounds were identified that increase crop plant resistance to pathogen infection.

This synthetic elicitor research aims to identify chemicals that will induce the inherent plant immune system with the prospective goal of applying them toward the development of improved crop protection strategies. In 2012, over 24 thousand tons of microbial pesticide (fungicide and bactericide) active ingredients were used in the US alone (http://www.fao.org/faostat/en/#data/RP/visualize). Traditional agricultural pesticides often rely on direct antibiotic or biocidal activity (Casida, 2009). Many are designed to kill a target pathogenic organism by inhibiting key metabolic steps. Such biocidal pesticides can be carcinogenic or act as toxicants to developmental, nervous or reproductive systems in non-target organisms (Aktar *et al*., 2009; Kroschwitz, 1996; Vidhyasekaran, 2004). Agricultural application and runoff of such pesticides can introduce toxins into the air, soil and water, while residue on consumable crop products generates consumer health concerns (Aktar *et al*., 2009). Microbes can develop resistance to pesticides resulting in their inefficiency and the need to create new biocidal compounds (Hollowmon, 2015). In addition, large-scale measures, including pesticide application, solarization, etc., applied to eradicate a given pathogen in the field, can be expensive. Whether the synthetic elicitors used in this research are directly applicable as non-biocidal alternatives to pesticides or they are solely used to gain relevant knowledge of plant defense mechanisms, this work is important because of its potential to aid in protecting the environment by reducing the amount of biocidal pesticides used in agriculture.

Plant defense induction

The screen to identify synthetic elicitors employed knowledge of a specific plant defense pathway involving the plant hormone salicylic acid (SA). SA, jasmonic acid (JA) and ethylene (ET) are principle plant defense hormones, (and are discussed throughout this dissertation), but are not the only phytohormones with immune functions. Auxins, brassinosteroids (BL), gibberellins (GA), cytokinins (CK) and abscisic acid (ABA) can also affect plant immunity (Robert-Seilaniantz *et al*., 2011). Additionally, SA, JA and ET have roles that extend beyond defense, but those functions will not be emphasized here. Several characteristic marker genes are known which are transcriptionally up-regulated upon activation by one or more phytohormones. Synthetic elicitor-responsive expression of such marker genes can be examined by RT-PCR. Phytohormones engage in crosstalk to regulate each other, mobilize signaling cascades and activate or repress regulatory targets as part of a complex signaling network. Generalized oversimplification of crosstalk studies point to SA–JA and SA–auxin signaling pathways as antagonistic and JA–ET, SA–GA, SA–CK and SA–BL as positive or synergistic (JA/ET–ABA could be positive or negative), but direct and indirect hormone interactions are far more complex *in planta*. When a pathogen is recognized by the plant, phytohormones stimulate local and/or systemic responses involving signal transduction via protein-protein and protein-gene interactions toward the activation of defense genes. Major components of

hormone signaling pathways are known and can be exploited to further the understanding of plant immunity. For example, the *Calcium Binding Protein 22* (*CaBP22*) gene, a known component of the SA pathway, was utilized to create a reporter system that could detect activation of the gene *in planta*. A transgenic Arabidopsis line containing a GUS reporter gene driven by a 333 bp fragment of the *CaBP22* promoter (*CaBP22*$^{-333}$::*GUS*) was used in the screen to identify 114 synthetic elicitors that activate a particular branch of the SA signaling pathway (Knoth *et al*., 2009). The work described here utilized the most robust of these compounds to induce SA-mediated immunity in plants.

Plant immunity is multifaceted, including preformed chemical and physical barriers as well as pathogen-induced hormone signaling and genetic reprogramming. The induced defense response is activated upon recognition of a pathogen either through direct interaction with a microbial molecule or through detection of cellular damage caused by the pathogen. Basal defense is the generalized response to a microbe-associated molecular pattern (MAMP) or a damage-associated molecular pattern (DAMP). MAMPs are highly conserved (across *taxa*) regions of microbial molecules that are recognized by plant pattern recognition receptors (PRRs) (Chisholm *et al*., 2006). DAMPs, also recognized by PRRs, are plant cell molecules that are released into the intercellular space when cells are damaged (Choi and Klessig, 2016; Seong and Matzinger, 2004). PRRs typically are cell surface localized receptor kinases or receptor-like proteins that

perceive MAMPs and DAMPs through ligand binding which transduces the interaction via an intra- and intercellular signaling cascade (Zipfel, 2014). Consequently, a plant immune response, termed pattern-triggered immunity (PTI), is activated that can limit pathogenic activity (Chisholm *et al*., 2006). Many pathogens have evolved proteins, called effectors, that suppress PTI resulting in a compatible plant-pathogen interaction in which the pathogen is virulent and the plant is susceptible to infection (Hoefle and Hückelhoven, 2008; Jones and Dangl, 2006; Panstruga, 2003). Such compatible plant-pathogen interactions result in the development of disease symptoms in the plant.

Plants evolved a secondary, more robust (Tao *et al*., 2003), immune response in which plant disease resistance (R) proteins recognize (indirectly or directly) pathogen effector proteins and activate a swift, strong immune response that is termed effector-triggered immunity (ETI) (Cui *et al*., 2015; Eulgem, 2005; Hoefle and Hückelhoven, 2008; Jones and Dangl, 2006). Activated downstream defense gene responses may include release or generation of secondary metabolites, cell wall fortification and the hypersensitive response (HR), a form of programmed cell death typically occurring in plant cells directly in contact with the attacking pathogen (Dangl *et al*., 1996; Malinovsky *et al*., 2014). HR cell death is an efficient immune response against biotrophic pathogens, which are dependent on living plant tissue. During ETI, the plant is resistant, the pathogen is avirulent and the interaction is referred to as incompatible (Lamb, 1994). ETI is a manifestation

of gene-for-gene resistance or race-specific resistance, (although a single R gene can recognize effectors from different microbial *taxa*), a concept based on observations made by plant breeders (Chisholm *et al*., 2006; Flor, 1971; Jones and Dangl, 2001). A plant species that can generally serve as a host for a given host-adapted pathogen species can have different varieties or cultivars that are either resistant or susceptible to distinct races or isolates of the respective pathogen species. The outcome of such race-specific interactions is governed by a pair of (typically dominant) genes in the host and in the pathogen. Certain plant R genes can mediate specific recognition of genetically complementary avirulence (avr) genes of the pathogen (Jones and Dangl, 2001). Only if R and avr genes "match", is a full defense response triggered in the plant resulting in incompatibility. If either the R gene or avr gene of a matching pair is missing in a given plant-pathogen interaction, the result is compatibility and the pathogen can utilize the host, resulting in plant disease symptoms. Plant R genes often encode NLR proteins, a class of immune receptors with C-terminal leucine-rich repeat domains, a central nucleotide biding site and a variable N-terminal domain (Jones *et al*., 2016, Meyers *et al*., 2003). NLRs are known, in many cases, to directly or indirectly recognize pathogen effectors, which serve as the avr component in this concept. Pathogen effectors not recognized by R gene-encoded NLRs contribute to pathogen virulence.

During plant-pathogen interactions, the two-tiered immune system described above, which is controlled by host PRRs and R gene products (i.e. NLRs), is typically inducible by cognate MAMPs or pathogen effectors, respectively, of these receptors. However, it can also be activated by exogenously-applied synthetic elicitors. In either case, a wide variety of defense reactions is triggered, which includes complex transcriptional reprogramming and accumulation of defensive proteins and metabolites. Typically, successful pathogen resistance depends on multiple components of such comprehensive defense response profiles (Nimchuk *et al*., 2003).

In addition to such local immune responses, which are often restricted to pathogen infection sites, plants can activate systemic defense responses that are efficient against a broad spectrum of pathogens. One type of systemic immune state is referred to as systemic acquired resistance (SAR) (Durrant and Dong, 2004). SAR is mediated by SA and can be induced by local compatible or incompatible pathogen interactions. This immune response can also be induced by exogenous application of either SA or SA- analogous synthetic elicitors (Bektas and Eulgem, 2015; Ward *et al*.,1991).

Plants and pathogens

Over fifty years of extensive studies of Arabidopsis immune responses (Provart *et al*., 2016; Nimchuk *et al*., 2003) have made the model plant a valuable

research tool. Arabidopsis is a small, ephemeral (life cycle is less than three months), eudicotyledonous, diploid angiosperm of the family Brassicaceae, with ca. 1,135 known accessions throughout much of the world (Alonso *et al*., 2016), many of which can be easily grown and genetically transformed (Clough and Bent, 1998; Meinke *et al*., 1998). Its genome has been sequenced (Kaul *et al*., 2000) and a large set of Arabidopsis mutants covering almost its entire complement of genes, including many genes controlling immunity, are known and available from stock centers (Alonso *et al*., 2003; Sessions *et al*., 2002). Such mutant lines have been used to identify interaction points of synthetic elicitors within known defense signaling pathways (Bektas *et al*., 2016; Knoth *et al*., 2009; Rodriguez-Salus *et al*., 2016) and to functionally characterize individual genes that are transcriptionally activated at plant-microbe interaction sites (Campisi *et al*., 1999; Schroeder *et al*., 2015). This research utilized Arabidopsis and two crop plant species, *Solanum lycopersicum* (tomato) and *Vigna unguiculata* (cowpea) to study plant immunity.

Tomato is a diploid, eudicot, angiosperm member of the Solanaceae family native to western South America (Bergougnoux, 2014). Its genome has been sequenced and it is considered a major model organism for plant research (The Tomato Genome Consortium, 2012). Not only are tomato plants valuable research tools, tomato fruits are an important food crop. The fruit is popular in multiple countries around the world and is considered highly nutritious

(Frusciante *et al*., 2007). Tomatoes are a major commodity in California, which produces 99% of all processing-tomato products consumed in the US (http://www.californiacountry.org/features/article.aspx?arID=620). In 2014, over 170 million tonnes of tomatoes were produced worldwide (FAOSTAT, http://www.fao.org/faostat/en/#data/QC/visualize). One focus of this work was the protection of tomato plants from bacterial speck disease. As general plant defense inducers, synthetic elicitors will likely be effective against a wide variety of plant pathogens and may provide broad spectrum disease resistance to tomato including bacterial speck. The commercialization of such synthetic elicitors would specifically benefit the California tomato industry and therefore, positively impact the local economy.

Cowpea is a diploid, eudicot angiosperm of the Fabaceae family, native to Africa (Timko *et al*., 2007). Its genome has been recently sequenced (Muñoz-Amatriaín *et al*., 2017) and shares synteny with other agriculturally important legumes, like soybean (Lucas *et al*., 2011). The plant's grains, dried or fresh cowpeas or pods, as well as leaves, are grown and sold for human consumption and stems and leaves are also used as animal fodder (Timko *et al*., 2007). Although cultivated in various regions worldwide, including the southwestern US, parts of Asia, South America and throughout Africa, it is a critical crop for subsistence farmers in developing countries of sub-Saharan Africa, where it provides an important source of dietary protein for consumers (Timko *et al*., 2007). According

to FAOSTAT, over 5 million tons of dry cowpeas were produced worldwide in 2014, 95.7% of which were grown in Africa (http://www.fao.org/faostat/en/#data/QC/visualize). The plants have a high tolerance for drought, heat and unfavorable soil conditions, rendering them invaluable in regions where other crops cannot grow (Muñoz-Amatriaín *et al*., 2017). Breeding programs have been important for the development of cultivars with multiple desirable traits including early maturation time and insect and pathogen resistance (Timko *et al*., 2007). Multiple cowpea cultivars have been developed by researchers within the University of California system, including the two used in this work, 'California Blackeye 5' (CB5) and 'California Blackeye 46' (CB46) (Helms *et al*., 1991). University of California, Riverside- (UCR) based cowpea studies take place in campus greenhouses, at the UCR Agricultural Experiment Station and at field stations in the Central and Coachella Valleys. Over 30 years ago, emeritus Professor Anthony Hall established the cowpea research program to benefit farmers and breeders in both CA and Africa, effectively making cowpea one of UCR's legacy crop plants. Today, scientists in UCR's cowpea research group maintain partnerships with National Agricultural Research Services in Africa, combining genetic studies with breeding strategies to identify plant lines that grow best in the field. The work with cowpea described in this dissertation, has been an important introduction to the multifaceted importance of this crop plant.

Arabidopsis, tomato and cowpea plants are representative of three different plant taxa, being from the Brassicaceae, Solanaceae and Fabaceae families, respectively, thus providing a broad picture of plant defense responses that may relate to various eudicotyledonous agricultural crops. To study plant immunity in these plants, four distinct microbial phytopathogens were incorporated: an oomycete, a bacterium and two fungi.

Depending on their lifestyle and dependency on host plants, microbial pathogens may be categorized as either biotrophic, hemibiotrophic or necrotrophic. The interaction between a necrotroph and its host results in the eventual death of tissue or the entire plant whereas a biotroph requires that the host remain living to provide the pathogen with its nutritional needs. A hemibiotroph initially utilizes living plant cells but may kill plant tissue as the interaction progresses (Qutob *et al.*, 2002). Canonical theory in plant immunity tells us that in the model plant Arabidopsis, defense responses to necrotrophs are largely mitigated by JA and ET whereas biotrophic and hemibiotrophic pathogens elicit SA signaling pathways (Glazebrook, 2005). Several necrotrophic plant pathogens, as well as hemibiotrophs, have a biotrophic phase in their life cycles (Trail, 2009). This early phase may be affected by a synthetic elicitor, as these require fully functional plant cells for their defense-inducing activity. Similar defense mechanisms are activated that change plant gene expression patterns upon pathogen infection in response to both necrotrophs and biotrophs (Katagiri,

2004). According to one study, approximately 50% of the Arabidopsis genes responding to a hemibiotrophic bacteria and a necrotrophic fungus were the same (van Wees *et al*., 2003). Given the complexity of genetic signaling networks in plants, the putative overlap of defense responses to different pathogens and the biphasic lifecycles of some pathogens, it is possible that a synthetic elicitor that induces defense against one pathogen could also increase plant immunity against a very different pathogenic microbe.

The plants and microbes utilized in this research included a model plant, two crop plants, a biotrophic oomycete and a hemibiotrophic bacteria and two necrotrophic fungi (Table I.1), two foliar and two root-infecting pathogens, respectively. Together, the four plant-pathogen interactions formed a comprehensive approach to the study of compatible plant/pathogen interactions.

Hyaloperonospora arabidopsidis (*Hpa*) is an obligate biotrophic, oomycete pathogen of Arabidopsis (McDowell, 2014). Distinct *Hpa* isolates are compatible with particular Arabidopsis accessions, causing downy mildew disease in aerial plant tissues of those accessions. Several incompatible *Hpa*-Arabidopsis interactions have also been described (Parker *et al*., 1996). The genome of one *Hpa* isolate, Emoy2, was sequenced (Baxter *et al*., 2010) and serves as a reference model for downy mildew-causing pathogens and other obligate biotrophic oomycetes (McDowell, 2014). *Hpa* isolates have been valuable tools in the discovery of Arabidopsis resistance genes (Slusarenko and Schlaich,

2003). Although *Hpa* does not infect crop species, its interaction with Arabidopsis allows researchers to study the interaction at a level of molecular detail that is difficult to achieve in most crop plant pathosystems. This information potentially may be applied toward the management of oomycete-crop plant diseases (McDowell, 2014). The *Hpa*-Arabidopsis pathosystem provides a model Brassicaceae family plant in which to initially test candidate synthetic elicitor effects against a pathogenic oomycete, a downy mildew- causing agent and an obligate biotroph.

Pseudomonas syringae pathovar *tomato* (*Pst*), strain DC3000 (Cuppels, 1986), is a gram-negative, phytopathogenic bacterium that infects extracellular regions in aerial plant tissues of both tomato and Arabidopsis (and *Nicotiana benthamiana* if its *hopQ1-1* effector gene has been deleted (Wei *et al*., 2007)). *Pst* causes bacterial speck disease in tomato and the *Pst* – tomato interaction has been a model system for the study of plant defense and bacterial pathogenesis for over three decades (Xin and He, 2013). The relative ease of bacterial genetic transformation and the 2003 sequencing of the *Pst* genome (Buell *et al*., 2003) have made *Pst* a valuable microbial research tool enabling the characterization and identification of genes involved in host surface attachment, secretion systems, toxin formation, sugar or amino acid transport and stress tolerance (Xin and He, 2013). A key focus in virulence studies has been proteins (and the genes that code for them) translocated by the type III secretion system. *Pst*'s

type III secretion system, one of seven different secretion systems, is a protein construct through which *Pst*'s ca. 30 different type III effector proteins, T3Es (Wei *et al*., 2015), can be injected into plant cells to suppress the plant immune system. Variety among T3Es allows for interactions with multiple plant proteins and may provide an avenue for adaptation to new hosts (Wei *et al*., 2015). For example, in tomato, T3Es AvrPto and AvrPtoB interact with the protein kinase Pto and degrade the membrane-associated protein RIN4 resulting in HR (Scofield *et al*., 1996; Luo et al., 2009). AvrPtoB also obstructs plant defense signaling by interrupting Bti9, a protein kinase involved in PTI signaling (Zeng et al., 2012). A notable phytotoxin created and utilized by *Pst* is coronatine. Coronatine is a bacterial jasmonate mimic that has been shown to manipulate plant JA- and SA- response genes to promote pathogen virulence (Zhao *et al*., 2003). The JA-insensitive tomato mutant, *jai1,* is highly resistant to *Pst* and is unresponsive to coronatine, demonstrating a role for coronatine in the activation of JA signaling to foster pathogenicity (Zhao *et al*., 2003). In Arabidopsis, SA promotes systemic acquired resistance against *Pst* (Lawton *et al*., 1995) and since most of the synthetic elicitors we have identified and characterized so far promote activity of the Arabidopsis SA pathway, it is possible that the same compounds will be effective against *Pst* in tomato, especially given the general antagonistic nature of SA and JA and *jai1*'s resistance to *Pst*. *Pst* strain DC3000 has been extensively used in tomato pathogen studies (Oh and Martin, 2011) and was the strain used in this research.

Fusarium oxysporum formae speciales *tracheiphilum* race 3 (*Fot*3) is a soil-borne, necrotrophic fungus that infects root tissues resulting in vascular wilt disease in cowpea plants (Armstrong and Armstrong, 1981). *Fot* is known to have caused disease in cowpea plants in the southeastern U.S. and California, Australia, Brazil and Nigeria (Armstrong and Armstrong, 1981; Assunção *et al*., 2003; Pottorff *et al*., 2014; Summerell *et al*., 2011). Principal among four known *Fot* races, race 3 is widely distributed in California and gave rise to breeding programs for cowpea cultivars that were resistant to the pathogen (Pottorff *et al*., 2012; Smith *et al*., 1999). Of the two cowpea cultivars in this research, CB5 is susceptible to *Fot*3 and CB46 is resistant to race 3 (Ehlers *et al*., 2000). Here, the CB5-*Fot*3 compatible interaction was used to test synthetic elicitor efficacy in cowpea plants. The incompatible CB46-*Fot*3 interaction served as a control. The *Fot3-1* cowpea locus that confers resistance to *Fot*3 consists of four leucine-rich repeat serine/threonine protein kinases (Pottorff *et al*., 2012). One (or more) of these RLKs is likely an R gene (Pottorff *et al*., 2012) that is integral to the race-specific interaction. Over 120 *Fusarium oxysporum* (*Fo*) formae speciales, or strains of the pathogen specific to plant host species, are known (Armstrong and Armstrong, 1981, Katan and Di Primo, 1999). Research with *Fo* and Arabidopsis has added to knowledge of plant immunity against a root pathogen, focusing on phytohormones, molecular responses, secondary metabolites or beneficial rhizosphere-microbes (Berrocal-Lobo and Molina, 2008; Chen *et al*., 2014; Di *et al*., 2016).

Macrophomina phaseolina (*Mp*) is a necrotrophic, soil-borne fungus known to infect over 500 plant species, including forest trees (McCain and Scharpf, 1989; Su *et al.*, 2001). This broad host range pathogen is destroying crop plants worldwide (Kaur *et al*., 2012). In 2016, charcoal rot caused by *Mp* infection was one of the top five diseases resulting in soybean crop loss (yield suppression) in sixteen southern US states, accounting for 7.62 million bushels lost (Allen *et al*., 2017, http://www.mssoy.org/uploads/files/allen-dis-loss-survey-2016.pdf). Since the 2016 average soybean value was $9.20 per bushel (https://www.ers.usda.gov/topics/crops/soybeans-oil-crops/related-data-statistics/), that was an approximate $70.1 million loss for those states in 2016 due to *Mp* in soybean alone. *Macrophomina* is a monotypic genus (Sutton, 1980) whose single species is characterized as having dark mycelia with microsclerotia (an aggregation of 50-200 hyphal cells) imbedded in the hyphae (Kaur *et al*., 2012). Pycnidia, structures containing conidia (spores), may also be present, but were not utilized in this research. *Mp* hyphae penetrate plant epidermal cells, grow intercellularly in roots and other tissues, producing cell wall degrading enzymes (CWDEs) and phytotoxins (Kuti *et al*., 1997; Islam *et al*., 2012), and often colonizing vascular tissue resulting in plant wilting and death (Kaur *et al*., 2012; Mayek-Perez *et al*., 2002). *Mp*'s genome has been sequenced (Islam *et al*., 2012) providing information about potential CWDEs and mechanisms to surmount plant defense responses, important insight into the pathogenesis of this devastating pathogen.

Table I.1 Plant-pathosystems utilized in research

Chapter	Plant name	Pathogen name	Pathogen type	Plant advantages	Pathogen advantages	Experimental goals
I	*Arabidopsis thaliana* Arabidopsis*, *At* Columbia accession Col-0	*Hyaloperonospora arabidopsidis Hpa* isolate Noco2	biotroph	model plant**, T-DNA mutant lines	well characterized, used in our lab	increase synthetic elicitor-induced immune response
I	*Solanum lycopersicum* cultivar Money Maker tomato	*Pseudomonas syringae* pathovar *tomato* strain DC3000 *Pst*	hemibiotroph	global crop, ease of plant growth in chamber	well characterized	identification of effective synthetic elicitors, study tomato resistance to bacteria
I	*Vigna unguiculata* cultivars CB5, CB46 cowpea	*Fusarium oxysporum* f. sp. *tracheiphilum* race 3 *Fot*3	necrotroph	multi-national crop, greenhouse propagation	in-force crop pathogen	identification of effective synthetic elicitors, study cowpea resistance to soil-borne fungus
II	*Arabidopsis thaliana* Arabidopsis, *At* multiple Col-0 mutant lines multiple accessions	*Macrophomina phaseolina Mp*	necrotroph	model plant, several accessions and mutant lines	broad host-spectrum	create novel methods, study plant genetic response to *Mp*

*Abbreviations shown in bold
**Arabidopsis is a model plant because of the facility of its use, growth, genetic transformation, and available genetic information.

Chapter overview

Managing plant-pathogen interactions is of great importance for crop protection. Understanding the mechanisms at work during these interactions is crucial to creating improvements in crop loss and achieving crop security. Despite the presence of a complex plant immune system, many plant-microbe interactions result in plant disease. This is particularly poignant when crops are

lost. Common monoculture farming methods, often with plants of low genetic diversity (Wang, 1999), can provide an optimal environment for pathogen growth (Bruce, 2010; Bruce, 2011). Crop damage affects food availability, affects prices for consumers and results in a loss of revenue for farmers, from large agricultural organizations to small-scale growers in the U.S., to subsistence farmers in developing areas of the world. Plant immunity is often studied with the ultimate aim of reducing crop loss to disease. This goal requires research in multiple areas, from cellular and molecular mechanisms to agricultural field work.

The studies presented in this dissertation began in the laboratory with the model plant Arabidopsis and the pathogenic oomycete *Hpa*. The induction of molecular plant defense pathways in Arabidopsis using synthetic elicitors was being studied. Activation of specific plant defense pathways by synthetic elicitors was found to protect plants against infection by *Hpa* (Bektas *et al.*, 2016; Knoth *et al.*, 2009; Rodriguez *et al.*, 2016). The next step was to test the effect of these bioactive compounds in other phyto-pathosystems. Translational research is the practice of transferring findings from basic scientific studies in the lab to their application in the field (agricultural, medical or other) and was the impetus for Chapter I. Chapter I hypothesis: *Drug-like compounds that induce natural plant immune responses in the model plant Arabidopsis can provide protection to crops against agriculturally relevant diseases*. This chapter describes experiments in which synthetic elicitors protected tomato plants against *Pst*

bacteria and cowpea plants against *Fot*3 fungus. These two pathogens were chosen because of their ability to be used in reproducible, quantitative experimental assays and because they represent distinct disease processes. *Pst* mainly infects and reproduces in intercellular spaces in shoot tissues, whereas *Fot*3 invades through the roots, entering the shoot through vascular tissue and causing wilt. Chemical elicitors that promote broad spectrum disease resistance in crop plants have a potential application that is far more efficient than the use of pesticides that target one type of pathogen. Compounds induced defense responses in tomato and cowpea such that compound-treated plants showed lower levels of infection relative to untreated plants. Tests with the Arabidopsis-*Hpa* phytopathosystem and either combinations of synthetic elicitors or comparisons between compound derivatives sought more robust activation of immunity. Experiments were conducted in the lab and greenhouse. The results of these studies illustrate the potential power of synthetic elicitors as both tools for molecular/chemical biology research and for direct protection of agricultural crop plants.

Chapter II describes a novel approach to study plant defense responses to the broad host-spectrum pathogenic fungus *Mp*. Interactions between *Mp* and Arabidopsis have not been described or characterized in peer-reviewed literature. Chapter II hypothesis: *Novel techniques can be utilized to study plant defense responses to the broad host-spectrum pathogen Macrophomina*

phaseolina. Here, Arabidopsis plants were infected with *Mp*. Arabidopsis has been the principal source for understanding the molecular mechanisms of plant immunity (Povart *et al.*, 2016) and is unparalleled in stable genetic transformation capability among multicellular organisms (Somerville and Koornneef, 2002). I established methods to 1) quantify *Mp* microsclerotia in Arabidopsis roots and 2) assess *Mp*-induced Arabidopsis shoot disease severity to be able to utilize a large set of Arabidopsis mutants with well-characterized defects in regulatory processes controlling immunity (Povart *et al.*, 2016). Increased susceptibility to *Mp* in Arabidopsis defense mutants relative to wild type plants revealed plant defense mechanisms against *Mp* that may be potentially exploited and utilized in crop agriculture to protect against *Mp* or related broad host-spectrum pathogens. Long-term benefits include enhanced agricultural tools for subsistence farmers in developing countries such as those in sub-Saharan Africa where *Mp* crop damage is a severe problem (Muchero *et al.*, 2011).

This work studied four distinct plant-pathogen interactions to add to the understanding of plant immunity. Synthetic elicitor derivative and combinatorial tests were performed in pursuit of new avenues for stronger plant defense response induction. Potent plant defense elicitors that are active in the two tested crop plants (Chapter I) have the potential to benefit agriculture by promoting the design of new strategies to improve disease resistance in crops like tomato and cowpea. Defense-inducible genes with major roles in immunity against a broad

host-spectrum pathogen (Chapter II) could be used to create a plant line to screen for novel synthetic elicitors that are effective against *Mp* and pathogens like it. Broader impacts of this research are several-fold, from generation of new scientific knowledge to economic benefits and solutions for crop protection, food quality and environmental health. Future studies may use these synthetic elicitors to identify novel cellular targets that are key to plant immunity, further characterize known interactions in pathosystems or serve as leads for the development of new types of sustainable crop protection procedures that are less hazardous to the environment than current biocidal pesticides. Fewer toxic pesticides could result in safer working environments for field workers, reduced food safety concerns and improved air and water quality for organisms throughout the ecosystem.

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Chapter I: Synthetic Elicitors as Protectants against Microbe-Caused Diseases in Cowpea, Tomato and Arabidopsis Plants

Abstract

This research utilizes plant-pathogen interactions and chemical biology to study mechanisms of plant immunity. Chemical biology is the use of small molecule compounds (cell permeable yet of sufficient complexity to bind to proteins in biological organisms) to study biological processes. In 2009, a high-throughput chemical biology screening procedure identified 114 compounds, called synthetic elicitors, that induce defense reactions in the model plant *Arabidopsis thaliana* (Arabidopsis). Basic and translational research with three distinct synthetic elicitors, BHTC (2-(5-bromo-2-hydroxy-phenyl)-thiazolidine-4-carboxylic acid), DPMP (2,4-dichloro-6-{(E)-[(3-methoxyphenyl)imino]methyl}phenol) and DC-SAL (3,5-dichlorosalicylaldehyde) is described in this chapter. The efficacy of these compounds was tested in three dissimilar plant-pathosystems, 1) *Vigna unguiculata* (cowpea) and the fungus *Fusarium oxysporum* formae speciales *threchiephilum*, 2) *Solanum lycopersicum* (tomato) and the bacterium *Pseudomonas syringae* pv. *tomato* and 3) Arabidopsis and the oomycete *Hyaloperonospora arabidopsidis* (*Hpa*). Chemical elicitors that promote broad spectrum disease resistance in crop plants have a potential application that is far more efficient than the use of pesticides that target one type of pathogen.

Introduction

The research presented in this chapter aimed to induce the inherent immune systems of *Vigna unguiculata* (cowpea) and *Solanum lycopersicum* (tomato) plants to provide resistance to fungal and bacterial pathogens using potentially non-biocidal chemical alternatives to pesticides. Using chemical biology, the use of small molecule compounds to study biological processes (Stockwell, 2000; Smukste and Stockwell, 2005; O'Connor *et al*., 2011), the lab of Thomas Eulgem previously identified novel synthetic plant defense elicitors that induce immune responses in the model plant *Arabidopsis thaliana* (Arabidopsis) (Bektas *et al*., 2016; Knoth *et al*., 2009; Rodriguez-Salus *et al*., 2016). Described here, are the most robust of these synthetic elicitors tested in cowpea and tomato plants in the hope of transferring our laboratory findings to their application in the field of agricultural crop protection. In addition, combinations of synthetic elicitors were tested and comparisons between compound derivatives were performed with the Arabidopsis-*Hyaloperonospora arabidopsidis* (*Hpa*) phytopathosystem to ascertain more robust activation of immunity. The results of these studies demonstrated the potential power of synthetic elicitors as protectants for agricultural crop plants and as tools for chemical biology research.

The plant immune system has evolved to provide defense against a broad spectrum of pathogens. Yet, mechanisms for pathogens to invade plants without triggering strong defense responses frequently emerged during their co-evolution

with their hosts (Kessmann *et al*., 1994). Plus, centuries of plant breeding with a focus on yield have resulted in some cultivars that no longer efficiently recognize certain pathogens (Bruce, 2010), partially losing defense capabilities and rendering crop plants more vulnerable to disease. Furthermore, monoculturing, a common contemporary practice in large-scale agriculture, can lead to rapid spreading of pathogenic microorganisms. If the estimated annual USD 9.2 billion spent on crop-use pesticides in the United States actually saves USD 60 billion worth of crops (that would have been destroyed without pesticide use) (Gianessi and Reigner 2005; Gianessi and Reigner 2006; Gianessi 2009; Popp *et al*., 2013), then it is not surprising that growers invest in pesticides. However, conventional biocidal pesticides are typically designed to kill target pests and pathogens and, as such, act as neurotoxins, carcinogens or reproductive and developmental toxicants (Kroschwitz, 1996; Stenersen, 2004; Vidhyasekaran, 2004), posing risks to non-target organisms in the agroecosystem. Agricultural application and runoff of such pesticides can introduce toxins into the soil, water and air, while chemical residue on consumable crop products is cause for consumer health concerns (Aktar *et al*., 2009). Another problem with traditional pesticides is that microbes can develop resistance to them resulting in an ongoing need to create new biocidal compounds (Hollowmon, 2015). Chemicals with a low toxicity potential, that induce immunity in crop plants improving resistance to disease-causing pathogens, could reduce the need for biocidal pesticides.

An initial screen to find novel synthetic elicitors, identified 114 small drug-like compounds that activate a branch of the plant immune system controlled by salicylic acid-dependent signaling mechanisms, by interacting with cellular regulators of immunity and activating disease resistance in Arabidopsis plants (Knoth *et al*., 2009). The first of these compounds to be studied in depth, 3,5-dichloroanthranilic acid (DCA), was determined to be non-biocidal and of low toxicity, yet protected Arabidopsis seedlings against the pathogenic oomycete *Hpa*, as well as Arabidopsis and tomato plants against the bacterial pathogen *Pseudomonas syringae* pathovar *tomato*, strain DC3000 (*Pst*) (Knoth *et al*., 2009; *3,5-Dichloroanthranilic acid*; MSDS No. D56004; Sigma Aldrich: Saint Louis, MO, November 10, 2016.)

Next, research with the synthetic elicitor 2-(5-bromo-2-hydroxy-phenyl)-thiazolidine-4-carboxylic acid (BHTC) found that this compound not only activated a defense response in Arabidopsis plants against *Hpa* and *Pst* at doses of 100 - 200 μM, but it also had a hormetic effect at lower concentrations. Hormetic effects can be caused by a wide range of physical or chemical stimuli and are manifested by a decrease in biological performance in response to high doses/intensities of these stimuli, while low doses/intensities stimulate enhanced biological performance (Calabrese and Baldwin, 1997; Calabrese and Baldwin, 2002; Calabrese and Blain, 2009). At low doses (0.05 – 0.1 μM BHTC), BHTC increased wild type (Col-0) Arabidopsis primary root length, but inhibited root growth at 1 -

10 μM BHTC. Fascinatingly, low and high doses of BHTC triggered qualitatively different transcriptome changes. These results were published in a paper by Rodriguez-Salus and co-workers (Rodriguez-Salus *et al*., 2016). I added to this paper by testing the effects of BHTC on both bacterial-infected tomato and fungal-infected cowpea plants. BHTC significantly reduced severity of disease symptoms in both tomato and cowpea (Rodriguez-Salus *et al*., 2016). The data are presented both here, as well as in Rodriguez-Salus *et al*., 2016.

Of the 114 synthetic elicitors identified in the original screen, at least 30 featured a fundamental phenylimino-methyl-phenol (PMP), or related, skeleton (Bektas *et al*., 2016). These distinct PMP compounds displayed a wide variety of structural differences, yet all induced a plant immune response in the screen by activating expression of the pathogen-responsive *CaBP22-333*::*GUS* construct in transgenic Arabidopsis plants (Knoth *et al*., 2009). Thus, the general PMP skeleton can maintain biological activity even with varied chemical structures. Within such a large class of synthetic elicitors, it was hypothesized that there may be members that exhibit enhanced performance in tomato and cowpea plants. One PMP, 2,4-dichloro-6-{(E)-[(3-methoxyphenyl)imino]methyl}phenol (DPMP), was found to be substantially more potent than DCA and most other PMP derivatives in Arabidopsis tests. DPMP at 10 μM, 100 μM DCA and 100 μM BHTC induced a similar level of defense response in Arabidopsis seedlings against *Hpa*. It was further determined

that DCA was more robust than BHTC, but less so than DPMP (Bektas *et al*., 2016).

With DPMP as the most robust synthetic elicitor in Arabidopsis to date, it was anticipated that this compound would also exhibit higher potency in crop plant studies. DPMP treatment significantly reduced *Pst* growth in tomato plants at a lower dose than BHTC (50 μM DPMP versus 200 μM BHTC), but the reduction in tomato plants was less striking than the defense responses seen in comparable Arabidopsis pathogen assays (Bektas *et al*., 2016). DPMP treatment did not significantly reduce disease symptoms in cowpea plants. It is possible that a higher DPMP concentration may have been effective in cowpea. However, the phytotoxicity of the solvent this synthetic elicitor is applied with prevented those assessments. The data on effects of DPMP in tomato is presented both here and in Bektas *et al*., 2016.

Dissimilar species of plants may respond differently to each synthetic elicitor due to the variation of physical and physiological attributes between plants. Thicker tissues alone, in tomato and cowpea plants relative to Arabidopsis plants, may pose an additional barrier to the efficiency of synthetic elicitor absorption. Various physicochemical parameters are known to affect biological tissue absorption of compounds (Lipinski *et al*., 2001; Vidhyasekaran, 2004) and several studies have shown that derivatives of bioactive molecules can significantly differ in *in planta*-

efficacy (Asami et al., 2001; Lipinski *et al*., 2004; Surpin *et al*., 2005). Therefore, 3,5-dichlorosalicylaldehyde (DC-SAL), a derivative of DPMP, and other related compounds were tested in defense studies with cowpea, tomato and Arabidopsis plants. Application of DC-SAL significantly reduced severity of disease symptoms in all three plants. The effect of DC-SAL treatment was comparable to that of DPMP in tomato, but this derivative of DPMP was far more effectual than its parent compound in cowpea plants, providing a reduction of disease symptoms at just 100 μM DC-SAL. Furthermore, DC-SAL is much less expensive than DPMP. In an effort to further increase the potency of DC-SAL applications, multiple 1) concentrations of DC-SAL, 2) related compounds to assess the impact of individual chemical moieties and 3) combinations of both were tested in Arabidopsis and tomato plants. The results with DC-SAL have not yet been published and are exclusively described in this thesis chapter.

As discussed in the General Introduction, plant pattern-triggered immunity (PTI) is typically inducible by interactions of cognate microbe-associated molecular patterns (MAMPs) or damage-associated molecular patterns (DAMPs) with plant pattern recognition receptors (PRRs) during compatible plant-pathogen interactions (Chisholm *et al*., 2006). PTI can limit pathogen virulence and the fact that it can also be activated by exogenously-applied synthetic elicitors means that it can be exploited toward the goal of increased crop plant protection. Pathogens that are able to overcome plant basal defense could potentially face new obstacles

in the forms of stronger or reiterated plant defense responses when synthetic elicitors are applied in a crop setting. In addition to local defense responses, plants can activate systemic immune responses that are effective against a broad spectrum of pathogens. One such state is systemic acquired resistance (SAR) (Durrant and Dong, 2004). SAR is mediated by salicylic acid and can be induced by local compatible or incompatible pathogen interactions as well as by exogenous application of either salicylic acid or salicylic acid-analogous synthetic elicitors (Bektas and Eulgem, 2015; Ward *et al*.,1991). Synthetic elicitors may be xylem-mobile when applied to roots (as in our tomato root drench assays) and move throughout the plant with the water/transpiration system or possibly be phloem-mobile and distributed in a bi-directional manner (from the origin of application to remote tissues and vice versa).

Most synthetic elicitors and defense primers so far described belong to the following five classes: (1) salicylic acid analogs, (2) imprimatins, (3) sulfonamides, (4) adipic acid derivatives and (5) jasmonic acid analogs (Bektas and Eulgem, 2015). Typically, synthetic elicitors directly induce plant defense responses. Priming is when a stress-related stimulus (e.g., wounding or microorganism interaction) or compound increases the plant's capacity to activate an immune response, resulting in a faster and stronger defense reaction after pathogen infection (Bektas and Eulgem, 2015; Conrath *et al*., 2002). Salicylic acid analogs, such as DCA, BHTC, DPMP, BTH, 2,6-dichloro-isonicotinic acid (INA),

probenazole, tiadinil, isotianil, *N*-cyanomethyl-2-chloroisonicotinamide (NCI) and 3-chloro-1-methyl-1H-pyrozole-5-carboxylic acid (CMPA), may either mimic salicylic acid functions by molecularly interacting with salicylic acid receptors or trigger salicylic acid-related responses without interfering with physiological salicylic acid targets (Bektas and Eulgem, 2015). Imprimatins of classes A and B, prime immunity by increasing endogenous levels of salicylic acid, while class C imprimatins serve as weak salicylic acid analogs (Noutoshi *et al*., 2012b,c). Sulfonamides may act by priming (Noutoshi *et al*., 2012a) or direct defense induction (Schreiber *et al*., 2008). Adipic acid derivative mixtures (Flors *et al*., 2001) have been shown to activate plant immunity but lack thorough mode-of-action studies (Bektas and Eulgem, 2015). Jasmonic acid analogs increase transcript levels of genes naturally targeted by this plant stress hormone and induce signaling processes controlling responses to wounding, herbivory, necrotrophic pathogens and, also, various developmental processes (Pluskota *et al*., 2007).

Overall, data provided in this chapter provided proof-of-concept indicating that salicylic acid-analogous synthetic elicitors can provide, to some extent, protection against various diseases in the crop systems cowpea and tomato. The most promising compound studied was DC-SAL. This synthetic elicitor induced robust and strong immunity in both tested crop systems, but is much less expensive than its parent compound DPMP. DC-SAL could be used as a lead compound for the

design of commercial pesticide alternatives based on synthetic elicitors. The importance of the work presented in this chapter lies in its contribution to the understanding of plant-pathogen interactions and its potential to protect the environment from the harmful effects of traditional agricultural pesticides by providing leads for non-biocidal plant protection alternatives.

Results

Three distinct phyto-pathosystems were used in this research

The synthetic elicitor research included three different phyto-pathosystems: (1) cowpea plants and the soil-borne, necrotrophic fungus *Fusarium oxysporum* formae speciales *tracheiphilum* race 3 (*Fot*3), (2) tomato plants and the hemibiotrophic bacterium *Pst* and (3) Arabidopsis plants and the obligate biotrophic oomycete *Hpa*. The pathogen infection assays used for each of the three synthetic elicitors tested were as follows.

*Fot*3 infects cowpea root tissue resulting in vascular wilt disease (Armstrong and Armstrong, 1981). Of the two cowpea cultivars used in this research, ('California Blackeye 5' (CB5) and 'California Blackeye 46' (CB46) (Ehlers *et al*., 2000; Helms *et al*., 1991)), the CB5-*Fot*3 compatible interaction was used to test synthetic elicitor efficacy in cowpea plants and the incompatible CB46-*Fot*3 interaction (also synthetic elicitor-treated and untreated) served as a control (another control was chemically treated, but uninfected CB5 plants). *Fot*3 was media-propagated in the lab and its spores used to quantitatively inoculate 8-12 day-old cowpea roots using a greenhouse root clip and dip assay (Ehlers et al., 2009). To determine the optimal titer of *Fot*3 spores for the inoculum, resulting in visibly measurable disease symptoms within weeks, yet avoiding excessive infection levels, a dilution curve experiment was performed and the titer 10^4 *Fot*3 spores/ml was selected

(Figure 1.1A). Plants were grown in the greenhouse (Figure 1.1B) until disease symptoms became apparent, then each plant was assessed and given a whole plant score as a measure of disease severity. Percent vascular discoloration (visible vascular browning height in stem divided by total stem height) was also used to determine infection levels (Figure 1.1C).

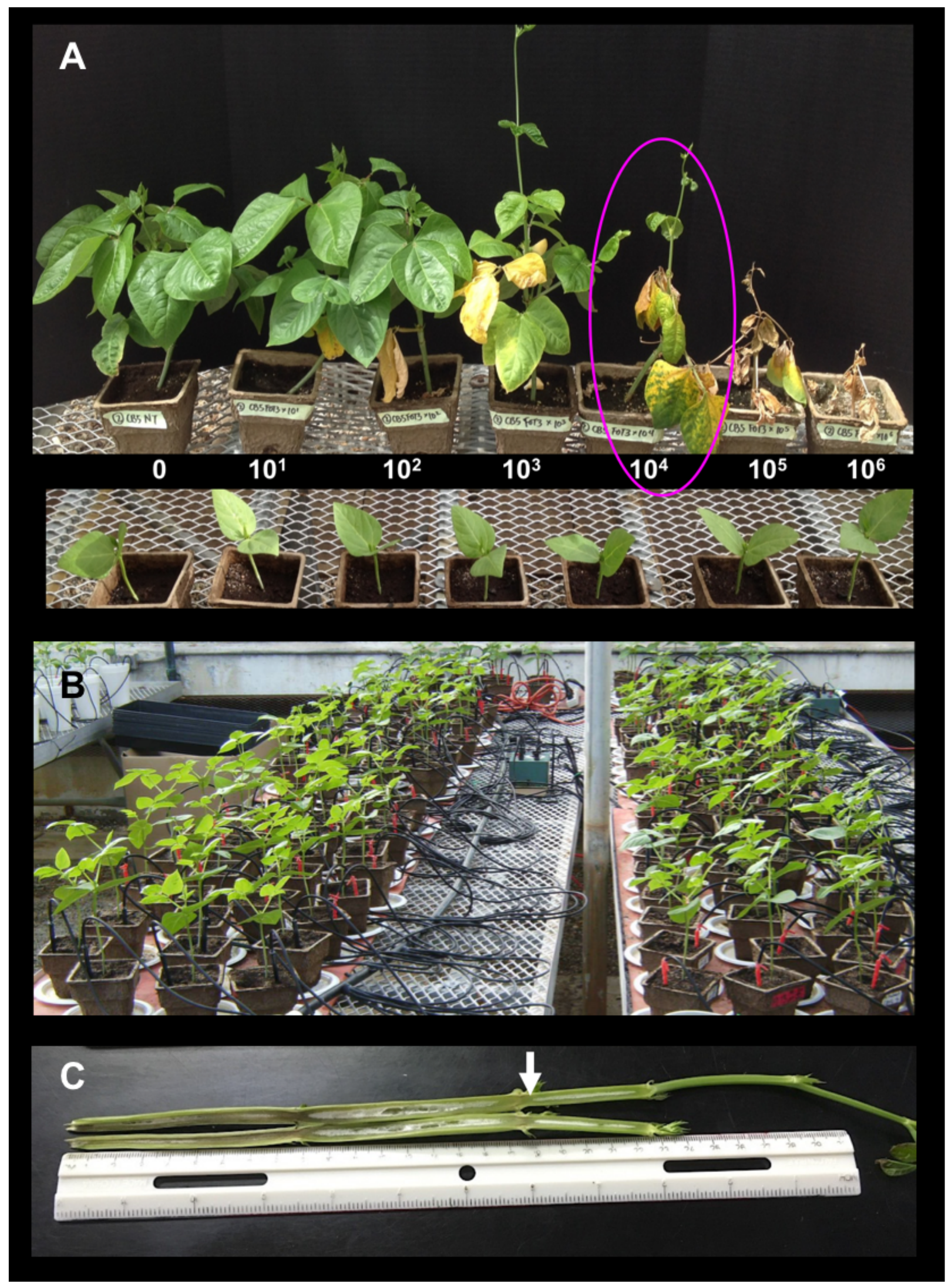


Figure 1.1. *Fusarium oxysporum* formae speciales *tracheiphilum* race 3 (*Fot*3) caused wilt disease in CB-5 *Vigna unguiculata* (cowpea) plants. A) Seedling roots clipped and dipped in water containing 10^4 *Fot*3 spores/ml showed clear, measurable disease symptoms five weeks post infection, circled in pink. **B**) Plants grew under greenhouse conditions until disease symptoms were apparent. **C**) A white arrow points to the visible end of vascular discoloration at a height of 17.8 cm in an infected stem (most leaves were removed prior to measurement).

Pst causes bacterial speck disease in tomato plants (Zhao *et al*., 2003) and infects and produces various disease symptoms in Arabidopsis plants (Schreiber *et al*., 2008; Whalen and Staskawicz, 1990; Whalen *et al*., 1991). In Arabidopsis, salicylic acid promotes systemic acquired resistance and local immunity against *Pst* (Lawton *et al*., 1995). Since BHTC and DPMP induce Arabidopsis salicylic acid signaling, it seemed possible that the same compounds would be effective against *Pst* in tomato. In the tomato-*Pst* assay, four week-old plants were first root-drenched with either the synthetic elicitor-containing solution or a mock, solvent only, solution, then, 24 hours later, shoot-dipped in *Pst* inoculum and finally, bacterial colony formation was counted three days post-infection. At the three-day leaf collection period, disease symptoms were apparent in the form of brown specks surrounded by a halo of yellow chlorosis on many leaves (Figure 1.2).

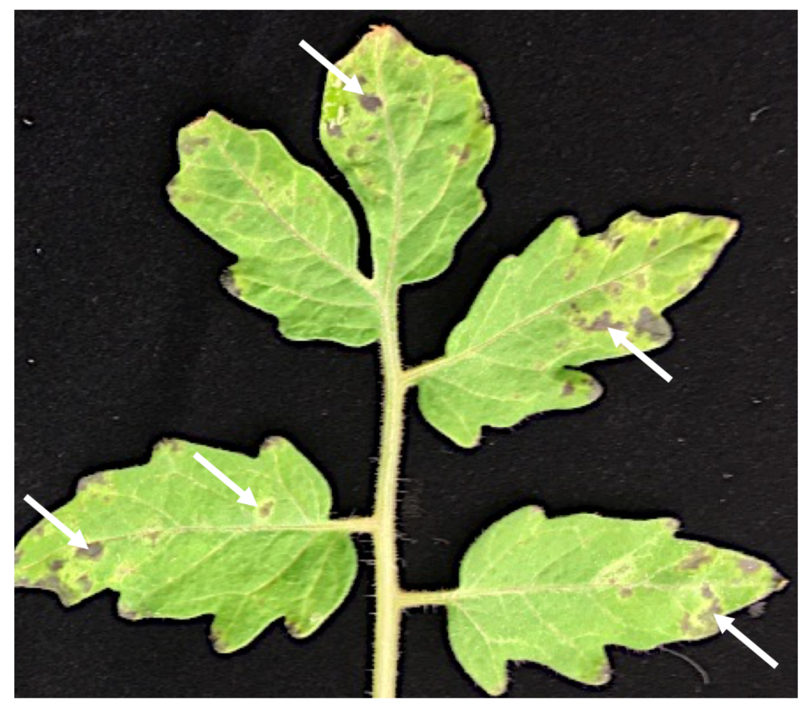


Figure 1.2. *Pseudomonas syrigae* pathovar *tomato* (*Pst*) caused bacterial speck disease in *Solanum lycopersicum* (tomato) plants. A four week-old (cv Moneymaker) plant leaf was photographed three days post *Pst* infection. White arrows point to brown areas of cell death surrounded by chlorosis; these "specks" are indicative of bacterial infection in the leaf tissue.

Hpa causes downy mildew disease in aerial Arabidopsis tissues (McDowell, 2014). Although *Hpa* is not known to infect crop plants, information on synthetic elicitor effects on the Arabidopsis-*Hpa* interaction may potentially be applicable toward the management of related oomycete-crop plant diseases, such as those caused by *Phytophthora infestans* in potato (Leesutthiphonchai et al., 2018), *Plasmopara viticola* in grape vines or *Peronospora effusa* in spinach (Buonassisi *et al*., 2017; Feng *et al*., 2013; McDowell, 2014). The Arabidopsis-*Hpa* system has been

extensively described in previous literature (Bektas *et al*., 2016; Holub *et al*., 1994; Knoth *et al*., 2009; McDowell, 2014; Rodriguez *et al*., 2016).

Synthetic elicitor BHTC reduced disease symptoms in cowpea and tomato plants

Aerial portions of cowpea seedlings were sprayed with approximately 1 ml of either 100 μM BHTC or a mock solution containing 0.15% of the solvent dimethyl sulfoxide (DMSO) 24 hours prior to *Fot*3 infection. To infect the plants, seedling roots were clipped and dipped in a *Fot*3 spore inoculum and individually planted in soil-containing pots. Plants grew under greenhouse conditions and were sprayed twice weekly with either BHTC or the mock solution until disease symptoms were apparent. As the seedlings matured, they were able to tolerate higher amounts of the DMSO solvent that was needed to keep BHTC dissolved in water. Therefore, higher concentrations of BHTC could be applied to the plants as they grew. During the first week after infection, plants were sprayed twice with either 100 μM BHTC or 0.15% DMSO, during the second week with 200 μM BHTC or 0.3% DMSO, during the third week with 500 μM BHTC or 0.75% DMSO, during the fourth week with 750 μM BHTC or 1.125% DMSO, and during the fifth week with 1 mM BHTC or 1.5% DMSO. Both BHTC and mock solutions contained the same percentage of solvent. BHTC was applied bi-weekly because BTHC-mediated immunity is only transiently active in Arabidopsis (Rodriguez-Salus *et al*., 2016). This application

regime was intended to allow for a long-lasting defense activation and to provide continual support if the pathogen was able to repress plant defense responses. Disease was visually evident when main plant stems obtained a faint brown hue and/or leaf chlorosis became visible. At this time, each plant was assessed and given a whole plant disease score. In two independent greenhouse experiments, (n = 100 individual plants per treatment in each experiment), a moderate, but significant difference in the average whole plant score was seen between BHTC- and mock-treated plants (Figure 1.3 A-B). BHTC application appeared to delay disease symptoms resulting in more plants with low disease scores and fewer dead plants than those plants that had been sprayed with the mock solutions (Figure 1.3C). This difference in whole plant disease scores between BHTC-and mock-treated plants was statistically significant at scores of “2” and “5” on the 0-5 scale, in which a healthy plant is scored as “0” and dead plants as “5” (Figure 1.3C).

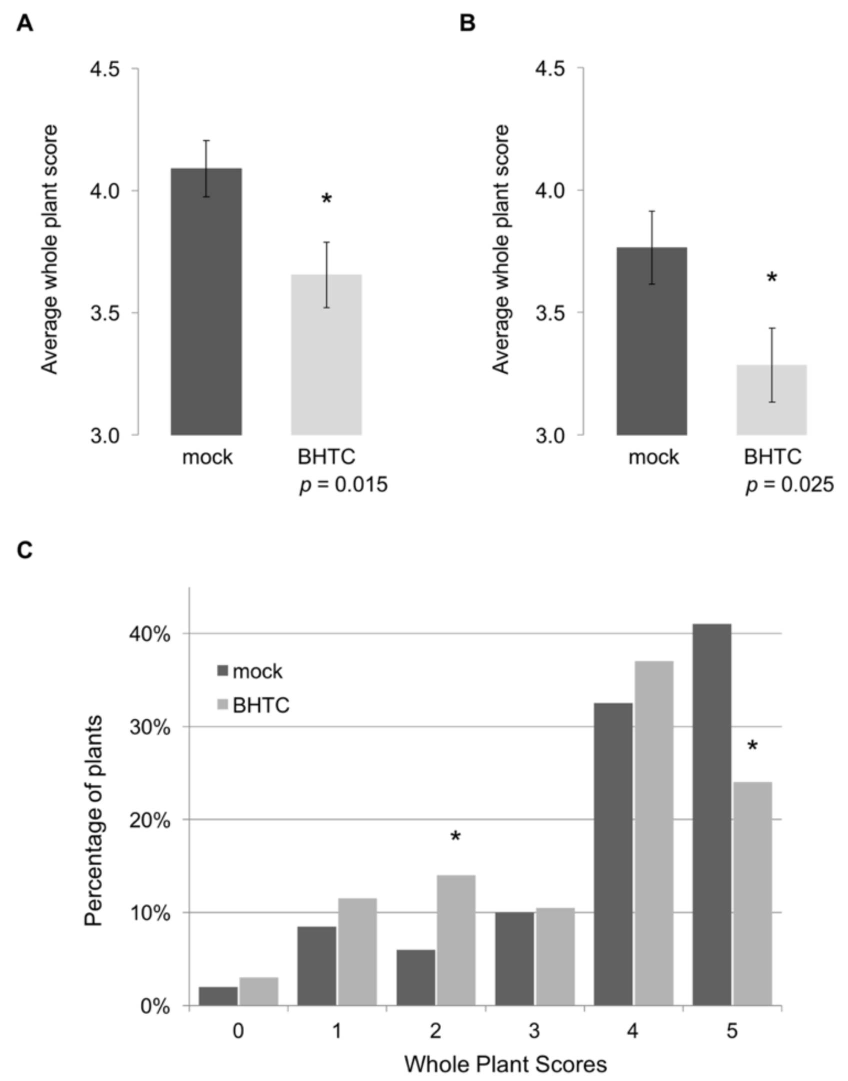


Figure 1.3. BHTC treatment reduced disease symptoms caused by *Fot*3 in cowpea plants. Plants were sprayed twice weekly with either BHTC or the mock solution. Two independent greenhouse experiments are shown, n = 100. **A**-**B**) Each experiment demonstrated a slight, but significant whole plant score difference. Asterisk; Student's *t*-test $p < 0.05$. **C**) The same two experiments combined, shown are the percentage of plants per treatment binned by whole plant score. More BHTC-treated plants had lower scores at the end of the infection period than mock-treated plants. Asterisk; chi-square test $p < 0.05$. The data shown are a combination of two independent experiments, each with 200 individual plants for each type of treatment.

BHTC treatment also reduced bacterial growth in tomato leaves. Tomato plants were root drenched with either 200 μM BHTC or the mock solution 24 hours prior to *Pst* infection. *Pst* growth was measured as colony forming units (cfu) per mg of tomato leaf fresh weight (FW) and compared on day zero (1 hour post infection) and on day three (3 dpi) in plants from each treatment group (Figure 1.4). *Pst* growth was significantly reduced (by approximately 0.5 log units) in plants that had received a root drench treatment containing BHTC (Figure 1.4).

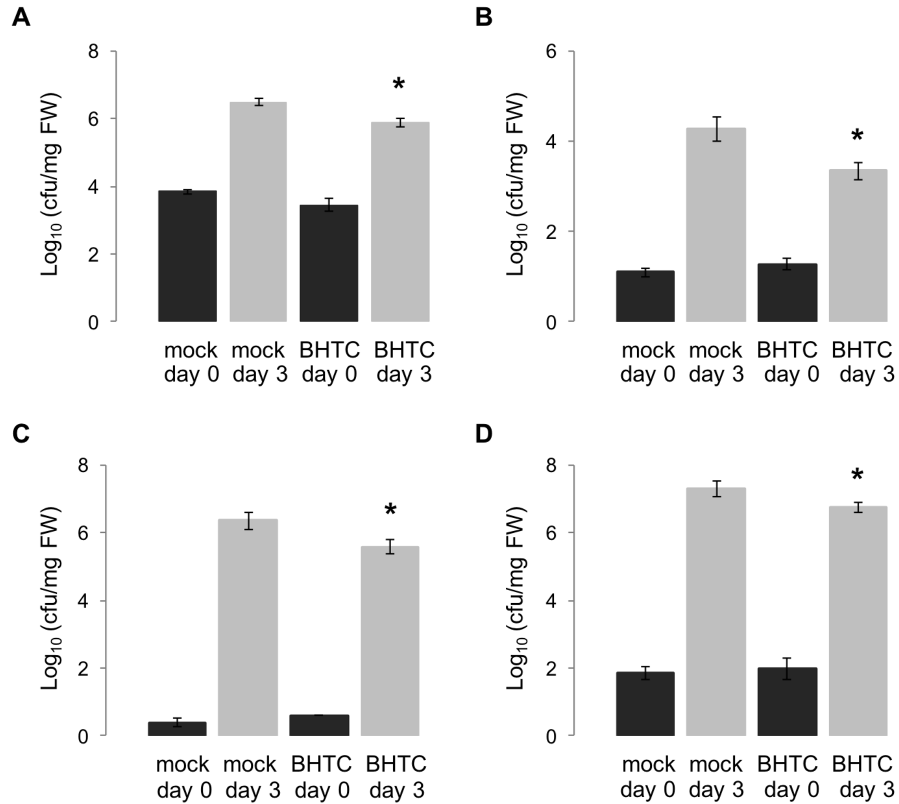
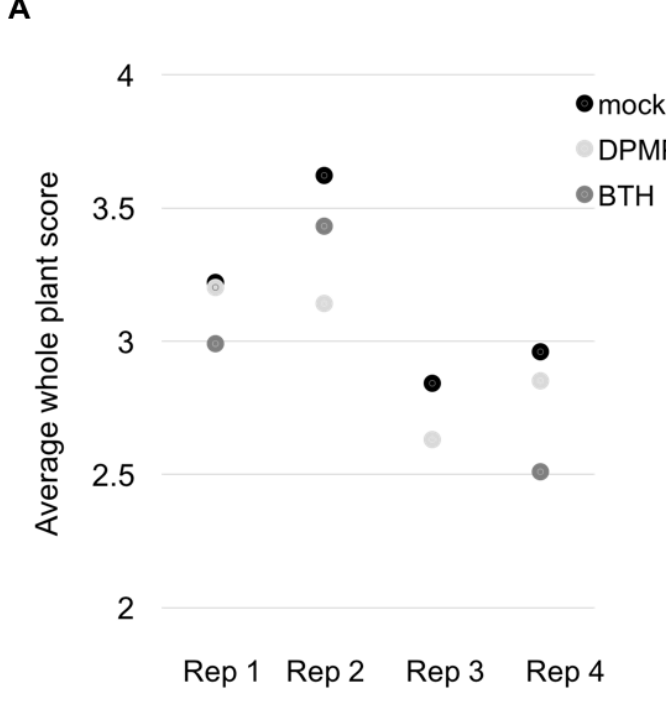


Figure 1.4. BHTC treatment reduced disease symptoms caused by *Pst* in tomato plants. Plants were root drenched with either 200 μM BHTC or the mock solution (solvent only) 24 hours prior to *Pst* infection. Independent replicates are shown (A-D). The error bars are based on technical replicates, n=3-6 pots, with 3 plants per pot. Asterisk; Student's *t*-test $p < 0.05$, cfu; colony forming units, FW; fresh weight of all leaves from plants in a pot.

Synthetic elicitor DPMP triggered immunity in tomato plants

Treatment of cowpea plants with 100 μM – 400 μM DPMP over the course of an experiment appeared to reduce *Fot*3-induced disease symptoms relative to mock-treated control plants (2 – 8 % DMSO). The difference in average whole plant score was not statistically significant, but a trend was visible over four independent experiments (Figure 1.5A). Interestingly, DPMP-treated cowpea plants also showed a trend of increased stem height, compared to mock-treated plants, in three out of those same four experiments (Figure 1.5B). BTH, a known salicylic acid analog has been studied (Maffai *et al*., 2011) and commercially used in crop protection as the product Actigard (Syngenta). A set of BTH-treated plants was included in three of the experiments. BTH-treated plants tended to have lower average whole plant disease scores and lower stem heights than mock-treated plants (Figure 1.5). However, these trends were not statistically significant.



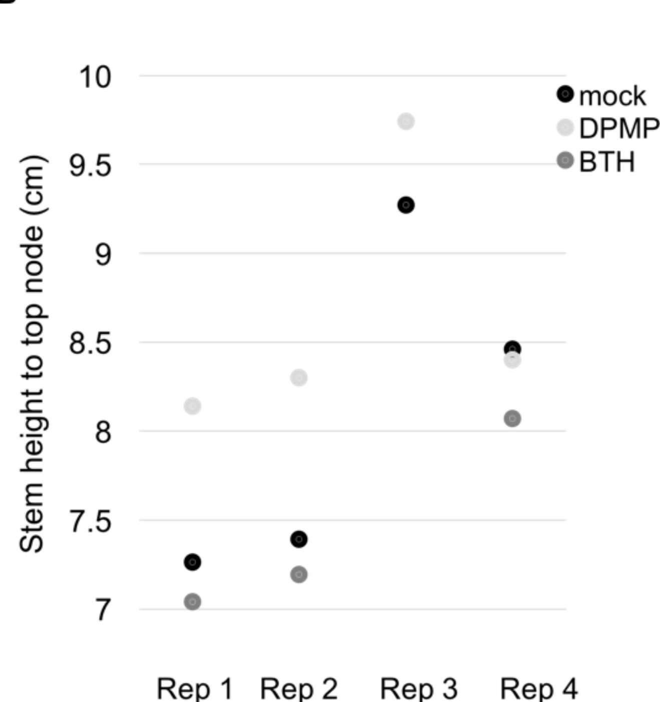


Figure 1.5. DPMP-treated cowpea plants showed *Fot*3-induced disease symptom reduction and increased stem height trends. A) DPMP-treated plants had lower whole plant scores than mock- treated plants. **B**) DPMP-treated plants were generally taller than than mock- treated or BTH-treated plants. Plants were sprayed twice weekly with either DPMP, BTH or the mock solution. Each dot is the average whole plant disease index score for a given treatment group in a biological replicate: black; mock-treated plants, light grey; DPMP-treated plants, dark grey; BTH-treated plants. Compound concentration ranges: 2-8 % DMSO (mock), 100 µM – 400 µM DPMP, 100 µM – 400 µM BTH. Rep; independent biological replicate. Rep 1; n = 50 plants per treatment, rep 2; n = 50, rep 3; n = 65, rep 4; n = 100.

DPMP-treated tomato plants, however, had significantly lower levels of *Pst* bacterial growth in leaves, compared to mock-treated plants (Figure 1.6). Three representative experiments, with a single 50 μM DPMP root drench (Figure 1.6A-C), and an additional experiment with a 100 μM DPMP root drench, are shown in figure 1.6. Both DPMP concentrations gave similar results, a significant reduction in bacterial growth relative to mock with a 0.3-0.6 log_{10} unit difference, between the two treatment groups. Experiments with a single 50 μM DPMP root drench were presented in figure 3C of Bektas *et al*., 2016. At 50 μM DPMP, and even at 100 μM DPMP, the synthetic elicitor concentration was lower than the 200 μM BHTC successfully applied in the tomato-*Pst* assay (Figure 1.4), designating DPMP as a more robust synthetic elicitor in tomato plants.

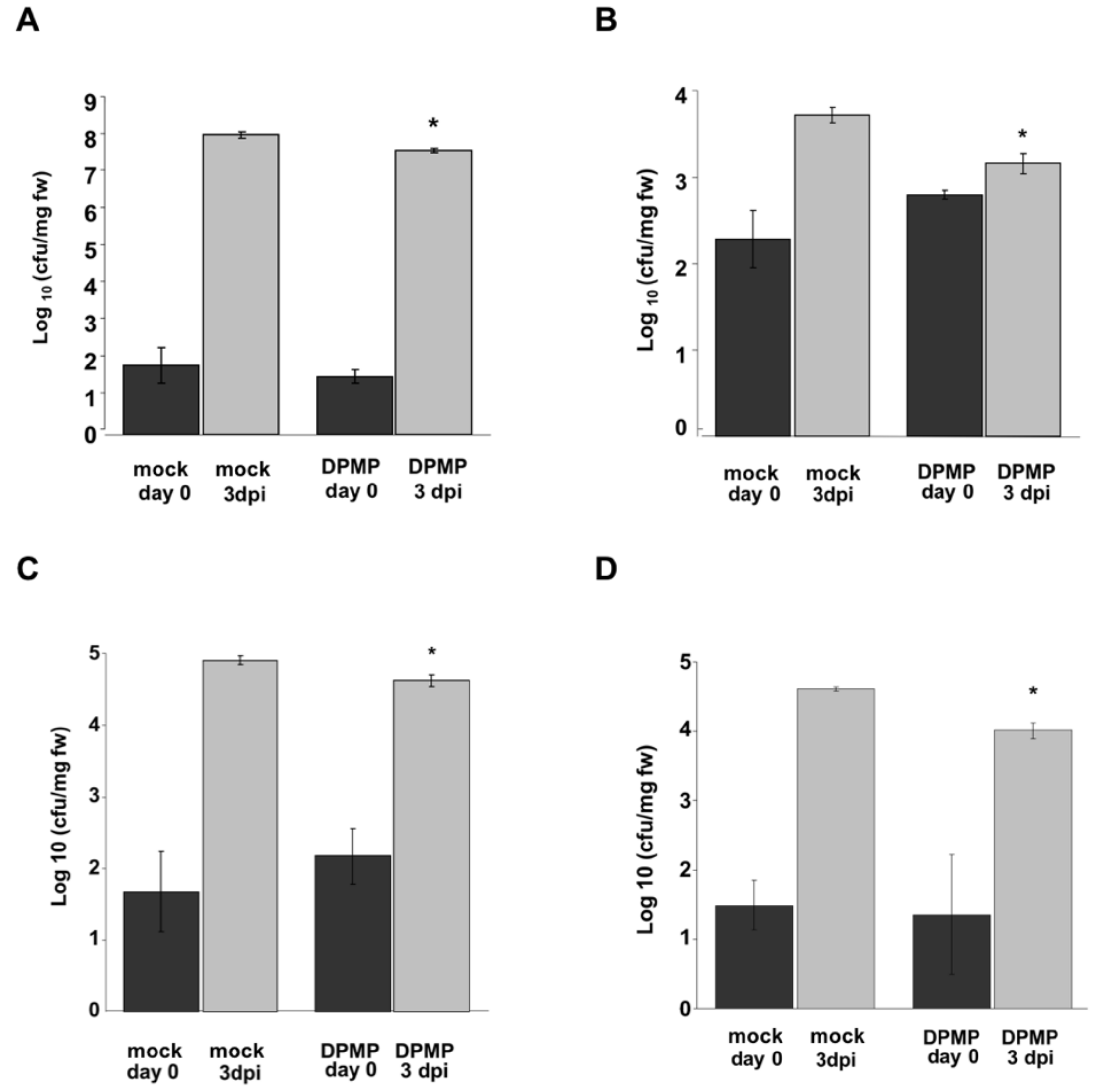


Figure 1.6. DPMP-treated tomato plants had lower levels of *Pst* bacterial growth in leaves. Tomato plants were root drenched with either 2-3 % DMSO (mock) solution or 50 µM DPMP (**A**-**C**) or 100 µM DPMP (**D**) 24 hours prior to being dipped in a *Pst* inoculum. Independent biological replicates are shown; **A**) n = 3 pots; Student's *t*-test $p = 0.026$, **B**) n = 5 pots; $p = 0.003$, **C**) n = 6 pots; $p = 0.031$, **D**) n = 4 pots; $p = 0.037$, each pot had 3 plants, dpi; days post infection. The error bars are based on technical replicates.

Real time quantitative reverse transcription PCR (RT-qPCR) (Figure 1.7) and semi-quantitative reverse transcription (sqRT-PCR) (data not shown) experiments were performed with leaf tissue from the tomato-DPMP-*Pst* experiments to analyze possible tomato gene expression level changes (relative quantification normalized to the reference gene *SlUbi3*) following DPMP and/or *Pst* application. At four weeks of age, plants received a single 50 μM DPMP root drench then were dipped in a *Pst* inoculum (or mock solution) 12 h later. Tissue was collected 24 or 84 hours after the root drench, with and without *Pst* infection. According to the RT-qPCR results, *Pathogenesis Related protein 1 b1* (*SlPR1b1*, Solyc00g174330 or Y08804) transcript levels were higher in DPMP-treated plants at both time points when no *Pst* was present, but *SlPR1b1* dropped to the level in mock-treated plants 72 hours following *Pst* dip (Figure 1.7). *SlPR1b1* is known to be induced by salicylic acid (Tornero *et al*., 1997) and serves as a marker for a salicylic acid signaling pathway. Beside showing that the general salicylic acid-responsive defense marker gene *SlPR1b1* was up-regulated by DPMP, these results supported two interesting conclusions: (1) DPMP seemed to act by directly inducing immunity and not by priming defense responses, as no increase beyond DPMP-induced *SlPR1b1* transcript levels were observed when treatment with the synthetic elicitor was followed by pathogen infection. (2) The reduction of *SlPR1b1* transcript levels in DPMP-treated plants observed 72 hours after *Pst*-infection may have been caused by bacterial effector activity counteracting the induction of immune responses by DPMP.

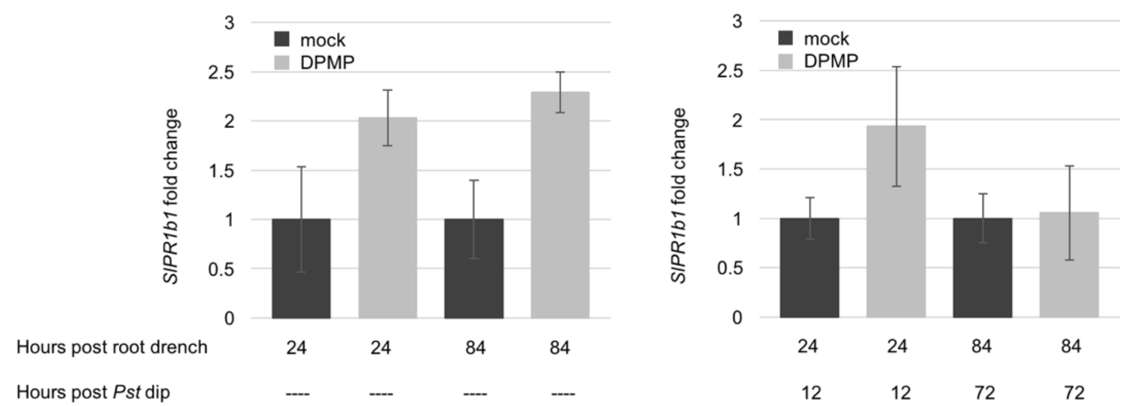


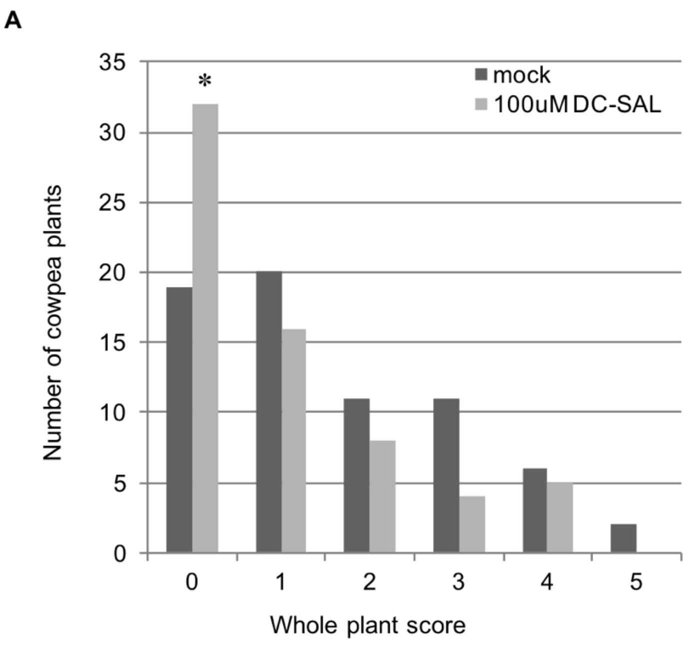
Figure 1.7. RT-qPCR data pointed to differential expression of *SlPR1b1* with DPMP or *Pst* treatments. Uninfected tomato plants showed an increase in *SlPR1b1* expression 24 and 84 hours post root drench with 50 µM DPMP (left). When plants were dipped in *Pst* 12 hours after the compound root drench, *SlPR1b1* increased at 24, but not at 84 hours post root drench. Error bars indicate technical replicates. *SlUbi3* served as the internal control gene.

DC-SAL aided cowpea, tomato and Arabidopsis plant immune responses

Of all of the synthetic elicitors tested in Arabidopsis, DPMP had the highest potency, triggering robust immunity against *Hpa*Noco2 at concentrations ten-fold lower than other compounds (Bektas *et al*., 2016). It was speculated that in plant tissues, DPMP degrades into two simpler compounds, one of which, DC-SAL, is of equal potency to its parent molecule DPMP. DC-SAL has a much simpler structure than DPMP and is substantially cheaper, (the latter making it a better candidate for larger scale crop plant treatment assays). Thus, this compound was tested for its ability to protect crop plants from diseases.

Cowpea plants treated with 100 μM DC-SAL showed a clear reduction in *Fot*3-induced disease symptoms relative to mock-treated plants. Aerial portions of plants were sprayed twice weekly with either 100 μM DC-SAL (a single concentration) or the mock solution until disease symptoms appeared. When whole plant scores were combined for three independent experiments, almost twice the number of DC-SAL-treated plants maintained a score of zero (a healthy-looking phenotype, like those of uninfected negative control plants) than mock-treated plants (Figure 1.8A). DC-SAL-treated plants also had significantly lower percent vascular discoloration (Figure 1.8B).

A



B

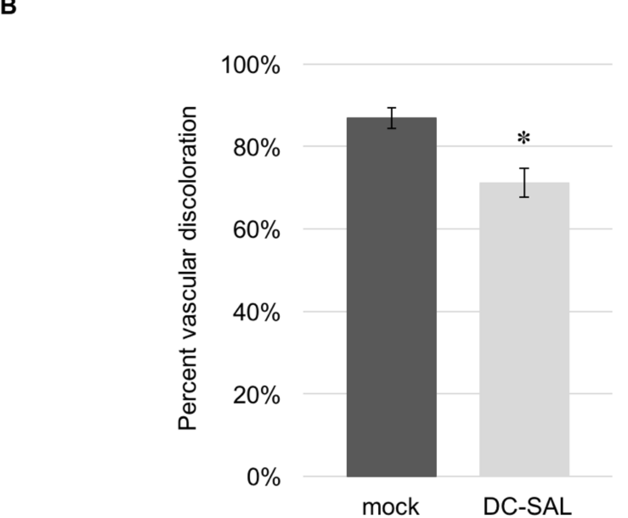


Figure 1.8. Cowpea plants treated with 100 μM DC-SAL showed reduced *Fot*3-induced disease symptoms relative to mock-treated plants. Plants were sprayed twice weekly with either 100 μM DC-SAL or the mock solution. The graphs show three independent greenhouse experiments combined, n = 20-25 per treatment in each experiment. **A**) Asterisk; chi-square test of independence for disease index score of 0 $p = 0.04191$. **B**) Vascular discoloration was measured in each plant stem and the height was divided by the total stem height (up to the fourth node). Asterisk; Student's *t*-test $p < 0.05$.

Only high concentrations of DC-SAL (250 μM-1 mM) inhibited growth of *Fot*3 when the fungus was grown on media containing the compound (Figure 1.9A). At 10 μM DC-SAL, *Fot*3 growth appeared unaffected (the same as no treatment) and when grown with 100 μM DC-SAL, *Fot*3 growth area was slightly reduced and partially discolored (Figure 1.9A). Thus, DC-SAL appeared far less toxic to the fungus than BHTC and DPMP. Despite the BHTC and DPMP tests having been conducted for seven days and the DC-SAL test for 10 days, the reduction in *Fot*3 growth at 100 μM BHTC and 100 μM DPMP (relative to growth on their DMSO solvent-only comparison plates) was clearly higher than on 100 μM DC-SAL (Figure 1.9). The known fungicide Prochloraz, used as a control, fully inhibited *Fot*3 growth in these assays at a concentration as low as 10 μM.

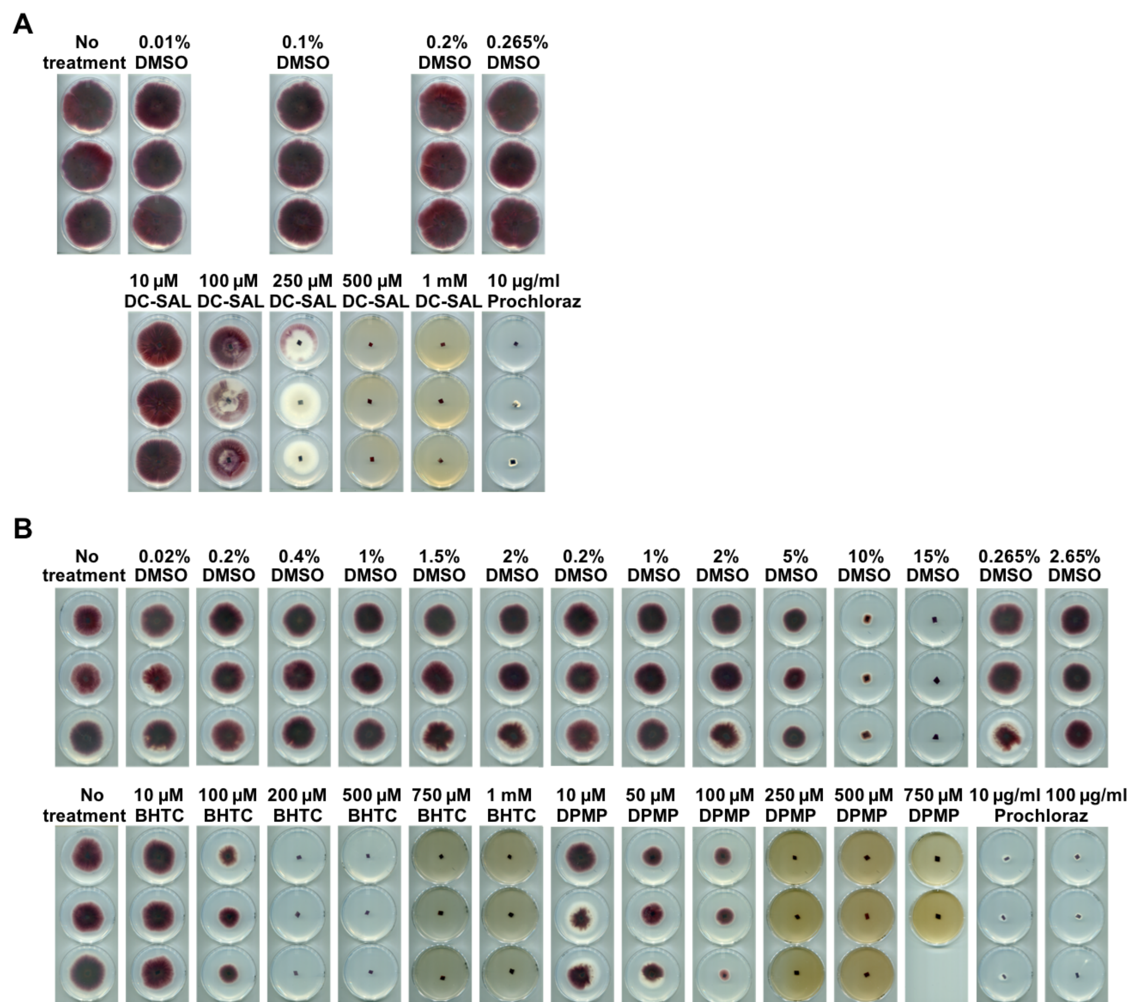
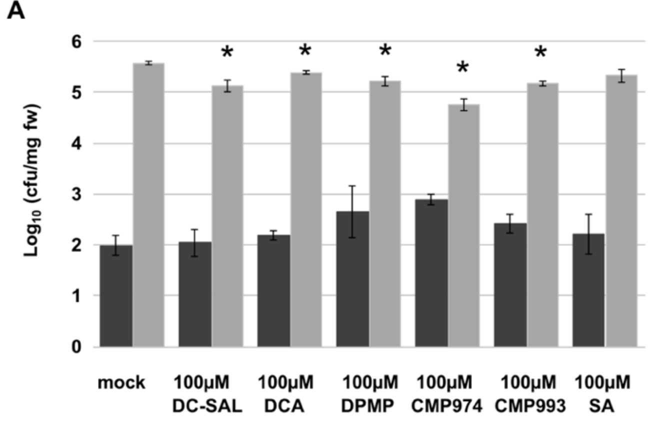
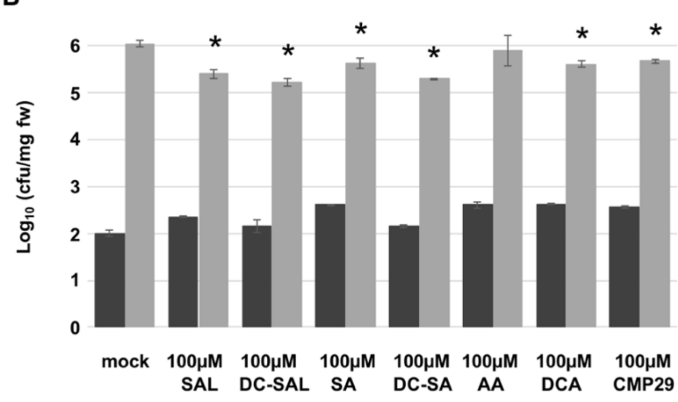


Figure 1.9. Low concentrations of BHTC, DPMP or DC-SAL were benign but high compound concentrations appeared to be detrimental to *Fot*3 growth. **A**) *Fot*3 was grown on PD agar plates with 10 µM – 1 mM DC-SAL or 10 µg/ml Prochloraz or the corresponding mock solution (containing DMSO) for ten days. DC-SAL was tested only once (shown). **B**) *Fot*3 was grown on PD agar plates containing 10 µM – 1 mM BHTC, 10 µM – 750 µM DPMP, 10 -100 µg/ml Prochloraz or the corresponding mock solution (containing DMSO) for seven days to test the direct effects of these compounds on *Fot*3. BHTC and DPMP were tested twice, with similar results; a single biological replicate is shown. Prochloraz, a fungicide, served as the negative control in this plate assay.

Various derivatives of the synthetic elicitors DPMP, DC-SAL and DCA were compared for their potency in protection of tomato against *Pst*. The PMP derivatives DPMP, CMP974 and CMP993, as well as DCA and DC-SAL (Figure 1.10A) and several compounds structurally related to them (Figure 1.10B) significantly reduced *Pst* bacterial growth in tomato plants relative to mock (solvent only) treatment. The leaves of DC-SAL-treated plants often showed visibly fewer disease symptoms than mock-treated plants (Figure 1.10C). Tomato plants were grown under high humidity to promote bacterial growth. Leaf tip tissue dieback was visible in all chemically-treated plants (Figure 1.10C, white arrowheads). It was unclear if this was caused by the solvent in the root drench or the extremely high humidity over the three-day infection period. RT-qPCR data indicated that DPMP may induce *PR1b1* in tomato 24 h and 84 h post root drench (Figure 1.7). As a derivative of DPMP, future determination of tomato defense gene expression conducted with DC-SAL, would be informative. Additional tomato genes to test include *Proteinase INhibitor II* (*SlPinII*, Solyc03g020080.2.1 or AY129402.1), which is induced by jasmonic acid and serves as a marker for a jasmonic acid signaling pathway (Fidantsef *et al*., 1999), *Allene Oxide Cyclase* (*SlAOC*, Solyc02g085730), which encodes an important enzyme in jasmonic acid biosynthesis (Ziegler *et al*., 2000) and *Aminocyclopropane Carboxylic acid Oxidase 1* (*SlACO1*, Solyc07g049530.2 or X58273) which encodes a key enzyme in ethylene biosynthesis (Jafari *et al*., 2013). *Pst* activates *SlPinII* expression

(Fidantsef *et al*., 1999) and so can serve as a control in synthetic elicitor gene activation experiments.





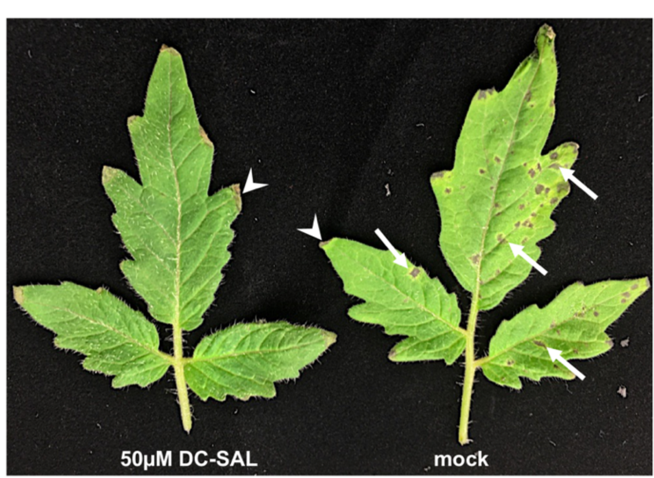


Figure 1.10. Initial experiments indicated that various PMP and di-chlorinated compounds may provide protection in tomato against *Pst*. A) PMP compounds (DPMP, CMP974 and CMP993), DCA and DC-SAL significantly reduced *Pst* bacterial growth in four week-old tomato plants. **B**) DC-SAL and several similarly structured compounds increased plant defense relative to mock (solvent only) treatment. Asterisk; Student's *t*-test $p < 0.05$, error bars indicate technical replicates. Salicylic acid; SA, anthranilic acid; AA. Black bars; day 0, grey bars; day 3. **C**) DC-SAL treated plant leaves (left) often showed fewer disease symptoms than mock-treated plant leaves (right). White arrows point to *Pst*-induced disease "specks". White arrowheads point to leaf tip dieback, possibly induced by the solvent.

As seen in these experiments (Figure 1.10), both DC-SAL and DCA aided tomato plants in defense against *Pst*. DCA has been shown to activate immunity in tomato and Arabidopsis plants previously (Knoth *et al*., 2009). To test for a synergistic response, the two compounds were applied together in both plant species. Arabidopsis seedlings were treated with either the mock solution, DCA, DC-SAL or DCA plus DC-SAL 24 hours prior to infection with *Hpa*Noco2 spores. Potential synergistic interactions between the two synthetic elicitors would have been revealed if combined application of two compounds had resulted in a "greater-than-additive" reduction of disease parametes compared to application of single synthetic elicitors. In each individual comparison, the total synthetic elicitor concentration remained constant (e.g. 10 μM) and the effects of single synthetic elicitors at this concentration (e.g. 10 μM) were compared to those of a mixture of the two compounds in which each was applied at half the total synthetic elicitor concentration (e.g. each at 5 μM). Significantly increased disease protection by the mixture compared to that of each single synthetic elicitor application would have indicated potential synergism.

Higher compound concentrations were initially tested (10 μM, 2 μM) (Figure 1.11A), but synergism was not evident. Next, lower compound concentrations were used to minimize the robust action of DC-SAL and putatively increase the sensitivity of the assay to see a more distinct response (1 μM) (Figure 1.11B). However, synergism between DC-SAL and DCA was not seen in any of the

experiments performed with the Arabidopsis-*Hpa* pathosystem. Synergistic activity was also tested in tomato plants root-drenched with either a mock solution, 100μM DCA, 100μM DC-SAL, or 50μM DCA plus 50μM DC-SAL 24 hours prior to being dipped in a *Pst* inoculum and *Pst* infection was then measured 3 dpi. Again, the synthetic elicitors were effective against *Pst* individually, but compound synergism was not evident (Figure 1.11C).

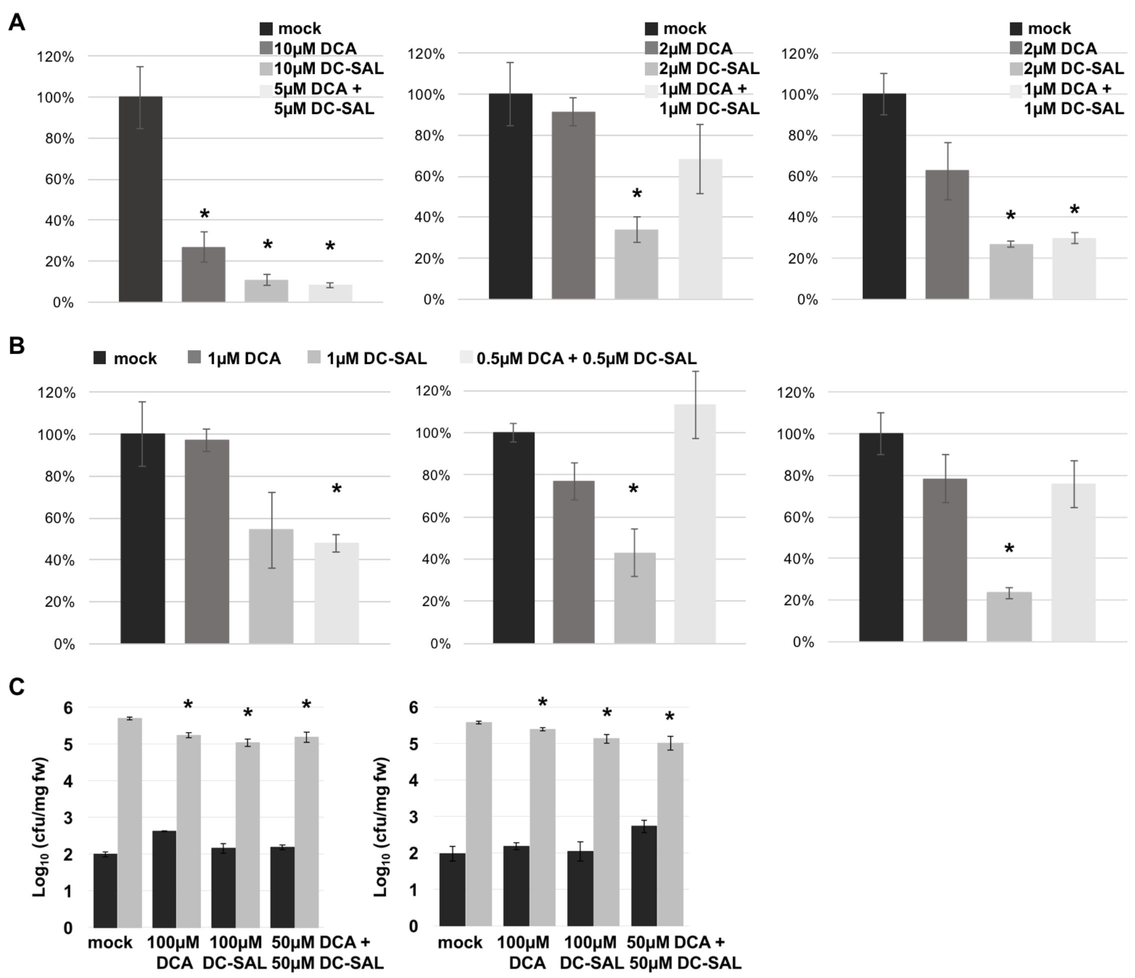


Figure 1.11. DC-SAL and DCA did not demonstrate synergism in Arabidopsis or tomato plants. A-B) Arabidopsis seedlings were treated with either the mock solution, DCA, DC-SAL or DCA plus DC-SAL 24 h prior to infection with *Hpa*Noco2 spores. Spores were counted 7 dpi and data is shown as a percent relative to the mock-treated plant spore levels. **A**) Higher compound concentrations were initially tested. **B**) Lower compound concentrations were tested in order to increase the sensitivity of the assay and possibly see a more distinct response. **C**) Tomato plants were root-drenched with either a mock solution or 100 μM DCA, 100 μM DC-SAL, or 50 μM DCA plus 50 μM DC-SAL 24 hours prior to being dipped in a *Pst* inoculum and *Pst* infection was measured 3 dpi. The synthetic elicitors were effective against *Pst* but compound synergism was not evident. Asterisk; Student's *t*-test $p < 0.05$ relative to the mock-treatment, error bars indicate technical replicates, black bars; day 0, grey bars; 3 dpi. Each graph represents an independent biological replicate.

At 1 μM, DC-SAL reduced *Hpa* growth in Arabidopsis seedlings by approximately 70% compared to the mock solution (Figure 1.12). To identify which moiety of the DC-SAL molecule was critical for the compound's activity, similar compounds, each varying in one aspect of DC-SAL's structure were tested in the Arabidopsis-*Hpa* pathosystem (Figure 1.12). Non-chlorinated forms and 3,5-diclorinated derivatives of three base skeletons often found in related and structurally simple plant defense-inducing molecules were compared. The base skeletons tested were salicylaldehyde (SAL; non-chlorinated form of DC-SAL), salicylic acid (SA) and anthranilic acid (AA; non-cholrinated form of DCA; Knoth *et al*., 2009). SAL and SA differ regarding the functional group attached to the carbon at position 1 (C1) of the aromatic ring. It is an aldehyde group in the case of SAL and a carboxyl group in the case of SA. Compared to SA, AA features an amino group at C2, while both SA and SAL have a hydroxyl group at this position.

Plants treated with 10 μM DC-SAL, 10 μM DC-SA or 10 μM CMP29 (a newly identified PMP-type compound) had the lowest levels of infection 7 dpi. The efficacy of DC-SAL and DC-SA at this concentration was indistinguishable. However, DC-SAL appeared to be of slightly higher potency than DC-SA, as this compound already mediated significantly reduced levels of susceptibility (compared to mock-treated plants) at a concentration of 0.1 μM, while DC-SA did not trigger any significant effects at this low concentration (Figure 1.12). CMP29 was included in these experiments to test if a new PMP-type compound (that was

not among those tested in the synthetic elicitor screen) elicited a defense response. At 10 μM, CMP29 significantly (relative to mock-treatment) protected Arabidopsis plants against *Hpa* (Figure 1.12).

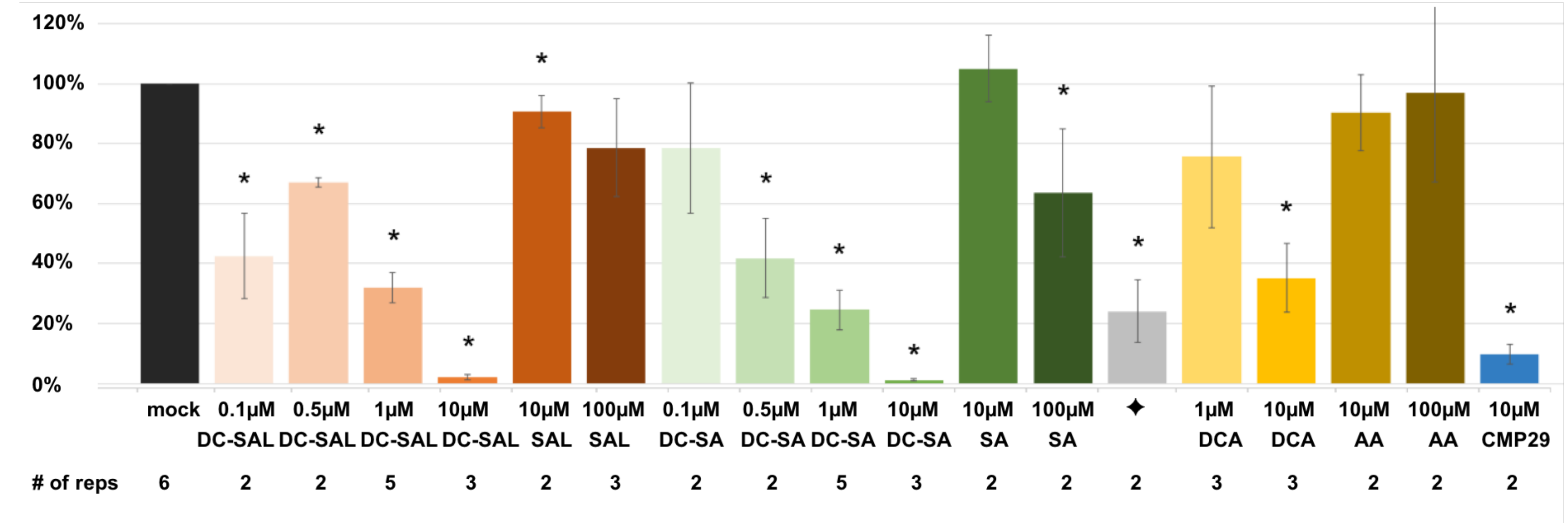


Figure 1.12. The 3,5 position chlorines of DC-SAL, not the aldehyde, increased the defense response in Arabidopsis plants. Arabidopsis seedlings were treated with compounds having structures similar to DC-SAL or DCA, but with varied moieties, 24 h prior to infection with *Hpa*Noco2 spores. Spores were counted 7 dpi and data is shown as a percent relative to the mock-treated plant spore levels. Plants treated with 10 µM DC-SAL, 10 µM DC-SA or 10 µM CMP29 had the lowest levels of infection 7 dpi. The mock solution contained 0.1% DMSO solvent. Diamond; 0.25 µM DC-SAL + 0.25 µM DCA, asterisk; Student's *t*-test $p < 0.05$ relative to mock-treatment, error bars indicate biological replicates and the number of biological replicates is given below the graph.

Taken together, the results of this part of the study showed that DC-SAL was a potent and efficient synthetic elicitor of remarkably simple structure. While exchange of its aldehyde group by the slightly more complex carboxyl group had only a marginal effect on potency, removal of both chlorines at the 3 and 5 positions resulted in a strong reduction of synthetic elicitor activity. DC-SAL's ability to mediate enhanced protection against three different types of pathogens (fungal, bacterial and oomycete) in three different plant species (cowpea, tomato and Arabidopsis), combined with its low price, make this molecule a prime candidate for the development of commercial pesticide alternatives.

Discussion

The data presented in this chapter support the hypothesis that synthetic elicitors that are operational in the model plant Arabidopsis, have the potential to provide protection to crop plants against agriculturally relevant diseases. BHTC and DC-SAL reduced disease symptoms in tomato and cowpea plants while DPMP was effective in tomato plants. For synthetic elicitors to be efficient components of crop protection strategies, they need to activate plant immunity at low concentrations. At 1 μM, DC-SAL reduced *Hpa* growth in Arabidopsis seedlings by approximately 70% compared to the mock solution (Figure 1.12). Although potency was lower in the tested crop plants than in Arabidopsis plants for BHTC and DPMP, DC-SAL showed potential for use at very low concentrations, indicating that structurally related derivatives of bioactive compounds may improve *in-vivo*-stability, uptake efficiency or other functional characteristics in crop plants.

Future experiments with DC-SAL defense induction may include additional phyto-pathosystems or different pathogens, since only one pathogen was tested in each crop plant assay. Multiple strategies for synthetic elicitor optimization are possible including combination of compounds toward activation of multiple plant defense pathways or synergism of compounds to suppress antagonistic signaling pathways, consequently magnifying the intensity of the defense response or to provide protection against a wider variety of pathogens. Additional structural

variation of synthetic elicitors may enhance bioactivity (Lipinski *et al*., 2001). Formulation with specific adjuvants, solvents or surfactants may increase efficacy (Seaman, 1990). The process of optimization could lead, not only to synthetic elicitors that are more effective in crop systems, but to a better understanding of plant immunity with the identification of specific cellular targets or signaling mechanisms.

Could synthetic elicitors harm pathogens directly?

One of the most important aspects of synthetic elicitor research is the potential for non-biocidal plant protection. Conventional pesticides seek to eliminate disease-causing pests and pathogens whereas synthetic elicitors activate the plant's innate immune system and need not be toxic to any organisms. In toxicity, often dosage is the key factor (Calabrese and Baldwin, 1997); even water causes harm in excessive amounts. In the studies described here, aerial portions of tomato plants were dipped in a *Pst*-containing inoculum 24 hours after receiving a root drench of compound or mock solution. *Pst* DC3000 survives for less than 48 hours on the surface of the leaf and so must enter the apoplast via stomata or wounds to infect plant tissue (Xin and He, 2013). If the bacteria came in direct contact with a synthetic elicitor, it seems unlikely that the concentration would have been as high as the root drench dose poured over the vermiculite 24 hours prior to the *Pst* dip. At a concentration of 100 μM, BHTC does not directly impair *Pst* growth levels over a 24 hour period (Rodriguez-Salus *et al*., 2016).

However, it is not known if 200 μM BHTC affects *Pst* growth or if the compound came in direct contact with *Pst* inside tomato plant tissue during the three day period. Since uptake through the roots was initiated 24 hours prior to infection, it is possible that BHTC had been metabolized in the plant tissue before it could come in contact with *Pst*. Alternatively, some compound might remain in the vermiculite throughout the experiment for continual use by the plant. (If applied in an agricultural setting, a compound could reside in soil for days before being taken up by the plant.) BHTC is active in Arabidopsis (aerial spray) against *Hpa* for up to three days (Rodriguez-Salus *et al*., 2016), so even though tomato plants are very different from Arabidopsis, it is possible that some BHTC came in contact with *Pst* in the apoplast.

When testing potential synthetic elicitor dosage toxicity in direct contact with *Fot*3 for multiple days, 100 μM DC-SAL was the least detrimental to *Fot*3, compared to 100 μM BHTC and 100 μM DPMP (Figure 1.9). This assay could be repeated with DC-SAL for a seven-day time period for a more accurate comparison to BHTC and DPMP (in case the compound breaks down after seven days and *Fot*3 was able to grow unhindered for three more days than in the BHTC and DPMP plate tests) (Figure 1.9).

DPMP was applied to cowpea plants, against *Fot*3, at 100 μM – 400 μM with 2 – 8 % DMSO. It is possible that DPMP may have had a significant effect on cowpea

plant immunity at higher concentrations, but due to the amount of DMSO solvent needed to keep DPMP in solution, higher DPMP concentrations could not be applied because the high percent DMSO burned plant leaves and stems. Solvent toxicity is a necessary consideration in synthetic elicitor formulation. In the case of DPMP, a possible advantage of being limited to lower concentrations was the observation of an increased stem height trend in cowpea plants. Given the hormetic effect of low levels of DPMP in Arabidopsis (see Introduction section; Rodriguez-Salus *et al*., 2016), it is possible that, although the levels were too low for a significant disease response in cowpea, they may have had a partial hormetic affect that was responsible for the trend of taller DPMP-treated plants in three out of four experiments (Figure 1.5B). It would be interesting to test if a lower dose of DPMP results in a significantly higher stem height in cowpea plants. Hormetic effects, either intentionally low-dosed or as a low concentration remainder in the soil surrounding plants following application, could benefit plant growth, an important support for plants under disease pressure.

Additional testing in cowpea roots and shoots could help determine if the aerial synthetic elicitor spray activates a salicylic acid signal that moves through the plant or if the original compound is mobile. Knowledge of whether salicylic acid or a synthetic elicitor is mobile in the plant and if it travels to the roots or slows progress of *Fot*3 in the chemically-treated shoot tissue could aid in formulation and application decisions in the field and help determine if and when compounds come in contact with pathogens.

Chlorines showed advantage over aldehyde and carboxylic acid moieties in plant defense induction

Salicylaldehyde (SAL) lacks the 3,5-positioned chlorines of DC-SAL and was approximately 89% less effective than DC-SAL when compared at 10 μM in the Arabidopsis-*Hpa* pathosystem (Table 1.1, Figure 1.12). Salicylic acid (SA) and anthranilic acid (AA) were approximately 104% and 55% less effective than their di-chlorinated analogs DC-SA and DCA, respectively (Figure 1.12). Together, the three comparisons exhibited increased potency and efficacy when di-chlorinated. However, the effect of 10 μM 3,5-dichlorosalicylic acid (DC-SA) which has a C-1 carboxylic acid, (whereas DC-SAL has an aldehyde at that position), was only marginally different from that of 10 μM DC-SAL (Figure 1.12). It is not clear if the carboxylic acid or the aldehyde made the synthetic elicitor more potent. At 10 μM, DCA, which has a C-2 amide group and, like DC-SA, has 3,5'-chlorines and a C-1 carboxylic acid, was less effective than DC-SA or DC-SAL (Table 1.1, Figure 1.12). If the C-2 amide were substituted with an alcohol, DCA would become DC-SA, and since DC-SA is a stronger protector of Arabidopsis against *Hpa* than DCA (Figure 1.12), the C-2 OH group is functionally more effective than the amide. These comparisons were easily made with the Arabidopsis data (Figure 1.12), but more experiments need to be performed in tomato to determine their effect in that plant against *Pst*. In the initial tomato test, DCA, DC-SA and DC-SAL were not significantly more effective than AA, SA and SAL, respectively, (Figure

1.10B). It should be noted that that all tomato experiments were performed with high pathogen titers, which do not reflect authentic infection conditions. Higher efficacy of synthetic elicitors observed in crop plants infected with much lower pathogen titers may enable better discrimination between the different derivatives tested.

Future work with DC-SAL could determine if DC-SAL activates a defense response or if it is involved in priming. DC-SA is a priming agent (Basson and Dubery, 2007; Kauss *et al*., 1993). According to Basson and Dubery (2007), DC-SA and DC-INA elicit higher levels of *CYP98A5* (a putative marker of SAR) mRNA than salicylic acid and INA, respectively, in bean leaves (*Phaseolus vulgaris*) indicating increased potency with the di-chlorinated derivatives. In a comparison of INA- and salicylic acid-derivatives, Conrath *et al*. (1995) determined that compounds having a six member conjugated ring with a carboxyl group can bind to and inhibit SABP catalase activity (preventing H_2O_2 degradation, thus leading to induction of defense-related gene expression) and those compounds with the highest levels of binding/bioactivity had halide groups substituting hydrogen atoms at 3, 4 and 5 positions (while hydroxyl groups there had the opposite effect). Based on the studies described in this chapter, is unknown if DC-SAL and its aldehyde moiety are involved in SABP binding like that of DC-SA (Conrath *et al*., 1995). DC-SAL was slightly more potent than DC-SA in the Arabidopsis assays given that DC-SAL provided more protection at the lowest concentration tested (0.1 μM) (Figure 1.12).

Bektas *et al*. (2016) showed that CMP974 and CMP993 induced a “strong” defense response in Arabidopsis against *Hpa* (Bektas *et al*., 2016), and an initial test in tomato indicated possible defense against *Pst* (Figure 1.10B). CMP974 and CMP993 each have a PMP skeleton and were identified in the high throughput synthetic elicitor screening. The central imine bond in these molecules that links two ring structures may undergo hydrolysis in plant tissue, creating metabolic products that may act by themselves as synthetic elicitors (Bektas *et al*., 2016). Like DPMP, CMP974 may break down into two compounds in the plant, one of which being DC-SAL. Another optimization technique for synthetic elicitors may be the structural combination of independent compounds that have distinct modes of action. The lab of Thomas Eulgem has used this strategy to link DCA- to DC-SAL-related moieties by an imine bridge, but did not observe any increased activity in Arabidopsis (Yuna Wang and Thomas Eulgem, data not shown). This is likely due to the fact that both DCA and DC-SAL are activating the same defense mechanisms and is consistent with the results from the synergism experiments. Perhaps linking compounds known to be active in different signaling pathways should be done and tested in the future.

CMP29 was purchased from a ChemDiv catalog and was chosen because of its PMP structure. CMP29 strongly protected Arabidopsis against *Hpa* (Figure 1.12) and was effective in a single experiment in tomato against *Pst* (Figure 1.10B). That a highly effective compound could be so quickly selected based on structural

similarity to other synthetic elicitors suggests that the Eulgem lab has identified specific moieties or functional groups that form key interactions with defense-related targets in plant cells and that progress in synthetic elicitor research is evident.

Table 1.1 Summary of compounds.

Structure	Name	Abbreviation	Results
	2-(5-bromo-2-hydroxy-phenyl)-thiazolidine-4-carboxylic acid	BHTC	As presented in Rodriguez-Salus *et al*., 2016, BHTC induced defense responses in Arabidopsis, tomato and cowpea plants.
	2,4-dichloro-6-{(E)-[(3-methoxyphenyl)imino]methyl} phenol	DPMP	As presented in Bektas *et al*., 2016, DPMP induced defense responses in Arabidopsis and tomato plants.
	3.5-dichlorosalicylaldehyde	DC-SAL	DC-SAL induced defense responses in Arabidopsis, tomato and cowpea plants.
	salicylaldehyde	SAL	SAL was less effective than DC-SAL against *Hpa* in Arabidopsis and an initial test in tomato indicated possible defense against *Pst*.
	3,5-dichlorosalicylic acid	DC-SA	DC-SA induced defense responses in Arabidopsis that appeared similar to induction by DC-SAL, except at 1μM where DC-SAL was more effective. An initial test in tomato showed similar results to those of DC-SAL.
	salicylic acid	SA	SA defense induction in tomato against *Pst* was variable and in Arabidopsis against *Hpa* was effective only at 100μM.
	3,5-dichloroanthranilic acid	DCA	Synergism with DC-SAL was not evident in Arabidopsis or tomato. As presented in Knoth *et al*., 2009, DCA induced defense responses in Arabidopsis and tomato plants.
	anthranilic acid	AA	AA was ineffective in both Arabidopsis and tomato plants.
	benzenesulfonamide, 4-[[(3.5-dichloro-2 hydroxyphenyl) methylene]amino]	CMP974	An initial test in tomato indicated possible defense against *Pst*. CMP974 appears to be worth further study. As presented in Bektas *et al*., 2016, CMP974 induced defense responses in Arabidopsis against *Hpa*.
	4-tert-butyl-2-[(5-chloro-2-hydroxybenzylidene)amino]phenol	CMP993	An initial testwith CMP993 in tomato indicated possible defense against *Pst*. As presented in Bektas *et al*., 2016, CMP993 induced defense responses in Arabidopsis against *Hpa*.
	4-chloro-3-{[(1E)-(3,5-dibromo-2-hydroxyphenyl)methylene]amino} benzoic acid	CMP29	CMP29 induced defense responses in Arabidopsis against *Hpa* and in an initial test in tomato indicated possible defense against *Pst*.
	benzo(1,2,3)thiadiazole-7-carbothioic acid S-methyl ester	BTH	BTH is the active ingredient in the commercial plant activator product Actigard® 50WG. It was tested in cowpea against *Fot*3 in experiments with DPMP.

As discussed above, future chemical lead optimization strategies may include combination of potentially active moieties in a single molecule. Moieties with synthetic elicitor activity could be linked to moieties potentially enhancing their uptake, in planta stability or inhibiting antagonistic mechanisms. Plant immunity is often studied with the ultimate goal of reducing crop loss due to pathogen-caused disease. Translational research studies described in this chapter, in which previous synthetic elicitor induction of Arabidopsis immunity in the laboratory was expanded to their use in the crop plants tomato and cowpea, is a step toward potential compound application in the field of agricultural crop protection. The current results provide important proof-of-concept, by showing that, conceptually, synthetic elicitors identified and characterized in Arabidopsis can induce immunity in certain crop systems and may be useful as leads for the design of innovative commercial pesticide alternatives. Aside from their potential in agricultural crop protection, synthetic elicitors can be useful as pharmacological probes to uncover new mechanistic details of the plant immune system.

Materials and Methods

Cowpea plant growth conditions and pathogen infections

Vigna unguiculata (cowpea) 'California Blackeye 5' (CB5) and 'California Blackeye 46' (CB46) seeds (University of California Riverside cowpea germplasm collection) were sown in vermiculite, sprayed (using Preval sprayers) with a synthetic elicitor or mock solution approximately 10 days later and inoculated 24 hours post compound treatment with *Fusarium oxysporum* formae speciales *tracheiphilum* race 3 (*Fot*3) using a root clip and dip inoculation method. The *Fot*3 had been isolated previously from infected cowpea plants in the San Joaquin Valley, California, (unpublished data, Shirley Smith) and was propagated as previously described (Pottorff *et al*., 2012). Seedling roots were rinsed (free of vermiculite) in water, clipped to a length of 10 cm and submerged in water containing 10^4 *Fot*3 spores/ml for 30 seconds prior to being individually planted in labeled four-inch fiber pots containing moist UC Mix 3 soil. Pots were randomized on greenhouse benches, and plants were fertilized with Miracle- Gro (14-14-14; Scott's Miracle-Gro Products), watered every other day and sprayed with either a synthetic elicitor or mock solution biweekly. Plants grew under greenhouse conditions (with day temperatures up to 35°C and night temperatures down to 16°C) until disease symptoms were apparent.

Disease was visually evident when main plant stems obtained a faint brown hue and/or leaf chlorosis was visible. Each plant was assessed and given a whole plant score followed by stem vascular discoloration measurements. For BHTC experiments, whole-plant scores were rated on a scale of 0 to 5, based on the percentage of the plant that displayed *Fot*3-induced symptoms: 0 = no disease symptoms; 1 = 10%; 2 = 25%; 3 = 50%; 4 = 75%; and 5 = 100%, as described previously (Pottorf *et al*., 2012). Chi-square tests of independence showed significant differences between BHTC- and mock-treated plants for scores of 2 (p = 0.012) and 5 (p = 0.003) (Rodriguez *et al*., 2016). For DPMP and DC-SAL, individual cowpea plants were assessed according to the following disease index scoring system:

Score	Disease symptoms
0	Plant is 100% healthy; no wilting, chlorosis or necrosis is visible.
1	Up to 20% of visible plant tissue is affected by wilting and/or chlorosis and/or necrosis.
2	20% - 50% of visible plant tissue is affected by wilting and/or chlorosis and/or necrosis.
3	>50% - <80% of visible plant tissue is affected by wilting and/or chlorosis and/or necrosis.
4	80% - 100% of visible plant tissue is affected by wilting and/or chlorosis and/or necrosis.
5	Plant is dead and there are no signs of living tissue.

Chi-square tests of independence showed significant differences between DC-SAL- and mock-treated plants for the whole plant score of zero and the Student's *t*-test was used to determine significant statistical difference in percent vascular discoloration.

*Fot*3 – synthetic elicitor plate growth experiments were conducted by adding a plug of *Fot*3 to a plate containing 10 ml potato dextrose agar (BD Difco), made according to the manufacturer's instructions, with either DMSO alone or the synthetic elicitor in the concentration listed. Plates were loosely wrapped in foil and left at room temperature for 7 – 10 days and then photographed. Addition of Prochloraz fungicide (Sigma Aldrich, Amini and Sidovich, 2010) served as a negative control.

Tomato plant growth conditions, pathogen infections and qPCR

Solanum lycopersicum (tomato) (cultivar 'Moneymaker'; Everwilde Farms) seeds were sown on autoclaved vermiculite. Plants were fertilized with Miracle-Gro Tomato Plant Food (18-18-21; Scott's Miracle-Gro Products) and 1) maintained at 25°C under 200 mmol m22 s21 light intensity for a 12-h-light photoperiod for 4 weeks for BHTC and DPMP experiments, or 2) maintained at 23°C under 100 µE m−2 s–1 light intensity for a 16-h-light photoperiod for 4 weeks for DC-SAL experiments.

Pseudomonas syringae pv *tomato* strain DC3000 (*Pst*) was cultured on King's B medium with 50 mg/ml rifampicin (Sigma-Aldrich) at 28°C. Aerial portions of tomato plants were submerged in a 10 mM $MgCl_2$ solution containing *Pst* (OD_{600} = 0.005) and 0.02 % Silwet L-77 (Lehle Seeds) for 30 seconds. Leaves were weighed, ground in 10 mM $MgCl_2$, diluted, and plated at 1 hour and 3 days post

infection. Colonies were counted 40 to 48 hours after plating. Statistical significance was determined using the Student's *t*-test.

Real-time quantitative reverse transcription PCR (RT-qPCR) was performed to evaluate transcript levels of *SlPR1b1* normalized to the reference gene *Ubiquitin 3* (*SlUbi3*, X58253). Total RNA was extracted from treated tomato leaves using TRIZOL (Invitrogen) and cDNAs were prepared as previously described (Atamian *et al*., 2012). The following primer pairs were used for *SlUbi3*; Ubi3F 5′-GTGTGGGCTCACCTACGTTT-3′ and Ubi3R 5′-ACAATCCCAAGGGTTGTCAC-3′ and for *SlPR1b1*; PR1b1F 5′-TTATACTCAAGTAGTCTGGCGCA-3′ and PR1b1R 5′-TTGCAAGAAATGAACCACCA-3′ (Atamian *et al*., 2012). For *SlPR1b1* RT-qPCR amplification, the CFX Connect detection system (Bio-Rad) was used with iQ SYBR Green Supermix (Bio-Rad), 40 cycles of amplification with the annealing temperature at 57°C, with three technical replicates. PCR products were electrophoresed on a 1% agarose gel containing 0.5 μg mL^{-1} ethidium bromide.

Arabidopsis plant growth conditions and pathogen infections

Arabidopsis thaliana (Arabidopsis) ecotype Columbia (Col-0) seedlings were grown on soil under fluorescent lights (16h of light/8h of dark, 23°C, 100 µE m−2 s–1). *Hyaloperonospora arabidopsidis* isolate Noco2 (*Hpa*Noco2) was propagated as previously described (McDowell *et al*., 2000). Seedlings were

spray-infected with a 3 x 10^4 spores/ml water *Hpa*Noco2 spore suspension, using a Preval sprayer, 24 hours post synthetic elicitor treatment. Plants were scored for *Hpa* growth 7 dpi by counting spores per seedling using a hemocytometer to determine the spore density of a suspension of 10 infected seedlings per 1 mL of water. Student's *t*-test was used to determine statistical significance.

Synthetic elicitor application

DPMP, DCSAL, SAL, DCA, DC-SA, SA, AA and CMP974 were ordered from Sigma-Aldrich (https://www.sigmaaldrich.com). CMP993 was ordered from Interchim (https://www.interchim.com). CMP29 was ordered from ChemDiv (https://www.chemdiv.com). BTH was ordered from Syngenta (Actigard® 50WG, https://www.syngenta.com). BHTC was synthesized following a previously described protocol (Khan *et al*., 2006; Rodriguez-Salus *et al*., 2016; Song *et al*., 2009). Stock solutions of synthetic elicitors were prepared in 100% (v/v) DMSO before dilution in water to a working concentration. Mock solutions contained identical amounts of solvent (and surfactant in cowpea experiments) as their synthetic elicitor counterparts. Arabidopsis plants were sprayed when 13 days old with 2-3 ml per pot, at the indicated concentration, with Preval sprayers, 24 hours prior to *Hpa* infection. Tomato plant root drench was performed as described (Rodriguez-Salus *et al*., 2016) for BHTC and DPMP studies. DPMP qPCR pots received a single root drench at the indicated concentration and time point. For DC-SAL tomato plant experiments, a single root drench with 40 ml solution was

performed 24 hours prior to *Pst* infection. As previously described (Rodriguez-Salus *et al*., 2016), aerial parts of cowpea plants were sprayed with either BHTC or a mock solution. Relatively mature plants could better tolerate higher concentrations of DMSO than younger plants, so the chemical concentrations increased over time and the amount sprayed increased for thorough coverage as plant size increased: 24 hours prior to inoculation = 100 μM BHTC, 2 mL per plant; 7 days post inoculation = 100 μM BHTC, 2 mL per plant; 14 days post inoculation = 200 μM BHTC, 3 mL per plant; 21 days post inoculation = 500 μM BHTC, 4 mL per plant; 28 days post inoculation = 750 μM BHTC, 4 mL per plant; and 35 days post inoculation = 1 mM BHTC, 4 mL per plant. In cowpea plant experiments with DPMP and BTH, compound concentration ranges were as follows over the course of approximately four weeks: 2-8 % DMSO (mock), 100 μM – 400 μM DPMP, 100 μM - 400 μM BTH. In cowpea plant experiments with DC-SAL, plants were sprayed with 100 μM DC-SAL or a 0.1 % DMSO-containing mock solution 24 hours prior to infection and then biweekly over approximately four weeks. In DPMP and DC-SAL experiments, solutions also contained 0.01% Silwet L-77 (Lehle Seeds) and 0.01% Triton X-100 (Sigma Aldrich).

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Chapter II: Quantitative Infection Assays Revealed Ethylene Signaling as Critical for Arabidopsis Resistance to the Broad Host-Range Fungus *Macrophomina phaseolina*

Abstract

Dubbed as a “global destroyer of crops”, the necrotrophic soil-borne fungus *Macrophomina phaseolina* (*Mp*) infects more than 500 plant species including many economically important cash crops. Host defenses against this powerful pathogen are poorly understood. Here, interactions between *Mp* and *Arabidopsis thaliana* (Arabidopsis) were established as a model system to quantitatively assess host factors affecting the outcome of *Mp* infections. Using agar plate-based infection assays, *Mp* biomass growth in roots of different Arabidopsis genotypes was quantified and ethylene signaling was found to play a critical role in host defense against this pathogen. By comparative profiling of host transcripts in roots of the wild type Arabidopsis accession Col-0 and *ein2/jar1*, an ethylene- and jasmonic acid-signaling deficient mutant that exhibited enhanced *Mp* susceptibility, expression changes in hundreds of genes potentially contributing to *Mp* defense mechanisms were uncovered. Many of these genes encode metabolic enzymes, cell wall altering components and signaling factors. These results established *Mp*-Arabidopsis interactions as a useful model pathosystem, which enables application of the vast genomics-related resources of this versatile model plant to

the systematic investigation of previously understudied host defenses against a "global crop destroyer".

Introduction

In order to manage plant-pathogen interactions toward agricultural crop protection, phyto-pathosystems need to be studied and understood. Such relationships have two (or more) distinct organisms, each with a high level of biochemical and genetic complexity, *intending* self-preservation at the cost of the other. Scientists research both plant immunity and microbial pathogenicity in the effort to improve crop security. However, crop plants can be difficult to study due to size, length of life-cycle, transformation limitations and lack of genetic information. The model plant *Arabidopsis thaliana* (Arabidopsis) is an invaluable research tool, both to gain information about plant defense and to analyze the infection process of a compatible pathogen. Arabidopsis has been a major source for understanding the molecular mechanisms of plant immunity (Provart *et al*., 2016). It is unparalleled in its stable genetic transformation capability among multicellular organisms (Somerville and Koornneef, 2002). Not only was the Arabidopsis genome fully sequenced over a decade ago, large sets of Arabidopsis mutants with well characterized defects in regulatory processes controlling immunity are easily attainable from stock centers (Provart *et al*., 2016). Furthermore, a large number of valuable genomics-related resources are available for Arabidopsis and have been extensively used by the scientific community for over 20 years. As a result, an extensive body of knowledge on the molecular genetics, biochemistry and physiology of this organism has accumulated. Plant

immunity biology, a very active area of research, has benefitted greatly from the Arabidopsis model system. Because of its advantages as a model plant, Arabidopsis was chosen to study plant interactions with the pathogenic fungus *Macrophomina phaseolina* (*Mp*).

Mp is a devastating soil-borne fungus that infects more than 500 plant species, from weeds to redwood trees (Baird *et al*., 2010; Fuhlbohm *et al*., 2012; McCain and Scharpf, 1989; Saleh *et al*., 2009; Su *et al.*, 2001). Several of these hosts are economically important crop plants including maize, soybean, canola, cotton, peanut, sunflower and sugar cane (Khangura and Aberra, 2009; Leyva-Mir *et al*., 2015; Mahmoud and Budak, 2011; Mahmoud *et al*., 2006; Saleh *et al*., 2009; Shanmugam *et al*., 2002). Often, microbial pathogens co-evolve with, and are specific to, a single plant host or a narrow range of host species. However, certain broad-spectrum host pathogens, like *Mp*, are capable of infecting multiple types of hosts. *Mp* is found throughout the world, predominantly in, but not limited to, warmer regions. Examples of *Mp* crop damage have been seen in countries spanning the globe, including Australia (Fuhlbohm *et al*., 2012), Benin (Msikita *et al*., 1998), Brazil (Sousa *et al*., 2017), China (Zhang *et al*., 2011), Czech Republic (Veverka *et al*., 2008), Egypt (Ahmed *et al*., 2010), India (Mahadevakumar and Janardhana, 2016), Japan (Fujinaga *et al*., 2002), Mexico (Leyva-Mir *et al*., 2015), South Africa (Zimudzi *et al*., 2017), Turkey (Yeşil and Baştaş, 2016) and the United States (Baird *et al*., 2010). Crop diseases caused by the pathogen are typically

associated with drought and heat stress (Muchero *et al*., 2011), though *Mp* can survive under multiple environmental conditions (Islam *et al*., 2012). Reports of crop damage caused by *Mp* from various parts of the world have increased in recent years (Avilés *et al*., 2008; Khangura and Aberra, 2009; Mahmoud and Budak, 2011) making efforts needed to combat this pathogen even more imperative.

In Figure 2.1, photographs from a cowpea field study performed by Philip Roberts' laboratory in a UCR Agricultural Operations field in Riverside, CA, show disease symptoms caused by *Mp* on cowpea plants. In addition to charcoal rot, wilt and chlorosis, the lethality of *Mp* infection is clear. Levels of host tolerance/resistance against *Mp* can vary substantially (Figure 2.1). As mentioned in the General Introduction, cowpea, while cultivated in multiple countries around the globe, is a critical crop in developing countries of sub-Saharan Africa, where it provides an important source of dietary protein (Timko *et al*., 2007). Cowpea's high tolerance for drought, heat and unfavorable soil conditions, renders it invaluable in regions where other crops cannot survive (Muñoz-Amatriaín *et al*., 2017). Heavy *Mp* field infestation has been known to cause complete cowpea crop loss in Senegal and Niger (Ndiaye *et al*., 2007). *Mp* disease research and cowpea breeding programs, such as those conducted at UCR (Figure 2.1C) (Boukar *et al*., 2016), aim to prevent such devastating crop losses in the future.

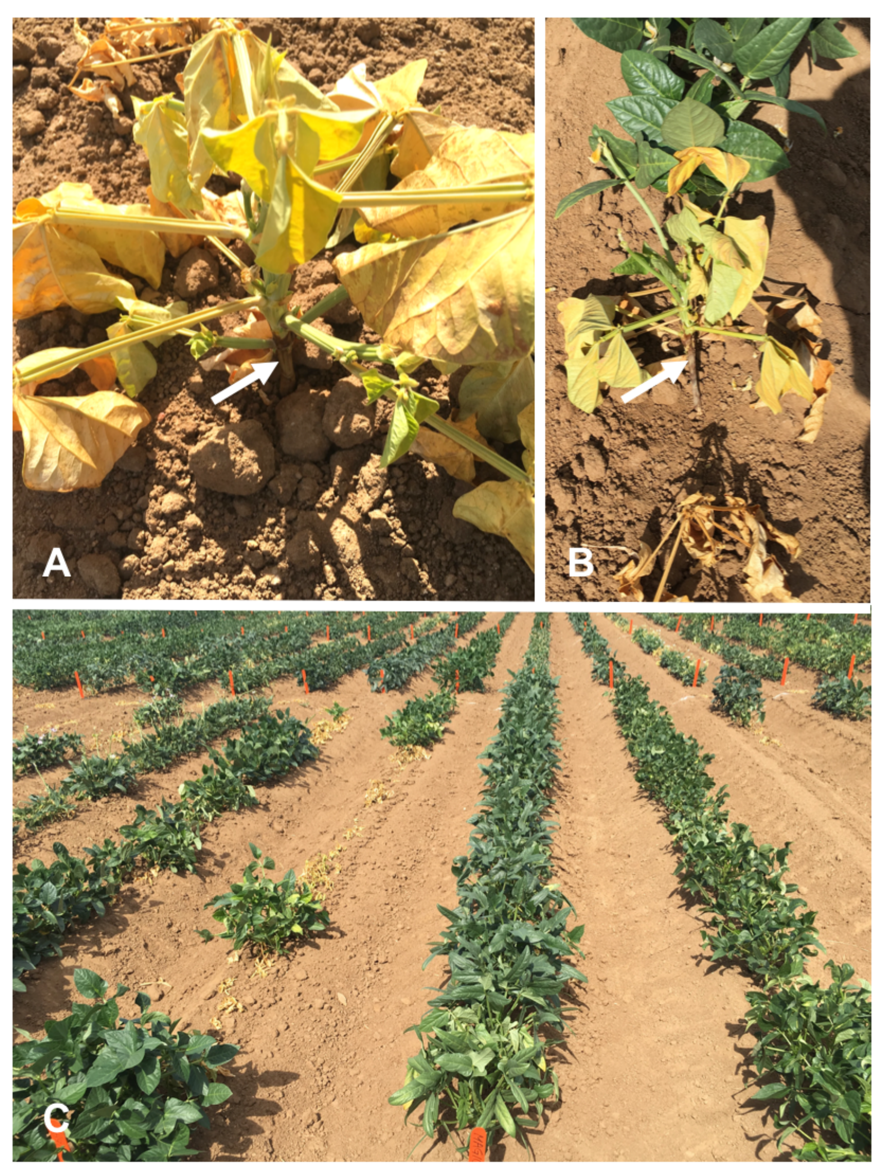


Figure 2.1. Cowpea plants revealed *Macrophomina phaseolina* (*Mp*) infection in the field. (**A**) A charcoal rot lesion at the root-stem junction (white arrow), wilting and chlorosis were visible on an *Mp*-infected plant. (**B**) Three levels of susceptibility are shown. Healthy plants (top) and the dying plant (center) had reached the flowering stage. The dead plant (bottom) died prior to flowering. A white arrow points to a charcoal rot lesion. (**C**) A population of seven week-old recombinant inbred lines (RIL) growing on a naturally-containing-*Mp* field in Riverside, CA, revealed varying degrees of susceptibility to *Mp*. (**A**, **B**, **C**) Cowpea field study by Philip Roberts' laboratory at UC Riverside Agricultural Operations.

Mp consists of mycelia with microsclerotia imbedded in the hyphae (Figure 2.2F) (Kaur *et al*., 2012). Pycnidia, structures containing conidia (spores), may also be present, but were not utilized in this research. A microsclerotium is an aggregation of 50-200 hyphal cells that form a compact mass and is generally brown to black in color when fully formed. Microsclerotia are asexual (non-sporic) propagation structures that may remain dormant for extended periods of time (depending on environmental conditions) prior to germination. Hyphae extend from microsclerotia, form an appressorium upon contact with host plant tissues and penetrate plant dermal cells growing intra- and inter-cellularly in roots and other tissues (Ammon *et al*., 1974). *Mp* hyphae produce cell wall degrading enzymes (CWDEs) and phytotoxins (Kuti *et al*., 1997; Islam *et al*., 2012) and typically colonize vascular tissue resulting in plant wilting and often death (Kaur *et al*., 2012; Mayek-Perez *et al*., 2002). *Mp* microsclerotia can persist in soil for weeks to several years and are extremely difficult to eradicate (Mah *et al*., 2012; Short *et al*., 1980; Vibha 2016). Such a wide variation in survivability is thought to be due to regional soil and climate conditions as well as agricultural techniques and presence of host plants. A high temperature and an arid climate are most favorable for *Mp* (Olaya *et al*., 1996). Microsclerotia present in the soil or on previously-infected residual plant tissue in soil, on neighboring weeds or on seed coats of newly planted crops may be the source of infection (Begum *et al*., 2005; Kaur *et al*., 2012). Infection may be visible at the seedling stage (Arabidopsis, sesame), at flowering stages or post senescence as *Mp* can also be saprophytic (Kaur *et al*.,

2012). Diseases caused by *Mp* vary by host and include charcoal rot (partial or complete disintegration of plant roots), canker (necrotic lesion on stem or other organ), damping off (collapse and death of young seedlings) and wilt (loss of turgidity in shoot tissues) (Agrios, 2005). At a cellular level, however, descriptions of the mode of infection are similar and tend to involve germination of a microsclerotium resulting in the formation of an appressorium that enters plant cells and hyphal intrusion into plant cell walls using CWDEs (Ammon *et al*., 1974). Hyphopodia, structures similar to appressoria yet arising from mature hyphae, may also penetrate plant cell walls (Bressano *et al*., 2010; Howard, 1997). Enzymatic degradation of cell wall tissue via hyphal secretion of CWDEs is a likely method of entry into plant cells given the high number of CWDEs identified in the *Mp* genome, however, mechanical breakage may also be involved (Ammon *et al*., 1974; Islam *et al*., 2012).

Macrophomina, (kingdom: Fungi, phylum: Ascomycota, family: Botryosphaeraceae) is a monospecific genus, having the single species *phaseolina* (Baird *et al*., 2010; Philips *et al*., 2013; Sutton, 1980). Early categorization of *Mp* isolates was based on host specificity, chlorate sensitivity or other phenotypic observations prior to the use of genetic data (Babu *et al*., 2011; Pearson *et al*., 1987; Saleh *et al*., 2009). More recently, Baird and co-workers (Baird *et al*., 2010), compared variability in 109 genetically distinct *Mp* isolates collected from 15 states throughout the continental United States using twelve

simple sequence repeat (SSR) loci and determined that neither host nor geography accounted for the genetic variation between collected isolates. For example, 79 of the 109 *Mp* isolates were from the host soybean (Baird *et al*., 2010). All isolates were determined to be genetically 99% identical via internal transcribed spacer region (ITS) sequencing, supporting the theory that all tested isolates belonged to a single species (Baird *et al*., 2010). In 2012, the *Mp* genome sequence was published (Islam *et al*., 2012) providing detailed information about potential CWDEs and mechanisms to surmount plant defense responses. Multiple studies have reported on CWDEs secreted by *Mp* as well as physiological plant responses to *Mp* (Kaur et al., 2012). CWDEs include proteases, pectinases, xylanases, polygalacturonases and endoglucanases (Islam *et al*., 2012; Khan *et al*., 2017; Wang and Jones, 1995).

Methods of *Mp* management include soil solarization, soil fumigation, no-tilling, crop rotation, flooding, fungicide and other soil amendments (including bio-agents and chemical additives) and seed treatments (Ahmed *et al*., 2010; Almeida *et al*., 2003; Almeida *et al*., 2008; Chamorro *et al*., 2016; Narayanasam, 2013; Ndiaye *et al*., 2007; Niem *et al*., 2013), but the reduction in pathogen development is often only partial. Costs of such treatments to large areas of crop land can be prohibitive or may only treat upper layers of soil. In addition, fungicidal treatments pose environmental health risks (Shanmugam, *et al*., 2002). Field observations have suggested that *Mp* infections may rise because of restriction of the use of methyl

bromide, (Chamorro *et al*., 2016; Koike, 2008; Zveibil *et al*., 2012) and alternative methods for *Mp* eradication are needed. Both composting (decomposition of plant debris prior to use as a soil amendment) and solarization (the covering of growing fields with tarps) raise the temperature of infected plant debris or soil, respectively, to temperatures that can destroy *Mp* microsclerotia (Narayanasam, 2013; Ndiaye *et al*., 2010). Solarization, in the form of polyethylene sheets applied to strawberry field plots in Turkey, for example, reduced viable *Mp* by 66% at a soil depth of 5 cm but was not effective against *Mp* at 10 cm or 20 cm (Yildiz *et al*., 2010). The same study showed that *Mp* microslerotia could survive for at least 18 days at 45°C, but for only 19 h at 50°C and 60 minutes at 55°C (Yildiz *et al*., 2010). Solarization plus soil amendments of millet residues or slaughtered animal paunch resulted in reduced *Mp* soil inoculum and reduced charcoal rot disease severity in cowpea crops (Ndiaye *et al*., 2007). Solarization is generally most effective against *Mp* in soils with high moisture content (Ndiaye *et al*., 2007; Sheikh and Ghaffar, 1987). A combination of mung bean seed treatment and soil application with the bacteria *Burkholderia* sp. strain TNAU-1 reduced root rot incidence to 16.7%, from 52.6%, a reduction that was similar to that caused by the application of the fungicide carbendazim against *Mp* (Satya *et al*., 2011). Additional biological agents, such as arbuscular mycorrhizal fungi that can increase plant tolerance to *Mp* (Marquez *et al*., 2018), may also be used to complement integrated disease management systems. Many organic farmers seek less susceptible plant varieties or completely different crops, which is difficult when faced with a broad host-

spectrum pathogen. Identification of specific genetic plant defense mechanisms in Arabidopsis that either enhance susceptibility or contribute to resistance to *Mp* may be applied in breeding efforts to generate crops that can grow in *Mp*-infested soils.

Immunity has been well studied in Arabidopsis and the functions of known, general root responses may also be active against *Mp*. Early defense responses in plant roots include reactive oxygen species (ROS) production which, in addition to being toxic to some pathogens, can trigger cell wall strengthening events in the form of cell wall compound crosslinking and lignin formation (De Coninck *et al*., 2015). ‘Casparian strips’, primarily composed of lignin, and the presence of suberin in the endodermis may delay pathogen entry into the vasculature (De Coninck *et al*., 2015; Ranathunge *et al*., 2008). Preformed anti-microbial compounds present in root cells may include pathogenesis-related proteins (e.g. chitinases), secondary metabolites (e.g. flavonoids, glucosinolates) or root exudates that may alter soil properties or attract beneficial microbes that can complement plant resistance (De Coninck *et al*., 2015).

Canonical theory in plant immunity tells us that wound responses and defense responses to necrotrophic microbes or chewing insects are largely mediated by jasmonic acid and ethylene whereas biotrophic and hemibiotrophic pathogens elicit defense reactions controlled by salicylic acid-dependent signaling pathways (Glazebrook, 2005). Jasmonic acid and ethylene often act together and are known

to antagonize salicylic acid at higher hormone doses, yet cooperative activity is also apparent in some cases (Mur *et al*., 2006, Pieterse *et al*., 2012). Faced with *Mp* as a necrotrophic pathogen, activation of ethylene-and jasmonic acid-responsive genes, and possible suppression of salicylic acid-directed genes, could be expected in Arabidopsis. However, recent studies in other plant species have indicated roles for salicylic acid signaling in *Mp* resistance. *Jc*WRKY-overexpressing (Agarwal *et al*., 2014) transgenic tobacco plants exhibited increased resistance to *Mp* via a salicylic acid-mediated balance of reactive oxygen species signaling (Agarwal *et al*., 2018). Seed soaking in salicylic acid reduced 1) *Mp*-induced pre-emergence damping off and wilt in *Hibiscus sabdariffa* L. field trials (24-h soak) (Hassan *et al*., 2014) and 2) *Mp*-induced stem lesion length in a sunflower (*Helianthus annuus* L.) green-house study (4-h soak) (Al-Wakeel *et al*., 2013). Nevertheless, roles for ethylene and jasmonic acid hormone pathway activation in relation to plant resistance to *Mp* have been demonstrated. Gaige and co-workers (Gaige *et al*., 2010) proposed that *Mp* interfered with ethylene and jasmonic acid signaling pathways in *Medicago truncatula* and showed an increase in resistance to *Mp* following stimulation of these pathways by exogenous application of ethephon and methyl jasmonate together. In sesame (*Sesamum indicum* L.), the disease index of *Mp*- infected plants was significantly lower in plants treated with jasmonic acid and ethylene together (39%) compared to control plants (69.93%) (Chowdhury *et al*., 2017). Also, both jasmonic acid and ethylene biosynthesis and marker genes were up-regulated during the

necrotrophic phase of *Mp* infection of sesame, whereas *SiJAZ,* a negative regulator of jasmonic acid signaling, expression decreased (Chowdhury *et al*., 2017). In *Medicago truncula*ta, jasmonic acid and ethylene-responsive genes *ERF* and *OPR-12* were up-regulated but *PR-5* and *PI-II* were not in infected root tissue (2dpi), leading the authors to propose pathogen interference of these hormone signaling pathways in the plant (Gaige *et al*., 2010). Additional plant hormone pathways have also been shown to be active during *Mp*-plant interactions, such as those controlled by auxin (Mah *et al*., 2012, Marquez *et al*., 2018) and abscissic acid (Biswas *et al*., 2014).

Given the importance of ethylene and jasmonic acid hormone-mediated defense against necrotrophic pathogens, key aspects of the Arabidopsis ethylene and jasmonic acid signaling pathways are outlined in the following paragraphs. Ethylene is a simple, gaseous molecule (C_2H_4) produced by plants that is known to have important roles in defense and other stress responses as well as in growth, abscission, senescence, fruit ripening and plant to plant communication (Dubois *et al*., 2018). Ethylene biosynthesis is a three-step process outlined as methionine → S-adenosyl methionine (SAM) (1) → 1-AminoCyclopropane-1-Carboxylic acid (ACC) (2) → ethylene (3) (Dubois *et al*., 2018). In the absence of ethylene, the ethylene receptors ETR1, ETR2, ERS1, ERS2, and EIN4 bind to and activate CTR1 (Constitutive Triple Response 1), a negative regulator of the ethylene signaling pathway (Ji and Guo, 2013). CTR1 represses EIN2 (Ethylene

INsensitive 2), but in the presence of high-enough levels of ethylene, CTR1/receptor complexes are degraded and EIN2 is released, dephosphorylated and cleaved, freeing a fragment of EIN2 to repress *EBF1* and *EBF2* (EIN3-Binding F box protein 1 and 2) (Dubois *et al*., 2018). EBF1 and EBF2 target the transcription factors EIN3 (Ethylene INsensitive 3) and EIL1 (EIN3-Like 1) for protein degradation (Potuschak *et al*., 2003), but in the presence of ethylene, EIN3 and EIL1 are principal regulators of ethylene response gene expression, including induction of genes encoding ERFs (Ethylene Response Factors) (Chao *et al*., 1997; Dubois *et al*., 2018).

Jasmonic acid and it's oxylipin derivatives, collectively called jasmonates, constitute a major phytohormone group and are known to have key roles in plant defense (e.g. against chewing insects and necrotrophic pathogens, regulation of secondary metabolites) and other stress responses, as well as in development (e.g. root growth inhibition, light signaling, flowering time, senescence) (Wasternack and Hause, 2013). Through a complex system of repressors and activators/de-repressors jasmonates are important regulators of the balance between defense and development (Song *et al*., 2014b). When jasmonates are absent, JAZ (JAsmonate Zim-domain) proteins, (bound to TPL [ToPLess] via NINJA [Novel INteractor of JAz]), inhibit jasmonate responses by associating with various transcription factors (Song *et al*., 2014b). In the presence of jasmonates, COI1 (COronotine Insitive 1) (as part of the SCF^{COI1} complex) targets JAZ proteins

for ubiquitination and degradation, allowing activation of the JAZ-inhibited transcription factors (e.g. *MYC2*) in jasmonate signaling pathways (Zhu and Lee, 2015). The brief description above explains a core jasmonate signaling mechanism, but only represents a portion of the pathways influenced by this phytohormone. JAZ8 and splice variants JAZ10.4 and JAZ10.3 have reduced affinity for COI1 (Moreno, *et al*., 2013; Shyu *et al*., 2012) making these proteins less likely to be degraded in the presence of jasmonate. Another inhibitory mechanism involves certain IIId subgroup bHLH transcriptional repressors, like JAM1, that competitively bind to JAZ protein targets and are thought to counteract excess jasmonate responses (Song *et al*., 2013; Song *et al*., 2014b).

As regulators of the plant defense response against necrotrophic pathogens, jasmonates and ethylene act in co-operative and antagonist manners. Jasmonates lead to the degradation of JAZ repressors of EIN3/EIL1 to activate pathogenesis-related genes (Song *et al*., 2014b) and a recent RNA-seq analysis found 25% of jasmonate-responsive genes to be regulated by EIN3/EIL1 (Zheng *et al*., 2017). On the other hand, MYC2 (also known as JIN1, Jasmonate Insensitive 1) and EIN3/EIL1 have been shown to be antagonistic (Song *et al*., 2014a). MYC2 represses EIN3/EIL1 and subsequent *ERF1* expression during the defense response to necrotrophic fungal pathogen *Botrytis cinerea* and EIN3/EIL1 inhibits MYC2 reducing expression of wound-responsive genes (Song *et al*.,

2014a). Crosstalk between phytohormone signaling pathways may provide alternative avenues for plants to respond to specific pathogens.

In the current study, accurate, quantitative methods were developed to examine the way *Mp* infects plants using the model plant Arabidopsis, which allowed systematic testing of known Arabidopsis defense mutants and natural accessions for their susceptibility to this debilitating fungus. Such a screening technique using Arabidopsis and *Mp* has not previously been described and applied previously. Arabidopsis mutants compromised in ethylene and jasmonic acid signaling exhibited enhanced susceptibility to *Mp*. This effect was highly significant in the *ein2/jar1* double-mutant which is deficient in both ethylene and jasmonic acid signaling processes. Profiling *Mp*-induced transcriptional responses in this line and its parental wild type background, Col-0, by RNA-seq, linked transcriptional up-regulation of multiple known jasmonate and ethylene regulators as well as numerous metabolic enzymes to immunity against this pathogen. Results described in this chapter will serve as a basis for more extensive systematic studies on *Mp* defense responses in the Arabidopsis model system and provide candidate genes to be further tested for their contribution to *Mp* protection in Arabidopsis and in crop plant systems.

Results

Design of a quantitative assay system for *Mp*-infected Arabidopsis roots

A critical step in the infection of plants by *Mp* is the establishment of microsclerotia in plant tissue. This process was observed in *Mp*-Arabidopsis interactions on agar plates. Microsclerotia added to ½ MS agar generated hyphae that grew throughout the agar, forming more microsclerotia as *Mp* grew. When Arabidopsis seedlings were laid down upon the *Mp*-laden agar infection plate, hyphae on the agar surface contacted the roots and grew toward them, surrounding and penetrating the root tissue (Figure 2.2 A-D). Microsclerotia were seen forming within the roots as early as 48 h post-contact (hpc) (Figure 2.2 E).

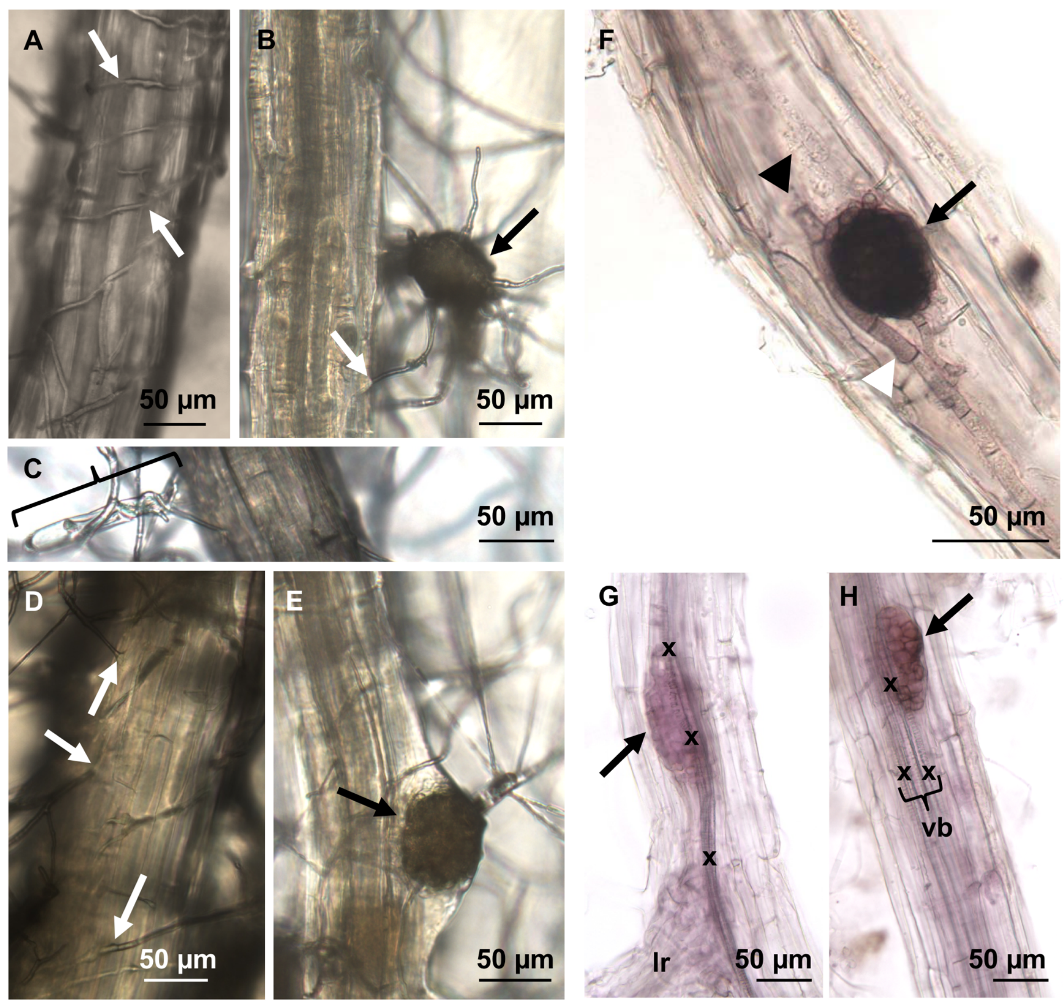


Figure 2.2. *Mp* infected Arabidopsis root tissue at 24 and 48 hours post-contact (hpc). (**A, D**) *Mp* hyphae surrounded and penetrated Arabidopsis roots at 24 (**A**) and 48 (**D**) hpc. (**B**) *Mp* microscerotium (black arrow), nearly in the microscopic focal plane with the root, shows relative size reference. (**C**) Bracket indicates a single root hair surrounded by hyphae at 24 hpc. (**E**) Microscerotia were forming inside root tissue at 48 hpc. White arrows point to locations of hyphal insertion into root tissue. (**F**) The black arrowhead points to hyphae emanating from the microsclerotium. The white arrowhead points to mature hyphae. (**G**, **H**) Acid fuchsin staining of *Mp*-infected Arabidopsis roots. *Mp* microsclerotia began to form as early as 48 hpc throughout root tissue and were often associated with vascular tissue. Black arrows point to microsclerotia. Bracket indicates vascular bundle, vb; vascular bundle, x; xylem cells, lr; lateral root.

The fact that critical events of the *Mp* infection process occured under controlled conditions on agar enabled design of a plate-based infection protocol to accurately quantify levels of host susceptibility (Figure 2.3). Arabidopsis seeds were sown on ½ MS agar plates (Figure 2.3A) and allowed to grow for ten days (Figure 2.3C) while *Mp* microsclerotia were added to separate plates (Figure 2.3B) and grown for six days (Figure 2.3D). The ten day-old seedlings were transferred to the six day-old *Mp* infection plates (Figure 2.3E). After 24 hours (h) of contact (Figure 2.3F), the seedling roots were infected by *Mp* hyphae (Figure 2.2A-C). By 48 h, hyphae penetration continued (Figure 2.2D) and microsclerotia could be seen forming in some Arabidopsis roots (Figure 2.2E). Root tissue was flash frozen in liquid nitrogen at 24 and 48 hpc for RNA-seq or at 48 hpc for qPCR analysis (Figure 2.3 H). Alternatively, at four-five days post-contact (dpc) microsclerotia density was determined by counting the number of microsclerotia per mm in each seedling's primary root (Figure 2.3G).

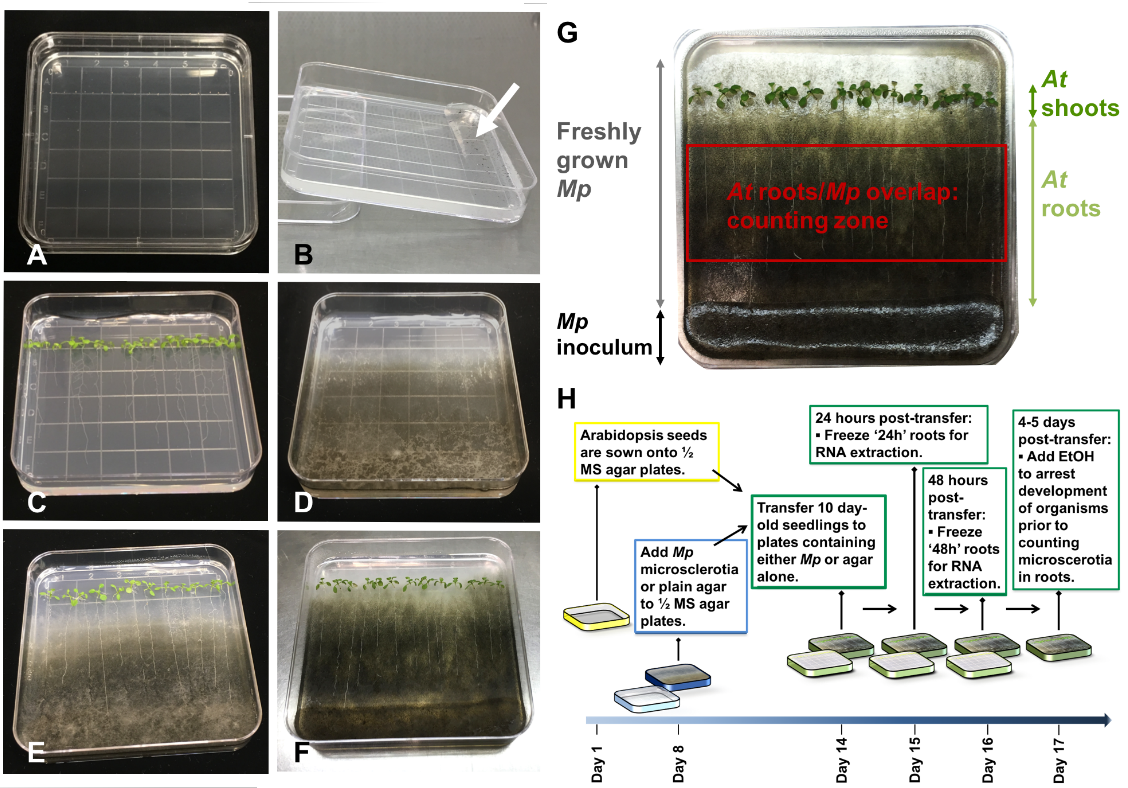


Figure 2.3. A plate-based assay system for quantitative analysis of *Mp* susceptibility levels in Arabidopsis roots. (**A**) Arabidopsis seeds were sown on ½ MS agar. (**B**) *Mp* inoculum, microsclerotia in ½ MS agar, was added to ½ MS agar plates and allowed to grow for six days, forming a *Mp* infection plate (**D**). (**C**) Arabidopsis seedlings grown for ten days prior to transfer to treatment plates are shown. (**E**, **F**) Arabidopsis seedlings 0.5 hours post transfer (hpt) (**E**) and 24 hpt to *Mp* infection plates (**F**). (**G**) A diagramed infection plate. (**H**) Assay time line.

Infection plates typically contained 12-14 seedlings, and the microsclerotia per mm density was based on the average of all seedlings per plant line (at least three plates per line) in a biological replicate. To gauge assay reproducibility, the average number of microsclerotia per mm root was compared from plate to plate, then all plates for each biological replicate were averaged and six independent biological replicates were compared to the average of all six as a percentage

(Figure 2.4). The variation between replicates was low with no extreme outliers observed within this limited data set. For two other common plant-pathogen defense assays that are routinely performed, I observed greater variation was observed between replicates with occasional extreme outliers (Figure 2.4).

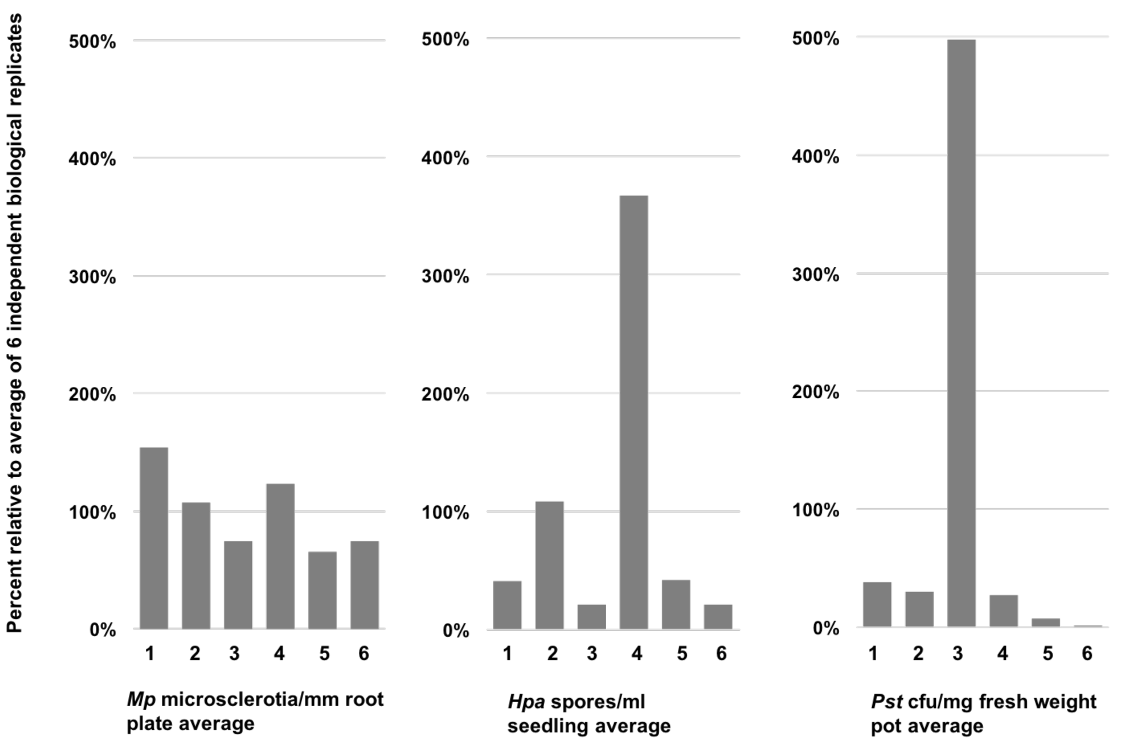


Figure 2.4. Assay variation comparison. For each of three dissimilar pathogen infection assays in Arabidopsis Col-0, the graphs show the average pathogen quantification for six independent biological replicates relative to the average of all six of those replicates as a percentage for comparison between replicates. *Mp* microsclerotia were counted under microscopy in individual seedling primary roots, 4-5 days post-infection (dpi), 4-12 plates per replicate. *Hyaloperonospora arabidopsidis* (*Hpa*) spores were collected and counted from seedling shoots 7 dpi. *Pseudomonas syringae* pathovar *tomato* (*Pst*) bacteria were quantified by counting colonies collected from shoot tissue 3 dpi.

Although Col-0 gave reproducible results, plant lines with extreme morphological or developmental phenotypes were determined to be ineffective for the microsclerotia density assay. For example, *ctr1*-1 plants, interrupted in *CTR1*, an important component in the ethylene signaling pathway, are very small with roots that were too short to use in comparison to Col-0. In addition, some variation of *Mp* susceptibility was observed in the natural accession Ksk compared to Col-0. Ksk had a lower microsclerotia density but preliminary qPCR results (not shown) indicated no significant difference in the amount of *Mp* tissue in Ksk roots relative to Col-0 roots.

Arabidopsis mutants compromised in ethylene signaling showed enhanced root susceptibility to *Mp*

To identify host defense mechanisms that affect levels of *Mp* susceptibility in Arabidopsis roots, a set of Arabidopsis mutants with defects in various defense pathways or other physiological processes was tested. JAR1 (JAsmonate Resistant 1 or Jasmonoyl isoleucine conjugate synthase1) is responsible for the conversion of (+)-7-iso-JA to the bioactive form (+)-7-iso-jasmonoyl-L-isoleucine (JA-Ile) (Fonseca *et al*.,2009) and *ein2* null mutants are completely ethylene insensitive (Ji and Guo, 2013). Only in the *ein2/jar1* double mutant, which is compromised in ethylene and jasmonic acid signaling, did we observe significantly increased density of microsclerotia in roots (Figure 2.5, Table 2.1).

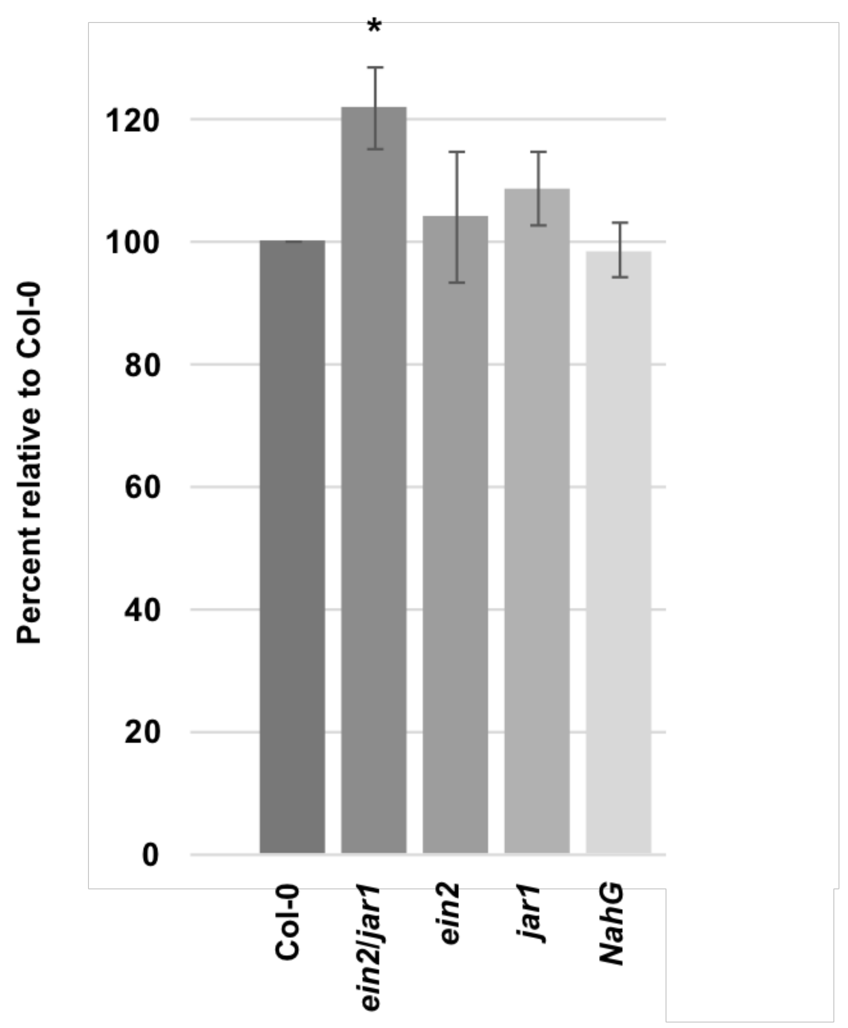


Figure 2.5. Novel agar plate pathosystem identified mutant plant line with increased susceptibility to *Mp*. *Mp* microsclerotia were counted under microscopy in individual seedling primary root, 4-5 days post-contact, 17-100 roots per line per replicate. Graph shows average percent microsclerotia per mm root relative to Col-0. Error bars represent standard error for three biological replicates. Statistical significance was determined by Student's *t*-test, asterisk; *p*-value <0.05.

Table 2.1. Average percent microsclerotia per mm root relative to Col-0.

Plant line	(%)	No. biological replicates	Category*
ein2-1/*jar1*-1	122	5	e,j
jar1-1	109	3	j
ein3/*eil1*	106	3	e,j
ein2-5	104	3	e,a,j
npr1,3,4	104	2	s
Col-0	**100**	**6**	**control**
NahG	99	2	s
Ler-0	96	2	A
Ws-0	96	2	A
axr1-3	96	2	a
Tsu-1	95	2	A
xi-k/myoB1,2,3,4	94	2	r
slac1-2	92	2	st
myb60-1	86	2	st
Ksk-1	76	2	A

Plant line	(%)	No. biological replicates	Category*
pap2	151	1	a
Ty-0	149	1	A
Mt-0	121	1	A
wakl22	120	1	cw
vad1-1	118	1	e
jin4	114	1	j
pin1-1	112	1	a,r
axr2-1	102	1	a
Col-0	**100**	**6**	**control**
tir1-1	93	1	a
amt1-1	90	1	e,r,a
ca1/*ca2*	89	1	CO_2
jin1	82	1	j

*Category abbreviations:
A = accession
a = auxin-related
CO_2 = carbon dioxide-related
cw = cell wall-related
e = ethylene-related
j = JA-related
r = root-related
s = SA-related
st = stomata-related

This finding was confirmed by comparing the abundance of Arabidopsis- and *Mp*-specific genomic DNA in Arabidopsis roots 48 h after *Mp* contact using quantitative PCR (qPCR) (Figure 2.6). For the qPCR assay, primer pairs were used specifically targeting the species-specific sequence characterized amplified regions (SCAR) of *Mp* (*MpSyk*) and Arabidopsis (Shaggy-related Kinase 11, *AtSK11*) DNA (Babu *et al*., 2011; Gachon and Saindrenan, 2004). The relative abundance of *Mp*-specific to Arabidopsis-specific DNA was clearly elevated in *Mp*-infected roots of the *ein2/jar1* line compared to Col-0 plants indicating a substantial increase of *Mp* biomass in this mutant. Contrary to the microsclerotia density assays (Figure 2.5), a significant increase of relative *Mp* DNA levels in the *ein2* single mutant compared to Col-0 was also observed (Figure 2.6). We attribute this to the fact that the SCAR-qPCR assays are more sensitive than microsclerotia density measurements and detect DNA from other *Mp* structures, such as hyphae. Taken together, enhanced levels of *Mp* growth were observed in the agar plate-based assays system in mutants compromised in ethylene signaling, strongly suggesting that this general stress signaling pathway contributed to host defense reactions against this pathogen.

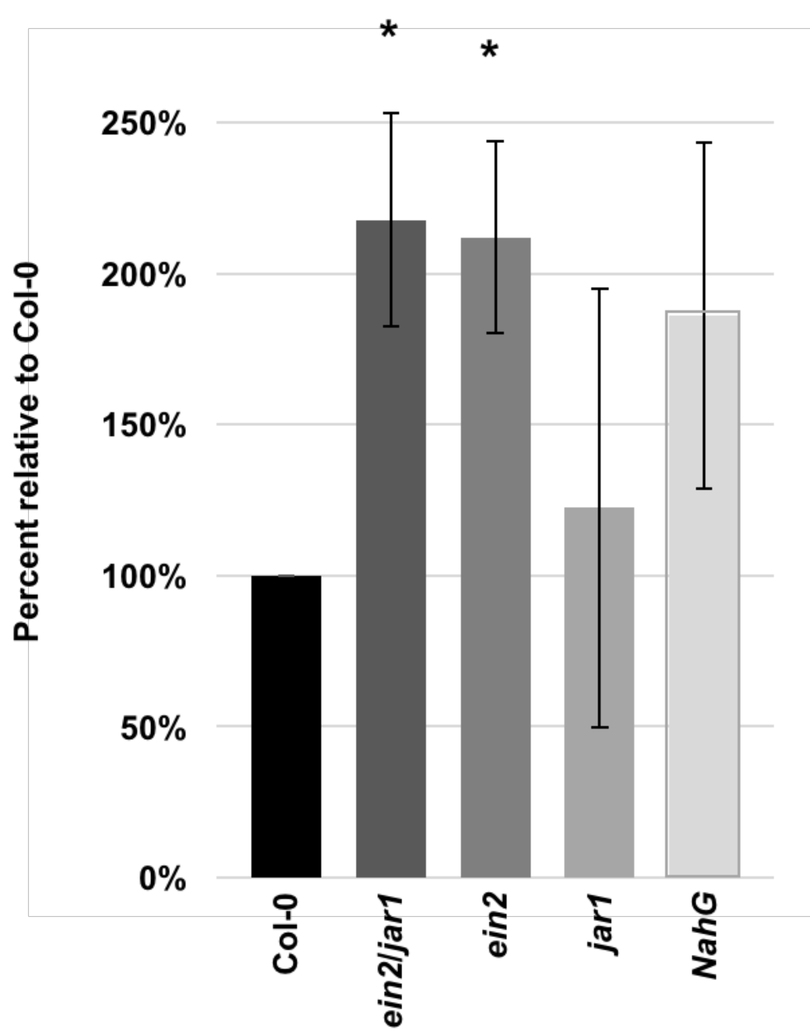


Figure 2.6. *Mp* growth quantification using SCAR-qPCR. The relative abundance of *Mp* and Arabidopsis genomic DNA was determined by the quantification of sequence characterized amplified regions (SCAR) of *Mp* (*MpSyk*) (Babu *et al*., 2011) and Arabidopsis (Shaggy-related Kinase 11, *AtSK11*) by qPCR using Arabidopsis roots 48 hpc with *Mp*. Error bars represent standard error for three biological replicates. Statistical significance was determined by Student's *t*-test, asterisk; *p*-value <0.05.

Arabidopsis mutants compromised in ethylene and jasmonate signaling showed enhanced susceptibility to *Mp* in shoots

Similar to the root assays, ten day-old Arabidopsis seedlings were transferred to *Mp* infection plates, however, plants were allowed to grow for up to three weeks with plates positioned horizontally/right side up, under growth room conditions. Seedlings were photographed during the infection period and their disease severity was determined. A disease index scoring system of "0", for healthy plants, through "5", for dead plants, was used to compare disease symptoms (e.g., chlorosis, necrosis, stunting) between Col-0 and mutant lines. All five of the mutant lines tested had more severe disease symptoms than Col-0 (wild type) after two weeks (Figure 2.7). After two weeks of *Mp* infection, *ein2/jar1* had the most severe disease symptoms and most of the *ein2/jar1, ein2 and jar1* plants were dying. *NahG* (transgenic plants that do not accumulate salicylic acid) and *tat3* shoot disease symptoms were less severe than those of the jasmonate and ethylene mutants, yet were also significantly higher than Col-0 plants at the two-week time point (Figure 2.7).

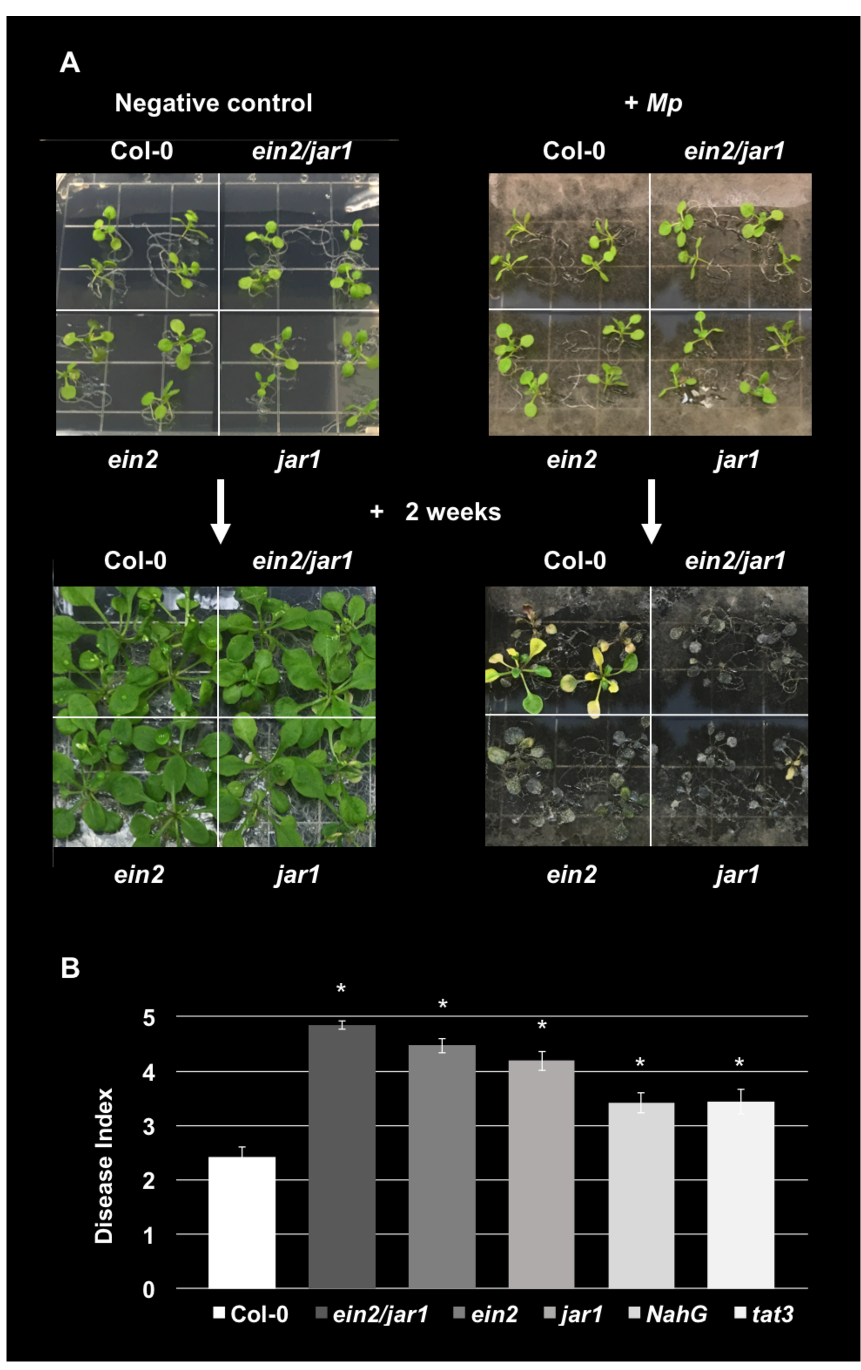
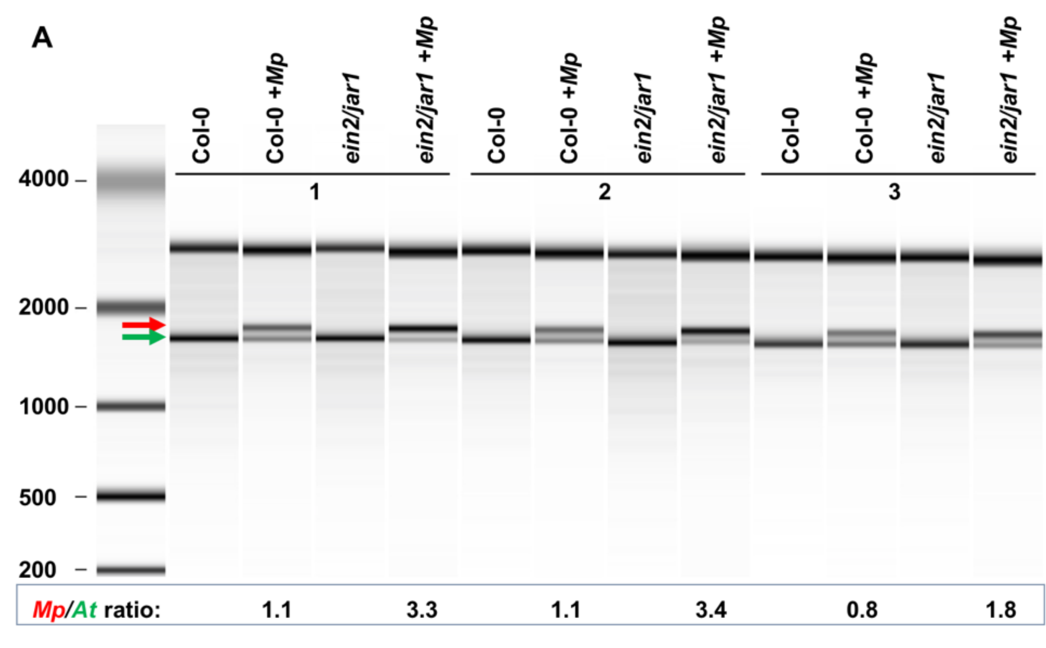


Figure 2.7. Arabidopsis shoot disease symptom severity was higher in mutants than in Col-0. Ten day-old seedlings were placed on *Mp* infection plates and scored two weeks post-contact for shoot disease symptom severity. (**A**) Representative plates are shown. (**B**) Scoring was based on a disease index of 0 (healthy) to 5 (dead). A representative biological replicate is shown. Asterisk indicates Student's *t*-test $p < 0.001$, $n = 40$ plants.

Profiling of *Mp*-induced transcriptome changes in Arabidopsis roots

In order to identify host transcriptome changes associated with enhanced resistance against *Mp*, an mRNA-seq study was performed with poly-A mRNA from agar plate-grown Arabidopsis Col-0 or *ein2/jar1* roots at 24 h or 48 h after *Mp* contact or control treatment. For each of the eight different experimental conditions two independent biological replicates were performed. Total RNA was isolated from roots, mRNA was processed and libraries were prepared using the NEBNext Ultra RNA library prep kit. Pooled libraries were sequenced on the Illumina HiSeq 2500 platform of the UC-Riverside Genomics Core facility and unique reads were mapped to the Arabidopsis genome (TAIR10) using STAR v 2.5.3a (Dobin *et al*., 2013). Reads in gene regions were counted using featureCounts (Liao *et al*., 2014) and the expression fold-change of each gene in pair-wise comparisons between treatments/genotypes (see below) was calculated using the R package DESeq2 v1.14.1 (Love *et al*., 2014) with the threshold for differentially expressed genes set to p value < 0.05. A comparison between levels of Arabidopsis (*At*) and *Mp*-specific 18S RNAs at 48 hors post-contact in samples used for this analysis indicated a higher abundance of *Mp* in *ein2/jar1* roots compared to roots of Col-0 (Figure 2.8).



B

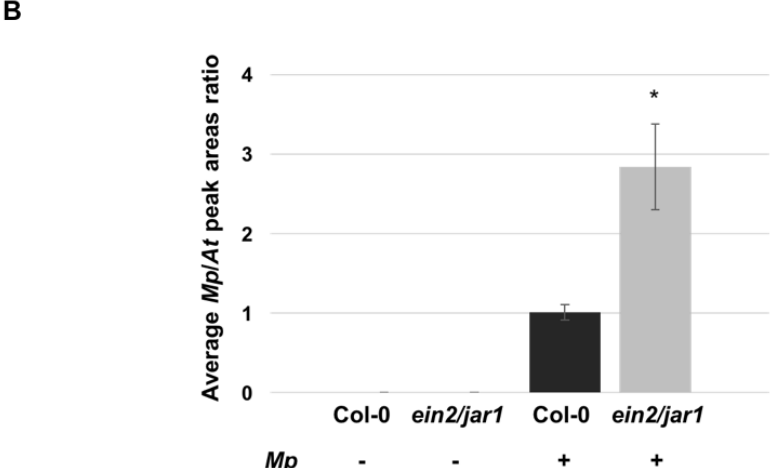


Figure 2.8. *ein2/jar1* roots contained more *Mp* RNA than Col-0 roots. (**A**) Bioanalyzer electrophoresis file run summary of Arabidopsis (*At*) root RNA samples with and without *Mp* 48 hpc. The red arrow points to *Mp* 18S RNA bands. The green arrow points to *At* 18S RNA bands. Band intensity is based on Bioanalyzer-generated data. The ratio of *Mp* 18S to *At* 18S band intensity was higher in *ein2/jar1* than in Col-0, indicating that *Mp* RNA levels were higher in *ein2/jar1* roots than in wild type roots in three independent replicates. (**B**) Graph of ratios between *Mp* 18S to *At* 18S bands quantified using ImageJ.

This observation further confirmed that the *ein2/jar1* double mutant exhibited elevated susceptibility to *Mp* and showed that the treatments for the transcript profiling experiment were successful. To identify gene expression changes potentially relevant for the induction of heightened resistance in Col-0, the following pair-wise comparisons were performed: (1) Col-0 24 h control vs. Col-0 24 hpc with *Mp*; (2) Col-0 48 h control vs. Col-0 48 hpc with *Mp*; (3) *ein2/jar1* 24 h control vs. *ein2/jar1* 24 hpc with *Mp*; (4) *ein2/jar1* 48 h control vs. *ein2/jar1* 48 hpc with *Mp*; (5) Col-0 24 h control vs. Col-0 48 h control (Figure 2.9A); (6) Col-0 24 hpc with *Mp* vs. Col-0 48 hpc with *Mp* (Figure 2.9B); (7) *ein2/jar1* 24 h control vs. *ein2/jar1* 48 h control (Figure 2.9C); (8) *ein2/jar1* 24 hpc with *Mp* vs. *ein2/jar1* 48 hpc with *Mp* (Figure 2.9D); (9) Col-0 24 hpc with *Mp* vs. *ein2/jar1* 24 hpc with *Mp* (Figure 2.9E); (10) Col-0 48 hpc with *Mp* vs. *ein2/jar1* 48 hpc with *Mp* (Figure 2.9F); (11) Col-0 24 h control vs. *ein2/jar1* 24 h control (Figure 2.9G); (12) Col-0 48 h control vs. *ein2/jar1* 48 h control (Figure 2.9H); (Tables 2.2, S1-S12). Transcript level differences in these comparisons with an adjusted *p* value of less than 0.05 (Table 2.2, Figure 2.9) were considered significant.

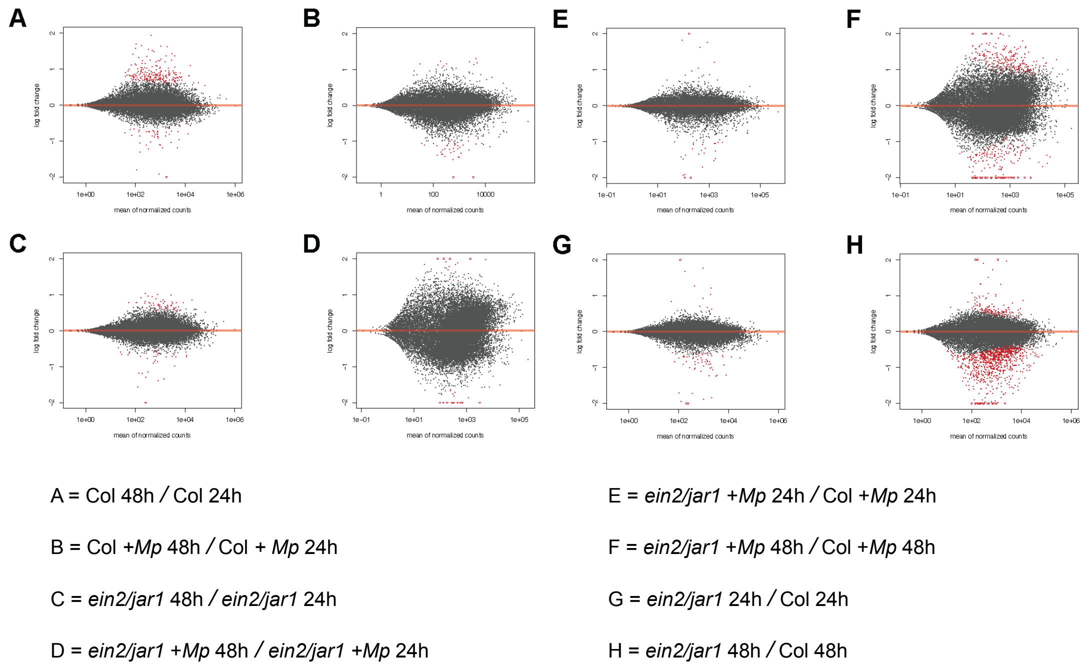


Figure 2.9. MA plots portrayed RNA-seq transcriptome trends. MA plots showed the $\log_2$ fold changes attributable to a given variable over the mean of normalized counts for the compared samples. Genes with similar expression levels in two samples appeared near the horizontal line y = 0. Points which fell out of the window were plotted as open triangles pointing either up or down. Red points, adjusted-*p* <0.01.

Table 2.2 RNA-seq twelve comparisons of significant gene expression changes.

Comparison	Total significant * DEGs	Up-regulated DEGs	Down-regulated DEGs
Col-0 24h control vs. Col-0 24hpi with *Mp*	335	260	75
Col-0 48h control vs. Col-0 48hpi with *Mp*	6,227	3,349	2,878
ein2/jar1 24h control vs. *ein2/jar1* 24hpi with *Mp*	421	393	28
ein2/jar1 48h control vs. *ein2/jar1* 48hpi with *Mp*	8,183	4,584	3,599
Col-0 24h control vs. Col-0 48h control	210	173	37
Col-0 24hpi with *Mp* vs. Col-0 48hpi with *Mp*	94	23	71
ein2/jar1 24h control vs. *ein2/jar1* 48h control	57	37	20
ein2/jar1 24hpi with *Mp* vs. *ein2/jar1* 48hpi with *Mp*	16	5	11
Col-0 24hpi with *Mp* vs. *ein2/jar1* 24hpi with *Mp*	24	5	19
Col-0 48hpi with *Mp* vs. *ein2/jar1* 48hpi with *Mp*	273	146	127
Col-0 24h control vs. *ein2/jar1* 24h control	56	10	46
Col-0 48h control vs. *ein2/jar1* 48h control	853	93	760

*Adjusted p value < 0.05

The set of Arabidopsis Col-0 genes up-regulated in response to *Mp* infection at 24 and 48 hpc seemed partially unique, as it showed only limited overlap with gene sets up-regulated by other pathogens, such as the necrotrophic fungi *Fusarium oxysporum* and *Botrytis cinerea* or the biotrophic oomycete *Hyaloperonospora arbidopsidis* (*Hpa*) (Figure 2.10A). A study by Zhu and coworkers (Zhu *et al*., 2013) provided a list of 116 genes that were up-regulated at both 1 day-post-inoculation (1 dpi) and 6 dpi of two week-old Col-0 seedlings by *F. oxysporum*. The seedlings were root-dipped in a spore inoculum and RNA-seq plus qPCR were performed on infected tissues at both time points, relative to un-inoculated plants (Zhu *et al*., 2013). Of the three comparisons in figure 10A, the *Mp*-induced transcript profile

in Col-0 roots most resembled that induced by *F. oxysporum*, as 68% (79/116) of Arabidopsis genes induced by this pathogen were also up-regulated by *Mp*. *F. oxysporum* and *Mp* are both necrotrophic, soil-borne fungal pathogens that often infect plants through their roots. Alternatively, just 28% (1,123/3,950) of Arabidopsis genes induced by *Hpa* (Bhattarai *et al*., 2010) were also up-regulated by *Mp*. The lower percent overlap was not surprising given that *Hpa* is a biotrophic oomycete that infects aerial portions of the plants, a very different lifestyle from *Mp*.

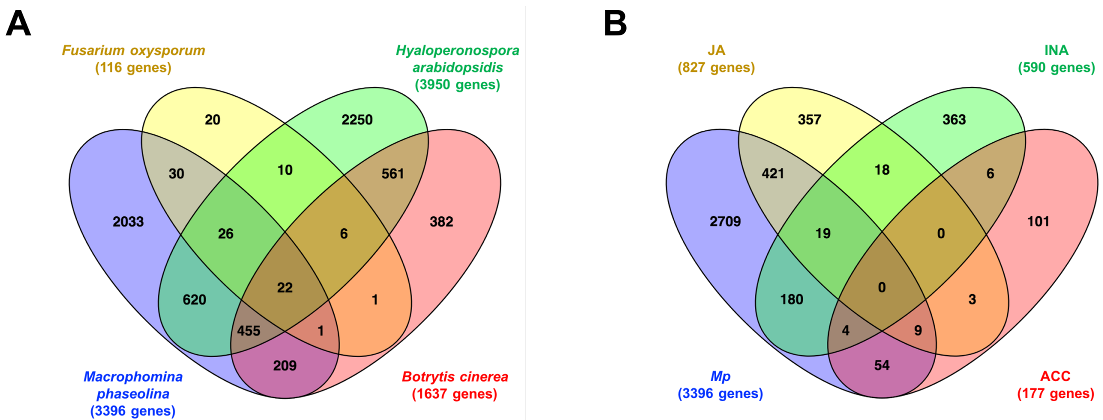


Figure 2.10. Pathogen and hormone treatment assays revealed overlaps of transcriptome up-regulation with that of *Mp*-infection. (**A**) Treatment of Col-0 seedlings with pathogens of varying lifestyles and in different plant tissues showed different degrees of transcriptome up-regulation compared to *Mp*-infection. *Fusarium oxysporum* (Zhu *et al.,* 2013) overlapped by 68% (79/116), *Hyaloperonospora arabidopsidis* (Bhattarai *et al.,* 2010) by 28% (1,123/3,950) and *Botrytis cinerea* (Sham *et al.,* 2017) by 42% (687/1637). (**B**) Hormone treatments with jasmonic acid (JA) (Zheng *et al*., 2017) overlapped by 54% (449/827), 1-aminocyclopropane-1-carboxylic acid (ACC) (Harkey *et al*., 2018) overlapped by 38% (67/177) and 2,6- dichloroisonicotinic acid (INA) (Knoth *et al*., 2009) overlapped by 34% (203/590).

Hpa-infected shoot tissue (7 dpi) was used for preparation of the ATH1 microarray data (Bhattarai *et al*., 2010) and the lower (28%) overlap may also be reflective of shoot versus root tissue analyzed in the *Mp* study. The biotrophic lifestyle of *Hpa* may also have contributed to the lower percentage of gene up-regulation overlap, especially since the necrotroph *F. oxysporum* gene set overlap was substantially higher, at 68%. Despite the fact that the relative overlap of these two gene sets was small, a large absolute number of genes was jointly up-regulated in both types of infections, reflecting that different defense signaling pathways can converge and target a common set of genes (Schenk *et al*., 2000). *B. cinerea* fungal spores sprayed onto five week-old Arabidopsis plants induced gene expression according ATH1 microarray data prepared and analyzed by Sham *et al*., 2017. Above ground tissue was collected 24 hpc and the wild type, Col-0, data set showed an overlap of 42% (687/1637) of up-regulated genes with the *Mp* data (Figure 10A). The 3,396 genes used in these comparisons are collectively up-regulated at 24 hpc and at 48 hpc in Col-0 by *Mp* ((260 24h + 3,349 48h) -213 duplicates = 3,396 genes) (Figure 2.11A).

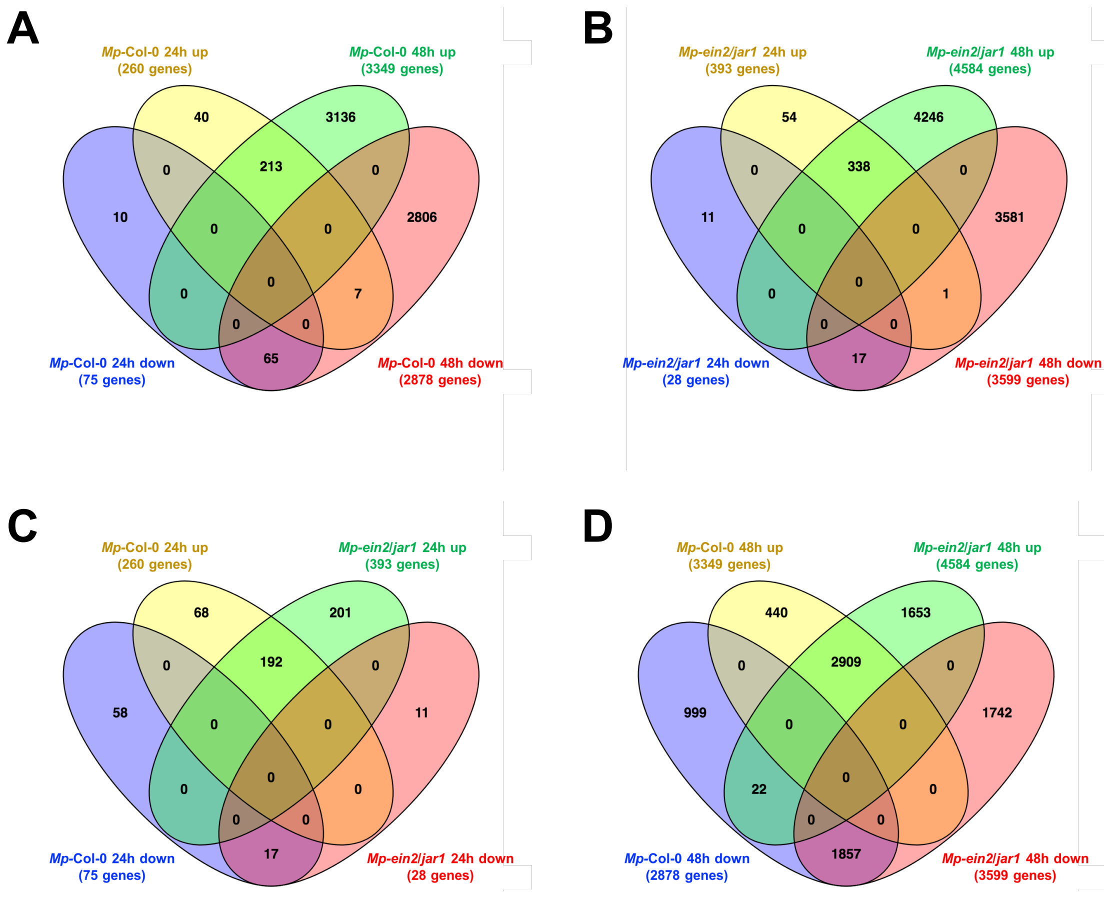


Figure 2.11. Transcript profiling comparison of Col-0 and *ein2/jar1* at 24 or 48 hpc with *Mp*. Venn diagram analysis illustrates differences and similarities between wild type and *ein2/jar1 Mp* infection. (**A**) Col-0 24 h control vs. Col-0 24 hpc with *Mp* compared to Col-0 48 h control vs. Col-0 48 hpc with *Mp*. (**B**) *ein2/jar1* 24 h control vs. *ein2/jar1* 24 hpc with *Mp* compared to *ein2/jar1* 48 h control vs. *ein2/jar1* 48 hpc with *Mp*. (**C**) 24 h Col-0 (from (**A**)) compared to 24 h *ein2/jar1* (from (**B**)). (**D**) 48 h Col-0 (from (**A**)) compared to 48 h *ein2/jar1* (from (**B**)).

Similarly, a comparison of the same set of *Mp*-induced up-regulated genes to hormone treated Arabidopsis revealed commonalities of *Mp*-induced responses to those activated by other defense stimuli (Figure 2.10B). RNA-seq-based transcriptional profiling revealed up-regulation of 827 genes when whole six day-old Col-0 seedlings were soaked in 100 μM jasmonic acid (JA) for four h (Zheng

et al., 2017). This gene set had a 54% (449/827) overlap with the *Mp* data (Figure 2.10B). Microarray analysis of root tissue from five day-old plants placed on agar containing 1 mM of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) for 24h showed 177 genes that were up-regulated relative to the 0 h time-point sample, based on averages of three replicates (Harkey *et al*., 2018). Of those 177 genes, 67, or ~38%, overlaped with the *Mp* –up-regulated gene set (Figure 2.10B). The SA analog 2,6- dichloroisonicotinic acid (INA) induced gene expression at two and six days-post-treatment in two week-old Col-0 seedlings (Knoth *et al*., 2009). Shoot tissue was used for microarray analysis in which 590 genes were up-regulated; 203 (or, 34%) of which overlaped with the *Mp* data set. These observations further confirmed a likely role of ethylene and jasmonic acid as critical regulators of immune responses against *Mp*, but also suggested that parts of the canonical salicylic acid-dependent defense system may, too, be activated in response to *Mp* infections.

Several prominent temporal patterns of transcript level changes were recognized. At 24 h a set of 68 genes (group I, Table 2.3) appeared only to be up-regulated in Col-0 and not in *ein2/jar1* plants (Figure 2.11C). Of these 68 genes, 50 were transiently up-regulated at 24 h, while 18 were sustained at the 48 hpc time point (Table S13). As expected, several members of group I were involved in ethylene and/or jasmonic acid signaling (e.g. several JAZ and ERF transcriptional regulators). The fact that these genes respond early to *Mp* and were only up-

regulated in the more resistant Col-0 line may suggest causal roles of their products in mediating critical defense reactions against *Mp*. An additional 422 genes (440 shown in Figure 11D minus 18 that are also up-regulated at 24 h) were up-regulated at 48 h exclusively in Col-0 (group II, Table 2.3). Group II genes might be considered “late responding”, but still possibly contribute to important defense reactions. GO terms enriched for group II included “triterpenoid biosynthetic process”, “negative regulation of ethylene-activated signaling”, “tetrahydrofolate biosynthetic process”, “cellular response to starvation”, “ethylene-activated signaling pathway”, and “transmembrane transport” (Tables 2.3, S14).

Group III, comprising 201 genes up-regulated only in *ein2/jar1* at 24 h (Figure 2.11C), as well as group IV, a set of 1,653 genes up-regulated only in *ein2/jar1* at 48 h (Figure 2.11D), may feature genes that reflect responses to enhanced stress suffered by the host – or alternatively, may be targeted/up-regulated by *Mp* effectors for the benefit of *Mp* (Tables 2.3, S15-S16).

Group V, 192 genes up-regulated in both Col-0 and *ein2/jar1* at 24 h (Figure 2.11C), and group VI, 2,909 genes up-regulated in both Col-0 and *ein2/jar1* at 48 h (Figure 2.11D), may reflect more generalized responses by the plant at early and later *Mp*-infection stages (Tables 2.3, S17-S18).

Group VII, A-C, gene sets had fewer genes than most other groups and, as such, were not enriched for GO terminology. Nevertheless, individual genes listed in this

group may be informative. For example, group VII-A (Table S19), a set of 22 genes that were up-regulated in *ein2/jar1* at 48 h, but down-regulated in Col-0 at 48 h, contained *WRKY70*, a transcription factor known to repress jasmonic acid-regulated genes (Li *et al*., 2006). Down-regulation of *WRKY70* and *WRKY54* together has been linked to enhanced resistance to necrotrophic pathogens (Li *et al*., 2017). Down-regulation of *WRKY70* in Col-0, yet up-regulation of this gene in *ein2/jar1*, at 48 h may be an interesting example for a transcriptional switch important for defense against *Mp*. Group VII-B (Table S19), comprising seven genes that were up-regulated in Col-0 at 24 h but were down-regulated in Col-0 at 48 h, contained three heat shock family proteins, *GI* (involved in circadian rhythm, 212 papers on TAIR including ethylene, drought or salt stress, but mostly circadian & flowering time & light) and *PIN5,* a regulator of intracellular auxin distribution. Group VII-B also contained genes encoding a serpin protease inhibitor and *LNK1.* While serpin protease inhibitors may have roles in plant defense (Fluhr *et al*., 2012), LNK1 is involved in phenylpropanoid biosynthesis and circadian rhythm (Rugnone *et al*., 2013; Zhou *et al*., 2017); both of which are processes potentially connected to plant immunity. Group VII-C (Table S19) consists of a single gene, Acyl-CoA N-acyltransferases (NAT) superfamily protein, that was up-regulated in *ein2/jar1* at 24h but down-regulated in *ein2/jar1* at 48h.

Down-regulated genes were also of interest in this study as they may also be an integral component of the defense response or possibly be manipulated by the

pathogen. Group VIII was a set of 58 genes down-regulated only in Col-0 at 24 h (Figure 2.11C) with GO term enrichment in processes of cellular growth and cell wall modification (Tables 2.3, S20). Group IX, 999 genes down-regulated in Col-0 at 48 h (Figure 2.11D), consisting of 980 genes that were newly down-regulated plus 19 genes that had sustained down-regulation since the 24 h time point in Col-0 (Table 2.3). Group IX GO terminology is enriched for multiple aspects of cell wall metabolism and a mixture of biosynthetic and catabolic cell wall processes (Table S21).

Group X had 11 genes that were down-regulated only in *ein2/jar1* at 24 h and were not enriched for GO terminology (Tables 2.3, S22). Individual genes ranged in function from RNA catabolism, toxin catabolism, detoxification, mitochondrial electron transport chain, terpenoid synthesis, translation initiation, negative regulation of a brassinosteroid pathway, to a UDP-glycosyltransferase that may be involved in jasmonate signaling (Chini *et al*., 2007; https://www.arabidopsis.org). Group XI, 1,742 genes down-regulated only in *ein2/jar1* at 48 h, was characterized by a high enrichment of RNA processing-related GO terms (Tables 2.3, S23). Group XII has 17 genes that were down-regulated only in both Col-0 and *ein2/jar1* at 24 h and was not enriched for GO terminology (Tables 2.3, S24). At a glance, this group contained cell wall-related genes (e.g. pectin-lyase like, expansin, pectinesterase), vacuole-related genes and stress- related genes.

Group XIII, 1,857 genes down-regulated in both Col-0 and *ein2/jar1* at 48 h, was second in size to group VI which listed 2,909 genes that were up-regulated in both Col-0 and *ein2/jar1* at 48 h (Tables 2.3, S25). Members of groups VI and XIII may play important roles in the activation or maintenance of defense reactions or be associated with the enhanced damage/stress in the host and/or enhanced pathogenicity of *Mp* in host tissues. Group XIII contained a wide variety of enriched GO terms. Certain GO terms appeared to involve logically down-regulated processes by plants under heavy attack by a root pathogen (e.g. phloem transport, chlorophyll biosynthesis, cell growth, cell-cell junction assembly).

Overall, the RNA-seq results provided an abundance of potentially interesting gene expression changes that may be relevant to the outcome of *Mp*-Arabidopsis interactions. As outlined in the discussion (below), some of these gene expression changes provided clues about processes likely to be important components of host defense or *Mp* virulence strategies. Reverse genetic studies on key genes representing certain themes will allow further testing of functional significance in the *Mp*-Arabidopsis interaction.

Table 2.3. GO terms significantly enriched in groups I - XIII.

Group	Description	No. genes	GO terms of enriched functional classes	Fold Enrichment	**Number of related categories also significantly enriched
I	24 h, up only in Col-0	68	response to wounding	13.9	0
			response to jasmonic acid	13.84	0
			defense response	3.85	0
			response to stress	3.21	0
			response to stimulus	2.05	0
II	48 h, up only in Col-0	422	triterpenoid biosynthetic process	22.58	1
			negative regulation of ethylene-activated signaling pathway	19.92	4
			tetrahydrofolate biosynthetic process	15.94	0
			cellular response to starvation	5.02	6
			ethylene-activated signaling pathway	4.52	8
			transmembrane transport	2.42	0
III	24 h, up only in *ein2/jar1*	201	cellular response to sucrose starvation	>100	0
			tryptophan catabolic process	52.62	19
			camalexin biosynthetic process	38.27	7
			jasmonic acid and ethylene-dependent systemic resistance	28.06	5
			response to absence of light	16.70	0
			response to hydrogen peroxide	14.44	7
			response to high light intensity	8.16	1
			response to heat	5.87	1
			defense response to bacterium	4.53	9
			response to ethylene	4.17	3
			response to acid chemical	2.99	2
IV	48 h, up only in *ein2/jar1*	1,653	defense response by callose deposition in cell wall	7.67	2
			response to chitin	4.41	3
			plant-type hypersensitive response	4.14	3
			autophagy	4.02	1
			response to oomycetes	3.97	0
			negative regulation of defense response	3.64	1
			regulation of response to biotic stimulus	3.29	0
			toxin metabolic process	3.21	0
			positive regulation of innate immune response	3.16	9
			regulation of response to external stimulus	3.09	1
			regulation of programmed cell death	3.02	1

			cell surface receptor signaling pathway	2.81	0
			defense response to bacterium	2.62	1
			defense response, incompatible interaction	2.61	4
			protein autophosphorylation	2.48	2
			response to wounding	2.30	0
			protein ubiquitination	2.07	2
V	24 h, up in both Col-0 and *ein2/jar1*	192	protein refolding	20.39	0
			cellular response to unfolded protein	17.92	3
			response to hydrogen peroxide	15.22	5
			response to virus	11.09	0
			cellular response to heat	10.87	2
			glutathione metabolic process	10.87	0
			toxin metabolic process	10.41	0
			response to toxic substance	10.33	11
			response to high light intensity	10.32	0
			antibiotic catabolic process	9.15	0
			cofactor catabolic process	7.46	1
			aging	6.76	0
			drug catabolic process	5.30	0
			response to cadmium ion	4.36	2
			defense response to bacterium	4.34	8
			response to acid chemical	2.52	1
			oxidation-reduction process	2.36	0
VI	48 h, up in both Col-0 and *ein2/jar1*	2,909	leucine catabolic process	8.46	5
			camalexin biosynthetic process	6.28	8
			protein localization to chromosome	5.92	0
			chlorophyll catabolic process	5.75	1
			cellular response to decreased oxygen levels	4.76	5
			jasmonic acid biosynthetic process	4.52	2
			regulation of jasmonic acid mediated signaling pathway	4.42	0
			defense response to insect	4.38	0
			defense response to bacterium, incompatible interaction	4.19	2
			trehalose metabolic process	3.86	1
			response to absence of light	3.76	0
			response to chitin	3.58	2
			response to nutrient	3.52	0
			tryptophan metabolic process	3.48	2
			fatty acid beta-oxidation	3.44	9
			autophagosome organization	3.39	3
			defense response to fungus, incompatible interaction	3.29	4
			response to oomycetes	3.16	0
			response to wounding	3.15	0
			response to salicylic acid	3.14	1
			plant organ senescence	3.06	2

			response to virus	2.96	0
			response to antibiotic	2.83	4
			response to drug	2.76	2
			response to jasmonic acid	2.72	0
			regulation of response to external stimulus	2.67	0
			cofactor catabolic process	2.65	4
			ethylene-activated signaling pathway	2.63	3
			regulation of response to biotic stimulus	2.59	0
			response to water deprivation	2.53	1
			auxin metabolic process	2.47	1
			response to starvation	2.39	4
			response to acid chemical	2.38	3
			regulation of defense response	2.29	0
			response to carbohydrate	2.21	0
			peptidyl-serine modification	2.14	0
			cell death	2.13	0
VII-A	48 h, up in *ein2/jar1* and down in Col-0	22	No statistically significant results		
VII-B	Col-0, up at 24 h and down at 48h	7	No statistically significant results		
VII-C	*ein2/jar1*, up at 24 h and down at 48h	1	No statistically significant results		
VIII	24 h, down only in Col-0	58	cell wall modification	14.89	2
			developmental growth involved in morphology	11.04	2
			cell growth	9.76	1
			cellular developmental process	5.02	0
IX	48 h, down only in Col-0	999	glucuronoxylan biosynthetic process	14.09	12
			xanthophyll metabolic process	10.06	0
			lignin catabolic process	9.39	1
			plant-type cell wall modification involved in multidimensional cell growth	7.31	6
			plant-type secondary cell wall biogenesis	7.17	4
			hydrogen peroxide catabolic process	3.68	4
X	24 h, down only in *ein2/jar1*	11	No statistically significant results		
XI	48 h, down only in *ein2/jar1*	1,742	‘de novo’ UMP biosynthetic process	16.07	28

			leading strand elongation	12.86	2
			DNA unwinding involved in DNA replication	12.50	3
			maturation of LSU-rRNA from tricistronic rRNA transcript	11.13	1
			snoRNA 3'-end processing	10.23	2
			histidine biosynthetic process	10.23	2
			rRNA pseudouridine synthesis	10.05	2
			ribosomal large subunit assembly	9.99	1
			endonucleolytic cleavage involved in rRNA processing	9.89	5
			ribosomal small subunit assembly	9.82	2
			rRNA 3'-end processing	9.18	0
			ribosomal small subunit biogenesis	9.06	2
			U4 snRNA 3'-end processing	8.77	5
			geranyl diphosphate biosynthetic process	8.66	1
			maturation of 5.8S rRNA from tricistronic rRNA transcript	8.46	3
			'de novo' pyrimidine nucleobase biosynthetic process	8.04	1
			lysine biosynthetic process via diaminopimelate	8.04	5
			arginine biosynthetic process	8.04	2
			cytoplasmic translation	7.81	0
			rRNA metabolic process	7.41	1
			geranylgeranyl disphosphate biosynthetic process	7.31	1
			nuclear-transcribed mRNA catabolic process, exonucleolytic, 3'-5'	6.89	0
			nuclear mRNA surveillance	6.89	2
			mitochondrial RNA processing	6.89	0
			leucine biosynthetic process	6.89	5
			mitochondrial gene expression	6.73	1
			establishment of protein localization to mitochondrial membrane	6.70	06.70
			cytidine to uridine editing	6.70	1
			ribonucleoprotein complex assembly	6.24	7
			chloroplast fission	6.12	1
			mitochondrial RNA metabolic process	6.06	3
			translation	5.91	6
			protein targeting to mitochondrion	5.87	11
			ncRNA processing	5.83	6
			DNA replication initiation	5.56	2
			glycogen metabolic process	5.36	1
			RNA secondary structure unwinding	5.02	0
			'de novo' protein folding	5.02	1
			embryo sac central cell differentiation	5.19	5
			regulation of DNA replication	4.52	0
			negative regulation of DNA metabolic process	4.50	1
			cell proliferation	3.76	0
			histone methylation	3.40	7
			nucleocytoplasmic transport	2.52	1

			ribonucleoside monophosphate metabolic process	2.52	3
			DNA metabolic process	2.41	1
			mitotic cell cycle	2.22	0
			DNA repair	2.17	1
			gene expression	2.16	1
			embryo development ending in seed dormancy	2.03	0
XII	24 h, down in both Col-0 and *ein2/jar1*	17	No statistically significant results		
XIII	48 h, down in both Col-0 and *ein2/jar1*	1,857	glycine decarboxylation via glycine cleavage system	12.83	6
			cell-cell junction assembly	11.97	1
			reductive pentose-phosphate cycle	8.31	3
			plant-type primary cell wall biogenesis	7.48	4
			gluconeogenesis	5.99	4
			phloem transport	5.82	1
			very long-chain fatty acid biosynthetic process	5.82	3
			cell wall pectin metabolic process	5.70	1
			negative regulation of actin filament polymerization	5.61	4
			suberin biosynthetic process	5.51	2
			chaperone cofactor-dependent protein refolding	5.39	3
			ATP synthesis coupled proton transport	5.34	1
			protein refolding	5.16	0
			mucilage metabolic process involved in seed coat development	4.99	0
			purine ribonucleoside triphosphate biosynthetic process	5.16	57
			ATP hydrolysis coupled proton transport	4.60	11
			cellulose biosynthetic process	4.55	6
			mucilage metabolic process	4.53	1
			purine ribonucleoside biosynthetic process	4.36	8
			pectin biosynthetic process	4.23	1
			photorespiration	4.16	1
			chlorophyll biosynthetic process	4.01	8
			pectin metabolic process	3.87	3
			response to unfolded protein	3.74	0
			tricarboxylic acid metabolic process	3.61	7
			response to karrikin	3.60	0
			flavonoid biosynthetic process	3.57	1
			acyl-CoA metabolic process	3.29	3
			plant-type secondary cell wall biogenesis	3.26	3
			cellular glucan metabolic process	3.25	8
			carbohydrate biosynthetic process	3.23	2
			response to cytokinin	3.14	0

			carbohydrate catabolic process	3.13	0
			phenylpropanoid metabolic process	3.08	2
			generation of precursor metabolites and energy	2.97	2
			cell wall organization or biogenesis	2.92	3
			steroid biosynthetic process	2.84	0
			sterol metabolic process	2.62	0
			microtubule cytoskeleton organization	2.59	2
			response to cadmium ion	2.47	1
			rhythmic process	2.43	0
			organic hydroxyl compound biosynthetic process	2.28	0
			cell growth	2.03	0

*The GO term may be a specific subclass or a parent term. GO terms with fold enrichment >2.00 were listed. When multiple terms were significantly enriched within the same functional class, only the GO term with the highest fold enrichment was listed.

** The number of GO term categories that were related to the listed term (within the same functional class, either as a subclass or parent class) and were also significantly enriched >2.00.

Discussion

Using novel agar plate-based assays for interactions between Arabidopsis roots and *Mp*, the *ein2/jar1* double mutant was found to be more susceptible to this pathogen compared to its wild type Col-0 parental background. Three different quantitative/semi-quantitative assays (microsclerotia density, SCAR-qPCR and shoot disease index) provided highly consistent results uncovering enhanced fungal growth and host damage by *Mp* in this double mutant. The *ein2* single mutant, but not the *jar1* single mutant, exhibited enhanced levels of *Mp* susceptibility in the SCAR-qPCR assay, while both single mutants clearly displayed enhanced shoot disease indices after *Mp* infection. These results implicate ethylene signaling as critically important for protection against *Mp* in Arabidopsis and attest a partial contribution to this by jasmonic acid signaling.

Ethylene- and jasmonate-dependent signaling processes have also been implicated in defense against *Mp* in *Medicago truncatula* and *Sesamum indicum* (Chowdhury *et al*., 2017; Gaige *et al*., 2010). Chowdhury and coworkers observed increased activation of jasmonic acid and ethylene genes in sesame plants in response to *Mp* infections (Chowdhury *et al*., 2017). Gaige and coworkers proposed *Mp* interference of jasmonic acid- and ethylene- directed responses in *Medicago trunculata* due to the low up-regulation of specific genes, particularly in root tissue (Gaige *et al*., 2010). They also saw an increase in length of survivorship in *Mp*-infected plants treated with methyl jasmonate and/or ethylene (Gaige *et al*.,

2010). Consistent with a role for ethylene- and jasmonic acid-signaling in *Mp* immunity, up-regulation of numerous genes related to these processes were observed in *Mp*-infected Col-0 plants. These included genes of numerous ERF transcription factors, jasmonate responsive JAZ transcriptional regulators as well as ethylene or jasmonic acid biosynthetic enzymes. Given that *Mp* is considered to exhibit a necrotrophic lifestyle, it is not surprising that Arabidopsis activated ethylene/jasmonic acid-dependent immune responses upon infection with this pathogen. As outlined earlier in the introduction, both of these stress hormones are known to control plant immune responses against necrotrophic pathogens (Glazebrook, 2005; Laluk and Mengiste, 2010). However, the results implicating ethylene and jasmonic acid as regulators of *Mp*-immunity in Arabidopsis served as critical “proof-of-concept” and showed that the new model pathosystem and defense assays developed here indeed allowed for the discovery of Arabidopsis processes controlling *Mp* infections. Furthermore, the results provided an opportunity to use an Arabidopsis line with elevated levels of susceptibility for a comparative transcriptomics study to identify gene expression changes associated with host defense reactions against *Mp*.

Surprisingly, a substantial overlap of the *Mp*-responsive Arabidopsis transcriptome with gene sets known to be inducible by salicylic acid was observed. Given the necrotrophic life-style of *Mp*, this was somewhat unexpected, as salicylic acid is known to control immune responses against biotrophic pathogens (Glazebrook,

2005; Yang *et al*., 2015). However, Chowdhury and co-workers (Chowdhury *et al*., 2017) recently proposed that in interactions with sesame, *Mp* employs a short biotrophic attack strategy prior to switching to a primarily necrotrophic phase. Based on *Mp* marker gene expression and morphological features of *Mp* hyphae they claim that between 24 h and 38 h after infection of sesame *Mp* transitions from a biotrophic phase to a necrotrophic phase. This "biotrophy-necrotrophy switch" (BNS) is accompanied by a change of physiological, biochemical and transcriptional responses of the host. Most importantly, BNS is associated with a transition from typical salicylic acid- to ethylene/jasmomic acid-response gene expression in sesame. Potentially defense-associated secondary metabolites (in particular flavonoids) are also more prevalent in the host after BNS.

The RNA-seq results were consistent with the observations and conclusions made by Chowdhury and coworkers. When interpreting the timing of transcriptional changes in pathogen-infected plants, it is important to consider that infection events do not occur synchronously in all cells/tissue areas of the host. At any time point, only the "average" of multiple non-synchronized events can be monitored. Thus, the apparent co-occurrence of salicylic acid and ethylene/jasmonic acid-responsive gene expression at the two time points (24 hpc and 48 hpc) studied here may, in fact, reflect two successive defense gene expression states in the host. While each individual Arabidopsis root cell may be engaged in either pre-BNS or post-BNS response activities, the tested tissue as a whole may represent

a mixture of response states. Chowdhury and co-workers made similar observations.

Importantly, enhanced levels of *Mp* susceptibility were observed not only in the *ein2/jar1*, *ein2* and *jar1* mutants, but also in *NahG* plants, a transgenic Arabidopsis line expressing a bacterial salicylate hydroxylase (Lawton *et al*., 1995), which converts salicylic acid into catechol, a derivative that does not exhibit the defense inducing activities of salicylic acid. In SCAR-qPCR assays *Mp*-infected *NahG* plants showed a trend toward greater susceptibility while their shoot disease index was significantly higher compared to Col-0 wild type plants. Thus, these results clearly supported some cooperation between salicylic acid signaling and ethylene/jasmonic acid signaling in mediating immunity against *Mp* in Arabidopsis. High resolution time course studies possibly monitoring gene expression events in individual Arabidopsis root cells may have to be employed in the future to determine if an early pre-BNS phase in *Mp* is countered by salicylic acid-mediated host defenses and if later post-BNS growth of *Mp* induces ethylene/jasmonic acid-dependent immunity in Arabidopsis roots.

Additional hormone-related processes suggested by the RNA-seq results included auxin-signaling. The presence of *PIN5* in group VII-B, a gene set which was transiently up-regulated by *Mp* in Col-0, is possibly of interest. *PIN5* encodes a regulator of intracellular auxin metabolism (Mravec *et al*., 2009). Auxin is known to inhibit xylem secondary cell wall deposition through repression of NAC-domain

transcription factors (Johnsson *et al*., 2018). Auxin metabolism was also one of the GO terms enriched in Group VI genes (genes up-regulated in both Col-0 and *ein2/jar1* at 48h). Furthermore, 28 of 2,909 genes in Group VI encoded NAC proteins or proteins related to these transcription factors, some of which may be potentially linked to auxin-controlled cell wall-related processes.

As indicated in the introduction, crop diseases caused by *Mp* are typically associated with drought and heat stress (Muchero *et al*., 2011). Thus, the induction of drought- or heat-tolerance mediating plant stress responses may be linked to immune responses against *Mp*. *Mp*-induced up-regulation of numerous Arabidopsis genes associated with drought and heat tolerance were observed, such as genes encoding several DREB (Drought-Response Element Binding Protein)-type transcription factors, drought tolerance associated LEA (Late Embryogenesis Abundant) proteins, as well as various heat shock transcription factors and heat shock proteins, in the RNA-seq data. Plant tolerance to drought stress is partially controlled by the phytohormone abscisic acid (ABA). Among genes up-regulated by *Mp* in Col-0 were genes encoding the ABA-responsive basic helix loop helix transcription factor BHLH17, the ABA-responsive protein ABR and AFP4 (a negative regulator of ABA-responses). Future forward genetic studies may be performed to test if auxin-signaling, ABA-signaling or other abiotic stress response mechanisms affect the outcome of *Mp*-Arabidopsis interactions.

In any case, the results point toward a complex interplay of multiple plant hormone-controlled signaling processes in immunity against *Mp*.

The pronounced presence of host genes related to cell wall metabolism in gene sets down-regulated by *Mp* was remarkable. With the on-going penetration of cell walls by *Mp* hyphae at 24h and 48h (Figure 2.2), it seemed as if the plant might have increased aspects of cell wall synthesis in defense against the pathogen. However, wall modifications typically performed for cell growth, like some of those represented by genes in group VIII (at 24h), might, theoretically, have been down-regulated by the plant if such modifications also facilitated hyphal invasion. The dynamic nature of cell walls makes it difficult to surmise if the down-regulation of cell wall-related genes in group VIII (at 24h) and in group IX (at 48h) was for the purpose of plant defense or if some of those genes might have been commandeered by the pathogen. Typical plant responses to necrotrophs include cell wall reinforcement and modifications that make the wall less susceptible to enzymatic digestion, a process that can be altered by the pathogen to, instead, increase wall digestibility (Houston *et al*., 2016).

Having established the Arabidopsis-*Mp* interaction as a lab model will allow for faster progress in uncovering plant defenses against this detrimental pathogen. In this study, advantages of the Arabidopsis system were clear. An abundance of well-characterized mutants with defined defects in signaling pathways enabled targeting of certain candidate processes (e.g. ethylene- and jasmonic acid-

signaling) for reverse genetic analyses. The existence of a well-annotated Arabidopsis genome sequence enabled extraction of detailed information from the RNA-seq based transcriptomics experiments. Furthermore, a wealth of existing RNA-seq and microarray data was available for a wide variety of biological conditions in Arabidopsis for comparison to the results.

Next steps in the use of the new model phyto-pathosystem include systematic testing of candidate genes identified by the RNA-seq study for their contribution to *Mp* defense responses using sequence-indexed T-DNA mutant collections, such as the SALK (Alonso *et al*. 2003), SAIL (Sessions *et al*., 2002) or Gabi-Kat (Kleinboelting *et al*., 2012) populations, which collectively cover almost 100% of all Arabidopsis genes. For example, *tat3*, a SALK line interrupting the tyrosine aminotransferase-encoding gene *TAT3*, had a significantly higher shoot disease index score than Col-0 (Figure 2.7B). This line was tested because *TAT3* showed the highest $\log_2$ fold change value (5.026) in *Mp*-infected relative to uninfected Col-0 plants at 24 hpc in the RNA-seq data (Table S1). The same reverse genetics resources can be used to test conserved QTL candidate loci potentially protecting crop species against *Mp*. Comprehensive collections of natural Arabidopsis accessions (ecotypes) can be tested against a panel of different *Mp* isolates to identify race-specific interactions with differential outcomes. Existing SNP resources for many Arabidopsis accessions or recombinant inbred lines (Weigel 2012) can be used to conveniently map loci responsible for the observed

differential effects. Finally, Arabidopsis has proven to be an excellent system for high throughput applications, such as forward genetic screens of large populations of randomly mutagenized individuals (Alonso and Ecker, 2006) or chemical screens (Raikhel and Pirrung, 2005; Knoth *et al*., 2009). Mutations or chemicals, respectively, affecting the outcome of plant-*Mp* interactions, can be identified this way. Members of the lab of Thomas Eulgem have begun some of these approaches.

Although artificial, the agar plate-based Arabidopsis root assays provided conditions for *Mp* to engage in its natural mode of host infection, as microsclerotia germinated and formed hyphae, which penetrated dermal root tissues resulting in the colonization of vascular bundles and the formation of new microsclerotia within the host. Thus, molecular host responses identified in these assays are likely to reflect authentic processes occurring during plant-*Mp* interactions under natural conditions. The new experimental system established here should allow researchers to make great progress regarding the identification of key genes affecting the outcome of plant-*Mp* interactions. Knowledge of such genes in Arabidopsis and their orthologs in crop species will facilitate the design of new molecular markers for precise marker-based breeding approaches in economically important plants. New synthetic elicitors may also be identified that specifically activate plant immune responses active against *Mp* and which could serve as leads for the development of new pesticide alternatives. Beyond benefiting

agriculture directly, discoveries made using the experimental pathosystem will allow for the gain of deeper insight into immune responses against *Mp*. The potential interplay of various hormone-pathways, which may be involved in controlling immunity against *Mp*, is a particularly appealing subject that could be approached using the simple agar-plate-based *Mp* infection assays designed here.

Materials and Methods

Plant and pathogen growth conditions

The Arabidopsis double mutant line *ein2*-1/*jar1*-1 has been described (Clarke *et al*., 2000). All mutants are in the Arabidopsis ecotype Columbia (Col-0) background. Growth room conditions were under fluorescent lights (16h of light/8h of dark, 23°C, 100 μE m−2 s–1). Seeds were surface-sterilized in 70% ethanol and a 0.02% Triton X, 20% bleach solution, for three and ten minutes, respectively, followed by sterile water rinses. Seeds were then plated on solid media containing ½ MS (Murashige and Skoog), 0.05% MES, 0.25% sucrose and 0.87% agar; pH was 5.7 prior to the addition of agar. All ½ MS-agar plates contained approximately 25ml of media. Plated seeds were stratified for two days (or six days for accessions other than Col-0) at 4°C before plates were positioned vertically under growth room conditions for 10 days.

Mp (originally isolated from UCR Ag Operations Field 11 by members of Philip Roberts' laboratory, see Muchero *et al*., 2011) was propagated by adding a plug to potato dextrose agar (BD Difco™, http://us.vwr.com) -containing plates (10ml per plate), incubating at 34°C for 10 days and then allowed to dry at RT for at least four weeks.

***Mp* infection assays**

Mp infection plates were created by adding *Mp* inoculum (approximately 2000 microsclerotia in 2-3 ml media for microsclerotia counting and RNA-seq roots and twice the amount for the shoot disease index assay) to plates, placing plates in a dark incubator at 34°C for three days and then transferring the plates to growth room conditions for an additional three days. The media used to pour plates and create inoculum contained ½ MS (Murashige and Skoog), 0.05% MES, 0.25% sucrose and 0.87% agar; pH was 5.7 prior to the addition of agar. Dried *Mp* propagation plate contents were ground in 10-20 ml sterile water with mortar and pestle, added to media and counted in 100μl drops on slides until the desired concentration was reached.

For the microsclerotia density assay, 10 day-old seedlings were gently transferred to *Mp* infection plates, with 12-14 plants per plate, and these plates were placed vertically in growth room conditions for four to five days. To arrest *Mp* development, 20-30 ml 75% EtOH was added to each plate. Microsclerotia were counted under bright field microscopy and root lengths were measured using ImageJ (Schneider *et al*., 2012). Magnified images were taken using a Leica DM/LB2 (Leica, Wetzlar, Germany) microscope equipped with an RT colour SPOT camera.

For the *Mp*-Arabidopsis shoot disease index, 10 day-old seedlings were gently transferred to *Mp* infection plates, with at least two lines per plate (10 plants per line), and these plates were placed horizontally in growth room conditions for up to three weeks. Images were taken of whole plates throughout the infection period to monitor progress of disease symptoms in plants. Individual plants were assessed according to the following disease index scoring system:

Score	Disease symptoms
0	Plant was 100% healthy and looked like the negative control. No stunting, chlorosis or necrosis was visible.
1	Plant was green and appeared healthy; stunted growth was the only visible symptom.
2	Up to 30% of visible plant tissue was affected by chlorosis and/or necrosis.
3	30% to 60% of visible plant tissue was affected by chlorosis and/or necrosis.
4	60% to 90% of visible plant tissue was affected by chlorosis and/or necrosis.
5	Plant was dead and there were no signs of living tissue.

All scores of 1 or higher may include stunting. All lines so far tested had a stunted phenotype relative to plants grown on the same media without the pathogen (negative control). Therefore, stunting was counted as a single point in the index.

Transcriptome profiling by mRNA-seq

For RNA-seq, 10 day-old seedlings were transferred to agar plates without *Mp* or to *Mp* infection plates, with 12-14 plants per plate, and these plates were placed vertically in growth room conditions for 24h or 48h. Plant roots were separated from shoot tissue using a blade and flash frozen in liquid nitrogen at 24 or 48 h post transfer. Total RNA was isolated from roots using TRIzol

(Invitrogen™, http://www.thermofisher.com). RNA was processed (74204 QIAGEN, http://www.qiagen.com, and AM1907 Invitrogen™, http://www.thermofisher.com) and libraries were prepared with the NEBNext Ultra Directional RNA Library Prep Kit for Illumina by following the manufacturer's instruction (E7490S, E7335S, E7420S, New England Biolabs, http://www.neb.com). Root tissues were separately analyzed for each line (Col-0 or *ein2*-1/*jar1*-1), treatment (+/- *Mp*) and time point (24h or 48h). Two independent biological replicates for each experimental condition were performed.

Libraries were pooled and sequenced on an Illumina HiSeq 2500 (Illumina, San Diego, CA, USA) platform at the UCR Genomics Core Facility. Reads that passed Illumina's quality control filters were further processed. The quality of sequencing reads was assessed using FastQC v 0.11.5 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The unique reads were mapped to the Arabidopsis genome (TAIR10) using STAR v 2.5.3a (Dobin *et al.*, 2013) with default settings and a known splice site file, built from Araport annotation file v11. Reads in gene regions were counted using featureCounts (Liao *et al.*, 2014). The expression fold-change of each gene was calculated using the R package DESeq2 v1.14.1 (Love *et al.*, 2014) with the threshold for differentially expressed genes set to p value < 0.05.

The MA-plots illustrate log-fold change (M-values, i.e. the log of the ratio of level counts for each gene between two samples) against the log-average (A-values, i.e. the average level counts for each gene across the two samples). They showed the log_2 fold changes attributable to a given variable over the mean of normalized counts for the compared samples. Differentially expressed gene points were red if the adjusted *p* value was less than 0.01. Genes with similar expression levels in two samples appeared around the horizontal line y = 0. Data comparison AGIs can be found in tables S5-S12. Read mapping/counting and the identification of differentially expressed genes were performed by collaborator Professor Tokuji Tsuchiya, Nihon University, Japan.

Comparisons between experimental gene sets and sets of genes responding to other stimuli were done using Venny 2.1, (http://bioinfogp.cnb.csic.es/tools/venny/index.html) (Figures 10-11).

Table 2.3 groups I-XIII GO terminology was compiled at http://go.pantherdb.org/ with the annotation version GO Ontology database, released 2018-04-04, analysis type: PANTHER Overrepresentation Test (released 20171205) and test type: Fisher's Exact with FDR multiple test correction displaying only results with a false discovery rate < 0.05. Table 2.3 groups I-XIII AGIs are listed in tables S13-S25.

Real-time PCR quantification of *Mp* biomass

Arabidopsis roots were harvested 48 hpc with *Mp* for genomic DNA extraction. Genomic DNA was extracted using the cetyltrimethylammonium bromide (CTAB) method modified from (Doyle and Doyle, 1990), 1% PVP (chloroform/isoamylalcohol) was added in the 2% CTAB extraction buffer prior to use. After RNase A digestion (19101 QIAGEN http://www.qiagen.com), 20ng of genomic DNA were used for qPCR amplification using the CFX Connect detection system (Bio-Rad) with iQ SYBR Green Supermix (Bio-Rad). Two pairs of species-specific primers were used; for *Mp* DNA amplification: MpSyk-F 5'-ATCCTGTCGGACTGTTCCAG-3' and MpSyk-R 5'-CTGTCGGAGAAACCGAAGAC-3'; for Arabidopsis DNA amplification: AtSK11-F 5'-CTTATCGGATTTCTCTATGTTTGGC-3' and AtSK11-R 5'-GAGCTCCTGTTTATTTAACTTGTACATACC-3'. Melt curve analysis was performed following 40 cycles of amplification with the annealing temperature at 60°C. The ratios of *Mp* and Arabidopsis genomic DNA were calculated by the standard curve method. Serial dilutions of *Mp* and Arabidopsis genomic DNAs were used for standard curve generation. The SCAR-qPCR assays used for figure 2.6 were performed by Dr. Yan Lai, a post-doc in the lab of Dr. Thomas Eulgem, with infected root tissues provided by Mercedes Schroeder.

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General Conclusions

Despite 10,000 years of human-directed crop plant evolution, microbial plant pathogens are a major cause of yield loss around the world. Wild plant species have retained defenses needed to protect against most pathogens, making diseases relatively rare compared to many agricultural crop plants. The plant immune system is, in itself, a guide for the design of crop protection strategies. Its complexity has evolved from innumerable random mutations that resulted in adaptations to environmental stimuli. Layers of intricate molecular signaling pathways allowed plants to engage in primary metabolism to fuel development, while simultaneously responding to various forms of stress. To better protect crops from phytopathogens, a comprehensive understanding is needed of the molecular mechanisms, or physical or chemical barriers, that resistant plants maintain to remain immune to a given pathogen in order to breed/generate plants with these traits. Such an approach, a speeding up of the evolutionary process, has been at work in agriculture over the last ten millennia, chiefly serving the purpose of increased food production, and the results have seen gains and losses of specific plant attributes.

Breeding crop plants for a specific disease-resistance phenotype can often be a complex trait, involving various genes (Collard *et al.*, 2005). When a single gene confers resistance in a crop cultivar, pests may more-rapidly evolve to surpass

incompatibility than if multiple genes were involved in resistance (Bruce, 2010). Multiple genes, with various functions, have been identified in the RNA-seq data generated from *Mp* infection of Arabidopsis roots (described in chapter II) that may be valuable in crop breeding efforts to increase resistance to *Mp*. To identify the genetic regions, or loci, responsible for resistance in crop plants, breeding studies are initiated in which a susceptible parent and a resistant parent are crossed and the genomes of progeny (and subsequent generations of progeny) are analyzed. Such studies against *Mp* have been performed for multiple crop plants including castor, cowpea, sorghum and soybean (Adeyanju *et al*., 2015; Coser *et al*., 2017; Muchero *et al*., 2011; Tomar *et al*., 2017). Quantitative trait loci (locations of traits on genetic maps) identified in these studies point to candidate genes and orthologous genes in Arabidopsis can be further studied using the *Mp*-Arabidopsis plate-based system described in chapter II of this dissertation. Current *Mp* control measures, (e.g. fungicide application) often provide inadequate protection (Coser *et al*., 2017; Mengistu *et al*., 2013). Although partial, but not complete, crop plant resistance to *Mp* has been reported, it is considered that continued efforts toward developing genetic resistance is the most sustainable way to manage *Mp*-caused disease (Coser *et al*., 2017). The Arabidopsis-*Mp* model system can be used to enhance and accelerate development of crop plants toward complete resistance to *Mp*. Arabidopsis has been used to study defense reactions to multiple pathogens and such studies identified putative defense genes in those systems that overlaped with the genes found in the Arabidopsis-*Mp* work described here.

These genes may be of use in determining the best candidate genes for breeding strategies in defense against multiple pathogens. The lab of Thomas Eulgem is also in the process of pinpointing Arabidopsis accessions with enhanced resistance to *Mp* to identify further genetic regions that provide protection against this pathogen. In addition, testing is on-going of T-DNA mutant lines of genes related to those identified with the microsclerotia density assays and RNA-seq data to gain a more comprehensive understanding of the plant defense response to *Mp*.

Next, using the same *Mp* plate-based system, tests in other plant species, including strawberry and tomato, are planned. *Mp*-infection of strawberry plants has been reported in the US, Spain and other countries and has been known to be highly aggressive in California (Avilés *et al*., 2008; Burkhardt *et al*., 2018; Koike *et al*., 2016; Zveibil *et al*., 2012). As of 2014, *Mp*-caused strawberry plant disease was present in every major California coastal strawberry production county and was associated with the discontinuation of methyl bromide + chloropicrin soil treatment in those fields (Koike et al., 2016). Strawberry and tomato are major cash crops in California, which produces nearly 90% of the US fresh strawberry market (https://www.smithsonianmag.com/science-nature/toxic-rise-california-strawberry-180967934/) and 99% of all processed tomato products consumed in the US (http://www.californiacountry.org/features/article.aspx?arID=620). While some host specialization of *Mp* isolates has been reported (Koike *et al*., 2016; Su *et al*., 2001), *Mp* (a monotypic genus with only one species) is still a broad

spectrum host pathogen and individual isolates can often infect multiple host species. The *Mp* used in our research with Arabidopsis was initially isolated from infected cowpea plants and is expected to infect other plants species as well, however, studies with a new isolate, taken from California-grown strawberry plants for example, could also be very informative. Although strawberry plants are typically propagated clonally, seeds for multiple cultivars and hybrids are available for purchase and could be tested using the described assays. Studies of tomato lines, including, for example, *epinastic* (*epi*), which has a distinct set of ethylene responses that are constitutively active (including an ethylene-inducible chitinase gene) (Barry *et al*., 2001), or *Never-ripe* (*Nr*), an ethylene-insensitive mutant (Lanahan *et al*., 1994), or *def1* (*defenseless* 1), a jasmonic acid-deficient line (Howe *et al*., 1996) may be used to initiate analysis of the roles of ethylene and jasmonic acid in this crop plant's defense response to *Mp*. Even tomato cultivars like Motelle and Moneymaker can be studied to compare plants with or without the *Mi* gene (R gene resistance against aphid and nematode; Kaloshian *et al*., 1995) against *Mp*. *Mp*-infected tomato plants (variety Nagina) were reported just two months ago in Pakistan (Hyder *et al*., 2018); a multi-tiered approach to research in plant defense responses to *Mp* is imperative.

In addition to defense gene/trait identification and breeding strategies, the use of synthetic elicitors to detect key cellular components of the plant defense process can assist in the development of improved crop protection strategies. As described

in previous chapters, DC-SAL application to tomato and cowpea plants has provided protection against microbe-caused diseases. The next steps in analysis with this compound include identification of related signaling pathway genes using Arabidopsis mutants and gene expression in tomato plants using qRT-PCR. Eulgem lab members are currently testing the chemical fate of DC-SAL in Arabidopsis Col-0 seedlings to determine if DC-SAL is converted into salicylic acid or if it activates salicylic acid production in plants. This study will uncover additional keys to the pathway of defense induction in the plant as well as whether DC-SAL serves as a primer, like DC-SA, or as a direct plant defense activator.

DC-SAL will also be sprayed on Arabidopsis plants that are infected with *Mp* in the shoot plate assay to determine its ability to delay disease symptoms. The discovery of the contribution of salicylic acid in the immune response against *Mp* suggests that DC-SAL may provide partial protection against this pathogen. Transgenic *NahG* Arabidopsis plants (which do not accumulate salicylic acid, Delaney *et al*., 1994) had statistically significant higher levels of shoot disease severity scores relative to wild type plants, indicating a role for salicylic acid in defense against *Mp*. Genes encoding products known to operate downstream of salicylic acid in salicylic acid signaling pathways could also be tested in this assay. Of particular interest may be NPR1-independent pathways, given the significant enrichment of camalexin-related GO terms for genes differentially regulated at 48 hpc for both Col-0 and *ein2/jar1* lines in the RNA-seq data. Camalexin

accumulation is not mediated by NPR1 according to studies of Arabidopsis with the necrotrophic fungi *Botrytis cinerea* and *Alternaria brassicicola* (Ferrari *et al*., 2003; Thomma *et al*., 1999).

Chemical modifications of salicylic acid are known to occur in the plant and include methylation, glycosylation and amino acid conjugation (Dempsey *et al*., 2011). Such conversions as well as other interactions in which binding occurs with salicylic acid's C1 moiety could, in theory, be more or less readily performed to an aldehyde than to a COOH, because breaking the C-H bond requires less energy than breaking the O-H bond or due to the more reactive nature of the moiety with an additional O atom. There is a higher difference in electronegativity between oxygen and hydrogen (and also between the C and the OH) than there is between carbon and hydrogen atoms. Interactions of DC-SAL with key defense-related target molecules could conceivably have contributed to DC-SAL's significant effect on Col-0 immunity against *Hpa* at the lowest dose tested ($0.1\mu M$) whereas DC-SA's effect was not statistically significant at that dose. However, many modifications inactivate salicylic acid and while methylation converts the compound into a membrane-permeable signaling molecule, glycosylation allows for vacuolar storage and amino acid conjugation is predicted to promote catabolism (Dempsey *et al*., 2011). Either of these processes may also apply to DC-SAL and DC-SA and the subtle chemical differences between these two synthetic elicitors may result in differences in their processing in plant tissues.

A new synthetic elicitor screen with reporter lines that contain a promoter sequence from *Mp*-responsive genes (identified by the RNA-seq study) is currently being developed in the Eulgem lab to test our most robust synthetic elicitors, and to identify new synthetic elicitors using available chemical libraries. This may result in the discovery of new types of synthetic elicitors efficiently protecting Arabidopsis (and other plants) against *Mp*. Synthetic elicitors that are effective as protectants against *Mp* may be used to identify novel cellular targets that are key to *Mp* defense, further characterize known interactions in the pathosystem or serve as leads for the development of new types of sustainable crop protection procedures that are less hazardous to the environment than current biocidal pesticides yet protect plants against *Mp*.

Globally, 3 billion kg of pesticides, costing nearly 40 billion USD, are applied in agriculture each year (Pimental and Burgess, 2014). Conventional biocidal pesticides that are designed to kill target pathogens pose risks to non-target organisms in the agroecosystem. Agricultural application and runoff of such pesticides introduces toxins into the environment (Aktar *et al*., 2009). In addition, microbes can develop resistance to these toxicants, resulting in an ongoing need to create new biocidal compounds (Hollowmon, 2015). The 2010-2014 estimated cost to create a new pesticide was 286 million USD (107 million USD on research + 146 million USD on development + 33 million USD registration) (McDougall, 2016). Chemicals with a low toxicity potential, that induce immunity in crop plants

improving resistance to disease-causing pathogens, could reduce the need for biocidal pesticides. The translational research, using BHTC, DPMP, DC-SAL and other compounds in tomato and cowpea plants as defense-inducing protectants against relevant microbial pathogens, showed the potential for synthetic elicitor application in agricultural crop disease management. DC-SAL was an efficient and potent compound, with a simple molecular structure, that is low in cost and was the most robust synthetic elicitor yet studied in the lab of Thomas Eulgem.

An important aspect of the research described in chapter I was the protection of tomato plants from diseases. As a general plant defense inducer, DC-SAL will likely be effective against a wide variety of plant pathogens and should therefore provide broad spectrum disease resistance to tomato. Thus, the use of DC-SAL and other effective synthetic elicitors in disease management strategies would specifically benefit the California tomato industry and therefore positively impact the local economy. Furthermore, the proposed *Mp*-strawberry phyto-pathosystem, plus synthetic elicitor application, may provide non-biocidal disease protection alternatives to methyl bromide fumigation for strawberry growers in the state. Methyl bromide, an ozone-depleting compound that was previously used as a pesticide soil fumigant, was phased out (with limited exemptions) on January 1, 2005, according to https://www.epa.gov/ods-phaseout/methyl-bromide. In addition, reduced-risk pesticide alternatives like DC-SAL, especially in an

affordable form, have the potential to benefit cowpea production, a critical component of food security in developing countries.

Together, the four phyto-pathosystems studied in this body of research, a model plant with a foliar, biotrophic oomycete and a root-infecting necrotrophic fungus, as well as two crop plants with a hemibiotrophic foliar bacterium and a root-infecting necrotrophic fungus, allowed for a comprehensive approach to the analysis of compatible plant-pathogen interactions. Arabidopsis (Brassicaceae), tomato (Solanaceae) and cowpea (Fabaceae) plants, representative of three different food/plant taxa, provided a broad picture of plant defense responses that may relate to various agricultural crops. Broader impacts of this research are several-fold, from generation of new scientific knowledge and a novel technique (that is of potential interest to plant pathologists, molecular biologists and plant breeders) to advances in crop protection strategies that could result in economic benefits and improved environmental health. In conclusion, the laboratory and greenhouse experiments and the analyses described in this dissertation, whether applying synthetic elicitors to activate an immune response or learning how to defend plants against *Mp*, provided important translational groundwork toward agricultural crop protection strategies.

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