UNIVERSITY OF CALIFORNIA
RIVERSIDE

Uncovering the Basis of Plant Root Immunity Against the Global Crop Destroyer *Macrophomina phaseolina* Using the Model Plant *Arabidopsis thaliana*

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

Doctor of Philosophy

in

Plant Biology

by

Miwa Shirai

June 2023

Dissertation Committee:
Dr. Thomas Eulgem, Chairperson
Dr. Linda Walling
Dr. Daniel Koenig

The Dissertation of Miwa Shirai is approved:

Committee Chairperson

University of California, Riverside

Acknowledgements

I would like to express my most sincere gratitude to my Ph.D. advisor, Dr. Thomas Eulgem for his support and mentorship over the years. Thank you for always seeing the cup half full, believing in my work, and guiding me with your wisdom.

To all previous and current members of the Eulgem lab, thank you for always being a supportive team. I will miss you all greatly. A special thank you to Dr. Mercedes Schroeder and Dr. Yan Lai for laying the foundation for the work in this dissertation, Dr. Jianqiang Wang for advising me on countless experiments, Ayat Riahi for assisting in the accession screen data collection, and Dr. Tokuji Tsuchiya for performing the alignment, read counting and differential gene analysis for the RNA-sequencing data.

I would also like to thank my dissertation committee members, Dr. Linda Walling and Dr. Dan Koenig, for their advice and guidance throughout the process of putting this project together.

To my family – Mom, Dad, Ian, Noah, and Nana; thank you for all that you have sacrificed for me, for always being my safety net, providing me comfort in the form of great food and laughs, in times when I needed it most. I am incredibly fortunate to be surrounded by such loving people (and cats).

ABSTRACT OF THE DISSERTATION

Uncovering the Basis of Plant Root Immunity Against the Global Crop Destroyer *Macrophomina phaseolina* Using the Model Plant *Arabidopsis thaliana*

by

Miwa Shirai

Doctor of Philosophy, Graduate Program in Plant Biology
University of California, Riverside, June 2023
Dr. Thomas Eulgem, Chairperson

The generalist soil-borne fungal pathogen *Macrophomina phaseolina* is known to cause a disease known as charcoal rot. Despite the widespread damage this pathogen has caused on over 500 species of plants including important crops, the mechanism of its virulence is still unclear, and effective methods of control are still lacking. In this study, *M. phaseolina* interaction with the model plant *Arabidopsis thaliana* is described using a plate-based infection assay which allows for image analysis-based quantitative assessment of disease progression. From a panel of *A. thaliana* inbred lines derived from natural populations, we observed significant quantitative deviation in growth inhibition caused by *M. phaseolina*. In order to gain insight to the genetic architecture behind *M. phaseolina* resistance, we sought to utilize the natural genetic variation represented in the panel of 87 natural *A. thaliana* accessions. Genome-wide association approaches combined with RNA-sequencing identified multiple genomic loci and candidate genes which could potentially contribute to the phenotypic variance. Seedling root

transcriptomes captured at three time points post-infection revealed that resistant and susceptible *A. thaliana* genotypes have distinct patterns in defense gene expression. Surprisingly, in the two resistant accessions examined, a rapid decline in up-regulation of many defense genes were observed between 12 and 48 hours post-infection. Genes previously associated with plant immunity, as well as genes involved in processes that have not been seen in *M. phaseolina* response, were identified in this study, providing a robust pool of candidate genes which will be a useful resource for increasing *M. phaseolina* tolerance in host plants.

Table of Contents

List of Figures

Chapter I:

Chapter II:

List of Tables

Chapter I:

General Introduction

Macrophomina phaseolina (Tassi) Goid. is a fungal pathogen that causes charcoal rot, a root and stem disease characterized by black specs that appear in the host tissue. It is distributed across the globe and is particularly problematic in arid climates (Kaur *et al*., 2012). Charcoal rot symptoms are known to be more severe under heat and drought stress (Fang *et al*., 2011; Mughogho & Pande, 1983). There are more than 500 documented host species susceptible to this pathogen, including many economically important crops including canola, maize, and sesame (Gaetán *et al*., 2006; Saleh *et al*., 2010; Su *et al*., 2001). Moreover, *M. phaseolina* is capable of human infection in immunosuppressed individuals, which demonstrates the range of environments it can thrive in (Arora *et al*., 2012; Tan *et al*., 2008). Such pathogens with a wide host range are described as “generalists”. *M. phaseolina* is difficult to eliminate from infected fields due to its production of microsclerotia, which are dark colored asexual propagation structures that can survive for extended periods of time in soil without a host Originally *M. phaseolina* was thought to be a necrotrophic pathogen, but detailed analysis of the early stages of infection in soybean revealed the presence of a distinct biotrophic phase of up to 36 hours preceding the onset of plant tissue collapse (Chowdhury *et al*., 2017). *M. phaseolina* is therefore often characterized as a hemibiotroph along the likes of *Phytophthora infestans* and *Fusarium*

oxysporum. The asymptomatic biotrophic infection phase further makes early identification of disease in the field difficult.

Agricultural impacts

Disease caused by *M. phaseolina* is most often referred to as charcoal rot, but this pathogen is also known to cause wilting, stem canker, seedling blight, rot, and damping-off (Kaur, Dhillon, Brar, Vallad, *et al*., 2012; Singh *et al*., 1990). These contribute to yield loss, reduction in seed quality and viability of infected plants (Mughogho & Pande, 1983). Moreover, the severity of symptoms are correlated with high temperatures and low water potential conditions, suggesting that plants growing in tropical, arid regions are at higher risk of succumbing to disease caused by this pathogen (Chamorro *et al*., 2015; Lodha & Mawar, 2020; Mayek-Pérez *et al*., 2002). Pandey and Basandrai reviewed the current literature on factors related to climate change that could potentially worsen *M. phaseolina* infestation (Pandey & Basandrai, 2021). Both the visible traits and genetic architecture of this pathogen, which are further described below, show that *M. phaseolina* as a species is well adapted to diverse climates and equipped to withstand environmental change.

Many important crops including grains and legumes are included among the wide host range of this pathogen (Pandey & Basandrai, 2021). Known hosts of *M. phaseolina* such as alfalfa, maize, canola, soybean, and sunflower have vast economic implications, while others like cassava, chickpea, and mungbean are critical for food security (Ammon *et al*., 1974; Bashir & Malik, 1988; Gaetán *et al*.,

2006; Kaiser & Das, 1988; Lakhran *et al*., 2018; Msikita *et al*., 1998; Pratt *et al*., 1998; Weems *et al*., 2011). The combined estimated loss of soybean yields in 10 countries due to charcoal rot exceeded 2 million metric tons during 1998, ranking third among the most common soybean diseases (Wrather *et al*., 2001). Charcoal rot also ranked second for economic losses per hectare in the U.S. across two decades and combined with soybean cyst nematode accounted for 33% of the total economic losses (Bandara *et al*., 2020).

M. phaseolina also poses a great threat to specialty crops, which include fruits, vegetables, nuts, and horticultural crops such as strawberry, pistachio, and sunflower (Bokor, 2007; de los Santos *et al*., 2019; Mertely *et al*., 2005; Nouri *et al*., 2020). Research on specialty crops is often underfunded and disproportionately lacking in comparison to the steady rise in value of production, despite the inherently high risk involved in marketing fresh produce (Alston & Pardey, 2008; Neill & Morgan, 2021). For example, strawberries ranked fourth among the top California crops produced in 2017, with a value of $3.10 billion (California Department of Food & Agriculture; Koike *et al*., 2016). Strawberries are highly susceptible to *M. phaseolina*; fumigation with methyl bromide and chloropicrin was a common method of pathogen control. In 2005, the agricultural use of methyl bromide was phased out. Since then, the relationship between yield and area of strawberry production reversed, as yields stagnated and new incidences of charcoal rot increased (Holmes *et al*., 2020).

Microsclerotia

M. phaseolina is most often characterized by their production of microsclerotia, or small sclerotial bodies 200 - 600 µm in diameter, which appear as dark specs in infected plant tissue (Jackson & Jaronski, 2009). Microsclerotia are dark, spherical clusters of hyphal cells, each with the ability to germinate and propagate when appropriate environmental conditions are met. They can remain dormant in dead host tissue or soil for an extended period of time, becoming the primary inoculum for the next growing season (Short *et al*., 1980). On potato dextrose agar (PDA), sclerotium formation can be identified as early as 24-30 hours post-inoculation (Wyllie & Brown, 1970). Pycnidia, which are asexual reproductive structures that form spores, have also been documented for this fungus, but are rarely observed *in vitro* (Ma *et al*., 2010). Therefore, in this investigation microsclerotia concentration is used as a proxy measurement for *M. phaseolina* inoculum concentration.

Disease cycle

Microsclerotia germinate at a temperature range of 28 - 35°C, as the emerging mycelium extends radially until it identifies host tissue (Ammon *et al*., 1974; Mihail & Taylor, 1995). Appressoria form when hyphal tips recognize host root cells, often penetrating the plant tissue between epidermal cells before colonization is established in the middle lamella and intercellular space (Ammon *et al*., 1974). Early stages of infection remain mostly intercellular, but during host

cell penetration the hyphae can invaginate the host plasma membrane creating an interface similar to that of haustoria in biotrophic pathogen infections. Moreover, formation of intracellular vesicles, which is another characteristic of early hemibiotrophic infection, has been observed in the cortical tissue of infected soybean (Chowdhury *et al*., 2017). Production of pectolytic and cellulolytic enzymes by the pathogen have been observed both *in vivo* and *in vitro*, but it is not certain whether secretion of such cell wall degrading enzymes is concurrent with the biotroph-like behavior during early stages of infection (Chan & Sackston, 1969, 1970; Radha, 1953). Development of thin, filamentous hyphae is thought to be an indicator of a biotrophy to necrotrophy switch (BNS), as subsequently tissue begins to necrose (Chowdhury *et al*., 2017). *M. phaseolina* mycelia eventually colonize the host's vascular tissue, and formation of microsclerotia in the xylem vessels leads to blockage and consequently wilting of the host (Abawi, 1990; Mayek-Pérez *et al*., 2002). A deeper understanding of the initial stages of *M. phaseolina* infection may provide us with clues as to which modes of plant defense are effective against this pathogen.

STRATEGIES FOR CONTROL

It has become apparent that *M. phaseolina* is adapted to survive in diverse environmental conditions, including sodium salt concentrations of 1 to 8%, and pH ranges of 3.5 to 10 (Islam *et al*., 2012). There is no universal, economically and environmentally reasonable protocol for eradicating this pathogen from an infected

field. What is known is that there is a clear relationship between inoculum concentration, disease occurrence and yield loss. Current approaches to controlling this disease can be grouped into four categories: agronomic practices, chemical control, host genetic resistance, and biological control. Countless strategies to suppress charcoal rot have been tested, and the results have been thoroughly reviewed by authors such as Lodha *et al.* (2020) and Marquez *et al.* (2021).

Agronomic practices

No-till strategy and disk tillage increased *M. phaseolina* microsclerotia density in soil layers at or near the surface (0 - 7.5 cm), but no significant correlations between tillage and disease severity were observed according to a report by Wrather *et al* (1998). Subsequent field experiments led to conflicting results as to whether a no-till strategy increases or decreases inoculum density (Almeida *et al*., 2008; Mengistu *et al*., 2009; Perez-Brandán *et al*., 2012). A 20-year study by Almeida *et al.* (2003) tested the effects of a no-tillage crop rotation system on *M. phaseolina* infested fields and revealed that tillage reduced pathogen density and disease incidence. The authors suggested that a no-till system conserves soil moisture thereby reducing fungal propagation and disease progression. This study also indicated that crop rotation preserved genotypic diversity of the pathogen (Almeida *et al*., 2008). Crop rotation also seems to increase microsclerotia density as reported by Singh *et al* (1990).

Solarization is a technique used to reduce microbe populations by heating soil surfaces. According to Sheikh and Ghaffar (1987), eradication of viable sclerotium can be achieved in wet soil at a temperature range of 50 - 60°C, but the lethality of the treatment decreases in dry soil. In Pakistan, mulching of wet, artificially infested soil increased temperatures up to 52°C, reducing viable sclerotial density in soil layers close to the surface (Sheikh & Ghaffar, 1984). Furthermore, the combination of irrigation, soil amendment with cruciferous residues, and solarization has been reported to eradicate (93 - 99%) *M. phaseolina* microscletotia in soil depths of up to 30 cm (Lodha *et al*., 1997). If soil temperature and moisture reach sufficient levels, solarization can effectively reduce inoculum concentration in the top soil layers.

Chemical control

To date, no fungicide has been registered as an effective control agent against charcoal rot. An *in vitro* assay testing mycelial susceptibility to common fungicides showed that *M. phaseolina* is highly sensitive to carbendazim and penflufen + trifloxystrobin, moderately sensitive to pyraclostrobin and iprodione, and insensitive to fluquinconazole, metalaxyl, tolyfluanid and thiram (Tonin *et al*., 2013). However, no treatment could suppress 100% of mycelial growth. Another study conducted *in vitro* and *in vivo* assays with green gram (*Vigna radiata*) and black gram (*Vigna mungo*), and found that benomyl, carbendazim and propineb contributed to plant survival (76.67%, 66.67%, and 63.33%, respectively) at 150 ppm (Iqbal & Mukhtar, 2020).

In the case of strawberry, reports of plant collapse due to charcoal rot in the U.S. began in 2005, and by 2014 this disease was confirmed in most strawberry-growing counties (Koike *et al*., 2016). This rise of strawberry field infestation is correlated with high temperatures and drought, as well as the ban on methyl bromide fumigation (Muchero *et al*., 2011). Although highly effective in controlling *M. phaseolina*, methyl bromide has been phased out throughout the world per the Montreal Protocol guidelines on Substances that Deplete the Ozone Layer, released in 1991 (Chamorro *et al*., 2016; Guthman & Others, 2017). Fumigation of strawberry fields with other chemicals continues in California, and in fact chloropicrin use has increased significantly in correlation to the drop in methyl bromide use, as it is less effective alone (Guthman & Others, 2017). Although alternatives to methyl bromide including chloropicrin have shown to reduce *M. phaseolina* density and plant mortality in the field, increased production costs have not been matched with consumer costs, and overall chemical use has increased (Chamorro *et al*., 2016; Guthman & Others, 2017).

Filho and Dhingra (1980) tested the effects of five herbicides, S-Ethyl dipropylthiocarbamate (EPTC), dinoseb, alachlor, fluorodifen and fluometuron, on *M. phaseolina* infested fields, and found that populations declined post-treatment with each chemical. It is important to note that dinoseb, alachlor, and fluorodifen are either banned or considered obsolete in the European Union and in the U.S. due to concerns of adverse health effects. The authors also point out that the herbicides may be reducing the pathogen population by two possible mechanisms:

a direct toxicity to *M. phaseolina*, or an indirect influence through changes in the soil microbial community. This raises interesting questions as to how the soil microbial community impacts *M. phaseolina* populations and whether it is sufficient to control disease.

Biological control

Dhingra *et al.* (1976) found that increasing organic matter in soil reduces *M. phaseolina* colonization of soybean and maize stems likely due to the increased presence of other microbes. The implication that *M. phaseolina* has low competitive saprophytic ability has been tested and confirmed with multiple fungal and bacterial biocontrol agents (BCAs). For example, several species of *Bacillus* have been reported to antagonize *M. phaseolina* growth both *in vitro* and *in vivo* and suppress disease symptoms in soybean, common bean and cowpea (Bojórquez-Armenta *et al*., 2021; Rangel-Montoya *et al*., 2022; Yasmin *et al*., 2020). Other rhizospheric bacteria such as fluorescent *Pseudomonas spp.* have also been identified as *M. phaseolina* BCAs (Das *et al*., 2008; Gupta *et al*., 2002). Many of these rhizobacterial species also have plant growth-promoting properties.

The genus *Trichoderma* is a well known fungal BCA (Lorito *et al*., 2010). Indeed, examples like *T. viride* and *T. harzianum* successfully inhibit growth of *M. phaseolina* and further have positive effects on plant growth (Khaledi & Taheri, 2016; Khalili *et al*., 2016; Sankar & Sharma, 2001). Interestingly, it has been shown that the combined application of *T. harzianum* and arbuscular mycorrhizal fungi (AMF) allows for AMF colonization in non-host *Brassica* plants, including

Arabidopsis thaliana, and increases silique numbers (Poveda *et al*., 2019). Additionally, AMF co-treatment with *M. phaseolina* showed a much lower number of upregulated genes in comparison to application of the pathogen alone. Pre-mycorrhized plants also exhibited greater expression of serine carboxipeptidase-like (SCPL) and lectin genes, which have been suggested to prime the plant for pathogen attack (Marquez *et al*., 2018, 2021).

Despite the growing interest in reducing agrochemical inputs, the biopesticide market constitutes only a small fraction (6.4% in 2018) of the total pesticide market (Pal & McSpadden Gardener, 2006; Regnault-Roger, 2020). Obstacles that remain to be addressed, such as storage, lack of commercialized products, narrow target application, and limited user awareness have hindered widespread adoption of biological control strategies.

Genetic engineering

Majumder *et al.* explored the potential of transgenic approaches for protecting crops from *M. phaseolina* in a study published in 2018. The *rice chitinase* (*chi11*) gene and *bar* gene, encoding phosphinothricin acetyltransferase (PAT), were incorporated into jute (*Corchorus capsularis* and *Corchorus olitorius*) genomes, in hopes that the antifungal activity of the chitinase and herbicide tolerance conferred by *bar* will improve yields. Transgenic jute lines showed significantly smaller lesion sizes and improved fiber yield in comparison to the wild type when infected with *M. phaseolina (Majumder et al., 2018)*. Efforts to combat *M. phaseolina* by transgenic crop lines have been scarce. This approach has been

used more so to identify genes and pathways that are important for host resistance (Dabi *et al*., 2020; Lygin *et al*., 2013).

M. PHASEOLINA TOOLKIT FOR PATHOGENICITY

Pathogen variability

Comparisons between isolates across host species and geographical distances have shown that high levels of morphological, physiological, pathogenic, and genetic variation are present in *M. phaseolina.* Despite this*,* the genus *Macrophomina* is monospecific, meaning that it refers to only one species. Classification of *M. phaseolina* isolates based on chlorate sensitivity was previously proposed by Pearson *et al*. (1986). Chlorate is an analogue of nitrate and can be toxic to plants and fungi when reduced to chlorite via the nitrate reductase pathway. Since *M. phaseolina* isolates from corn were chlorate resistant and those from soybean were chlorate sensitive, it was suggested that the presence or absence of the nitrate reductase pathway could be a marker for host-specific strains (Pearson *et al*., 1986, 1987). However, subsequent reports using this method have had mixed results. Mihail and Taylor (1995) were unable to confirm relationships between the chlorate phenotype and origin host species when comparing 114 isolates. Su *et al.* (2001) found that both host species and location (root or soil) of the isolate had effects on chlorate sensitivity, albeit at varying levels.

Furthermore, the authors reported here that random amplified polymorphic DNA (RAPD) analysis can be used to genetically differentiate isolates by host species (Su *et al*., 2001). Jana *et al.* (2005) also successfully used universal rice primers (URP), or markers derived from repeating sequences in the rice genome, to fingerprint and differentiate isolates from soybean, cotton, and chickpea, and simple sequence repeats (SSR) as markers to distinguish isolates from soybean and cotton. Baird *et al.* (2010) also surveyed 109 isolates across the United States using 12 SSRs and found that resulting clusters generally grouped isolates based on origin location and host, but exceptions were also not uncommon. No evidence for sexual reproduction has been confirmed in *M. phaseolina (Marquez et al., 2021)*. In a study that examined isolates across host plant families and geographical distances, hyphal fusions were observed frequently enough (64.3%) for the authors to conclude that there are no genetic barriers to nonsexual genetic exchange amongst *M. phaseolina* isolates (Mihail & Taylor, 1995). Potential for gene flow across populations, formation of microsclerotia, and movement of agricultural products including soil likely contribute to the variability seen in this species. As 3.98% of the annotated *M. phaseolina* genome encodes transposable elements, genetic transposition is another potential avenue for the introduction of novel mutations, gene duplications, and horizontal gene transfers (Islam *et al*., 2012). Understanding *M. phaseolina* variance is important for developing resistant crop genotypes, and highlights the importance of experimental design in studying this pathogen.

Host specialization

There are many conflicting reports regarding whether this heterogenous pathogen is adapted to infecting particular host species. To what degree the variability described above contributes to host specialization or preference is still unclear. For some examples of generalist necrotrophic fungi such as *Botrytis cinerea,* there is evidence to suggest that selective pressure maintains moderate virulence against a broad spectrum of host species (Caseys *et al*., 2019). In a study by Koike *et al.* (2016), *M. phaseolina* isolates from melon, thyme, and apple were tested against strawberry and failed to cause disease. Moreover, five cover crop species tested against strawberry-derived isolates also did not develop symptoms. However, results from Su *et al.* (2001) indicate that no specialization occurs for isolates from sorghum, cotton, or soybean, and while corn isolates seemed to colonize corn roots better than other isolates, it also was the most virulent against soybean. Similar cross-host species pathogenicity experiments have been carried out testing hosts against isolates collected across species and geographic distances, but no isolate had restricted virulence to a single host (Mayék-Pérez *et al*., 2001; Reyes-Franco *et al*., 2006). Although there is some evidence to suggest that host preference exists for certain isolates, it is not certain whether this is the result of evolutionary specialization or an artifact of environmental factors, which could alter the virulence of the pathogen against specific hosts**.** Recently, a large-scale analysis of three *M. phaseolina* isolates from three different hosts paired with 12 RNA-seq datasets from one of the isolates revealed large structural

rearrangements and SNPs, an indicator of genomic flexibility, although no pattern was necessarily associated with a particular host (Burkhardt *et al*., 2019). However, phylogenetic analysis indicated that isolates from jute and alfalfa were more closely related in comparison to the isolate from strawberry, and ten genes were identified to be exclusive to strawberry pathogenic isolates (Burkhardt *et al*., 2019). Whether these genes are the causal factor for the host-preference of these isolates has not been reported.

Advances in *M. phaseolina* -omics tools

In 2012, Islam *et al.* reported on the first draft *M. phaseolina* genome, isolated from infected jute. Cross-species homology analysis revealed its close synteny with another soil borne generalist fungi, *Fusarium oxysporum*, which shares 54.10% of the predicted genes in *M. phaseolina*. Additionally, it was found that 2.84% and 3.98% of the *M. phaseolina* genome is repetitive DNA and transposable elements, respectively (Islam *et al*., 2012). Transposons are potentially an important source of genetic variation for this predominantly asexually reproducing fungus. The authors identified 537 predicted genes in the *M. phaseolina* genome that are included in the pathogen-host interaction database (PHI-base), which is a collection of experimentally verified effectors from bacterial, fungal, and oomycete pathogens. Today, *M. phaseolina* isolates from a variety of hosts including jute, strawberry, alfalfa, sorghum, and castor have been sequenced (Burkhardt *et al*., 2019; Hossen *et al*., 2019; Islam *et al*., 2012; Marquez *et al*., 2021; Purushotham *et al*., 2020; Verma *et al*., 2018).

Sequencing and assembly of the *M. phaseolina* genome has led to comparative studies across isolates and geographic locations, and the development of new tools to elucidate biological processes involved in pathogenicity. Transcriptomic analysis was used to identify ROS, specifically superoxide (O_2-), as the key signaling factor during initiation of microsclerotia formation from hyphae (Liu *et al*., 2022). In fungi, redox signaling is an essential component of developmental processes, crosstalk with plant hosts, and lignocellulose degradation (Breitenbach *et al*., 2015). More recently, candidate reference genes for evaluating gene expression were identified through experimentation in three different growth conditions (Orrego *et al*., 2022). Reliable reference genes are crucial for accurate estimation of gene expression.

The first documented proteomic analysis on *M. phaseolina* was published by Zaman *et al*. (2020), which compared the expression of proteins with and without challenging the fungus with the jute endophytic bacterium *Burkholderia contaminans* NZ. A large number of hydrolyzing enzymes found in the *M. phaseolina* genome were confirmed through this study. Co-culture with a bacterial antagonist showed signs of decreased virulence, morphological changes and altered metabolic processes to switch to a more dormant state that prioritizes survival over growth. Characterization of the *M. phaseolina* mycelial proteome by Arafat *et al.* (2022) revealed a network of proteins which could be classified by their function including growth and reproduction, stress tolerance and virulence, nutrient synthesis, transcription and translation regulation, and more. These

proteomic studies provide the groundwork for potential functional studies of specific genes or gene groups involved in virulence.

Alteration of host cell wall and membrane permeability

A notable characteristic of the *M. phaseolina* genome is the significant presence of genes involved in cell wall degradation. Carbohydrate active enzymes (CAZymes) including glycoside hydrolases (GH), glycosyltransferases (GT), carbohydrate esterases (CE), carbohydrate binding modules (CBM), and polysaccharide lyases (PL), were predicted within the genome. *M. phaseolina* possess a higher than average number of GHs (four times more than GTs), and CEs (Islam *et al*., 2012), which is consistent with previous observations that this pathogen has greater cellulolytic activity than most other fungi (Kaur *et al.*, 2012). A β-1,4-endoglucanase produced by this pathogen was previously characterized and shown to be similar to plant-derived endoglucanase, which potentially allows for surreptitious hydrolysis of the host cell wall (Wang & Jones, 1995). A repertoire of genes associated with lignin depolymerization, including laccases, lignin peroxidases, galactose oxidases, chloroperoxidase, haloperoxidases, and heme peroxidases were also identified. The *M. phaseolina* genome encodes the highest number of laccases in comparison to seven other fungal species (Islam *et al*., 2012). Production of these cell wall degrading enzymes (CWDEs) has also been observed in *M. phaseolina* grown *in vitro (Ahmad et al., 2006; Ramos et al., 2016)*. The activity of phosphatidases, which hydrolyze phosphatide lipids and alter the permeability of cellular membranes, was also observed during *Brassica juncea*

infection with *M. phaseolina* isolates of various virulence. A strong correlation between host susceptibility, pathogen virulence, and phosphatidase activity was observed, which peaked at 18 days post-inoculation (Srivastava & Dhawan, 1982). The extensive collection of genes associated with cell wall and membrane disruption suggests that this pathogen is well equipped to breach diverse wall polysaccharide and lipid bilayer compositions, which differ between plant lineages (Kubicek *et al*., 2014). This perhaps explains, at least in part, *M. phaseolina's* wide host range.

Defense signal transduction

Eight homologs of the *Phytophthora parasitica* cellulose-binding elicitor lectin (CBEL) gene, which has a role in the perception of host cell wall components and is a known elicitor of host defense, were also found in the *M. phaseolina* genome (Gaulin *et al*., 2002; Islam *et al*., 2012). Pep-13 is another known elicitor motif in the cell wall transglutaminase gene from *Phytophthora sojae*, that triggers defense activation in host plants (Brunner *et al*., 2002). Three homologs containing Pep-13 are found in *M. phaseolina (Islam et al., 2012)*.

The *M. phaseolina* genome is also abundant in protein kinases and PTH11-like G-protein coupled receptors (GPCR), both of which mediate pathogen response to environmental signals and activate downstream metabolic pathways (Islam *et al*., 2012). Once a pathogen enters the host plant, it must acquire nutrients while evading and countering host defense responses. An unusually high number of amino acid transporters, which are likely to be useful for accessing host

protein degradation products, were also predicted in the *M. phaseolina* genome. There was also an enrichment of proteins involved in amino acid metabolism in the mycelial proteome (Arafat *et al*., 2022).

Production of free radicals and detoxification

This pathogen has a large set of detoxification genes, including dehydrogenases, acyl-coA N-acetyltransferases, monooxygenases, and cytochrome P450s (Islam *et al*., 2012). It has been reported that *M. phaseolina* infection causes nitric oxide (NO) accumulation in jute (*Corchorus capsularis*), but the fungus itself is also capable of NO production *in vitro* (Sarkar *et al*., 2014). NO is a key signaling factor in the early stages of the host hypersensitive response (HR). In plants, pathogen induced NO production is accompanied by a burst of reactive oxygen species (ROS) such as superoxide (O_2-) and hydrogen peroxide (H_2O_2) (Asai & Yoshioka, 2009; Van Baarlen *et al*., 2004). Balanced accumulation of both NO and H_2O_2 is required to trigger a hypersensitive defense response (HR) leading to cell death (Delledonne *et al*., 1998, 2001), although there are somewhat conflicting reports as to whether NO and ROS act synergistically (Delledonne *et al*., 2001) or antagonistically (Asai *et al*., 2010; Asai & Yoshioka, 2009) to induce a hypersensitive response (HR) during plant-pathogen interactions.

There is an interesting connection between ROS and fungi that form sclerotia-like survival structures, including *M. phaseolina*. As mentioned above, recent work by Liu *et al.* (2022) showed that genes involved in ROS-related functions were differentially expressed during microsclerotia formation, suggesting

that ROS, specifically O_2-, stimulates microsclerotia formation in *M. phaseolina.* Moreover, studies across multiple filamentous fungi have shown that ROS have functions in cell differentiation and development in fungi (Breitenbach *et al*., 2015). *In vitro* treatment of cultures with ROS further supported this hypothesis. In plants, pathogen attack and/or effectors trigger a rapid production of ROs (Doke, 1983), which acts as a diffusible signal for facilitating controlled cell death (Levine *et al*., 1994). Downstream outcomes of this oxidative burst and HR will be discussed further in later sections. It remains unanswered whether this defense-linked oxidative burst in the host contributes to *M. phaseolina* microsclerotia formation, and to what extent this affects host susceptibility.

Secondary metabolites

Fungi produce diverse bioactive natural products, some of which are used in human medicine, such as mycotoxins, and virulence factors (Chooi & Tang, 2012). A total of 75 putative secondary metabolite-related genes were identified in the *M. phaseolina* genome, which is more than double of what can be found in *Magnaporthe grisea*, *B. cinerea*, *Sclerotinia sclerotiorum*, and *Fusarium graminearum*. These genes include polyketide synthases (PKS), non-ribosomal peptide synthases (NPRS) and PKS-NPRS hybrids (Islam *et al*., 2012). Additional expression analyses and functional studies are required to confirm the role of these putatively identified genes. Examples of secondary metabolites produced by *M. phaseolina* include phaseolinone (Dhar *et al*., 1982; Siddiqui *et al*., 1979), phaseolinic acic (Mahato *et al*., 1987), (-)-botryodiplodin (Abbas *et al*., 2019;

Ramezani *et al*., 2007), succinic acid, tyrosol, (R)-mellein, *cis*-(3R,4R)-4-hydroxymellein, azelaic acic (Salvatore *et al*., 2020), koijic acid, moniliformin, orsellinic acic (Khambhati *et al*., 2020), macrollins A-c (Gao *et al*., 2021), phaseocyclopentenones A and B, and guignardone a (Masi *et al*., 2021). However production of such metabolites seems to be dependent on a number of factors including isolate variability and the presence of host substrates. To what extent virulence is affected by these metabolites is largely unknown. Phaseolinone has been documented to prevent seed germination, wilting, and leaf necrosis (Bhattacharya *et al*., 1992, 1994; Dhar *et al*., 1982). The compound (-)-botryodiplodin is a well-documented toxic metabolite prevalent in ascomycetes. When soybean plants were infected with *M. phaseolina*, it was isolated exclusively from root tissues displaying charcoal rot symptoms (Abbas *et al*., 2019; Ramezani *et al*., 2007), suggesting that it is produced during pathogenesis. In these assays however, phaseolinone could not be detected in any tissue examined. Shier *et al.* (2007) gives several possible reasons for this discrepancy, one being that Dhar *et al.* mistakenly characterized (-)-botryodiplodin as phaseolinone. Another possible explanation is that different isolates of *M. phaseolina* produce different “cocktails” of toxins. This is supported by the fact that mellein was identified in some, but not all, isolates of this pathogen recovered from symptomatic soybean plants collected in Mississippi, U.S.A. (Khambhati *et al*., 2020).

HOST DEFENSE

Plant basal defense

Plants have innate defense mechanisms that are widely conserved across species, which allows for protection against non-specific pathogens. Multiple efforts have been made to uncover the genetic basis of *M. phaseolina* resistance in crop species. Despite these recent advancements, molecular mechanisms that determine host resistance are still poorly understood.

One of the first lines of defense that plants possess is a physical barrier that simultaneously functions as a sensory organ - the cell wall. Together with the cuticle, which envelops the aerial epidermal cells, these physical barriers efficiently repel many pathogens, conferring non-host resistance. Polysaccharides of diverse compositions form a network that supports the structural integrity of the cell wall, alongside proteins, which have roles in remodeling and turnover of cell wall components. For pathogens that can breach and decompose this barrier, the cell wall may be a valuable nutrient source. However disruptions in the integrity of the cell wall, as well as enzymes that cause its degradation, including those that originate from the host itself, are known to trigger signaling cascades similar to that of biotic stress responses and contribute to disease resistance. Evidence for the role of cell wall maintenance in host defense are thoroughly reviewed by (Hamann, 2012). Molecular signals released as a result of cell wall damage can be perceived as damage-associated molecular patterns (DAMPs) by pattern recognition

receptors (PRRs) residing on the plasma membrane leading to local and systemic defense signaling (Hou *et al*., 2019).

PRRs can also detect conserved microbe- or pathogen-associated molecular patterns (MAMPs or PAMPs) directly derived from invading pathogens and activate a robust basal defense response called pattern triggered immunity (PTI) (Bigeard *et al*., 2015). For example, the *A. thaliana* PRR and receptor like kinase (RLK) FLS2 responds to flagellin, a bacterial elicitor, and confers resistance to these pathogens (Gómez-Gómez & Boller, 2000). Responses to PAMP recognition include ionic influxes in the cytosol, increased ROS and NO production, activation of mitogen-activated protein kinase cascades, and transcriptional reprogramming that entails induction of pathogenesis related (*PR*) genes (Nürnberger *et al*., 2004). In turn, some pathogens have adapted to counter host defenses through proteins and secondary metabolites collectively called effectors that can interfere with PTI. Effectors can serve as toxic agents themselves or as a means to evade and suppress host defense mechanisms (Presti *et al*., 2015). Although there are several characteristics that can be used to define an effector, such as size, species exclusivity, and expression profile, exceptions are also common. In terms of total secreted proteins, hemibiotrophs on average have the greatest number of effectors than any other fungal lifestyle group (Presti *et al*., 2015).

The perpetual coevolution of plant hosts and microbial pathogens, collectively described as the ‘zigzag model’, has contributed greatly to the

repertoire of defense mechanisms encoded by the genomes of both sides involved (Chisholm *et al*., 2006; Jones & Dangl, 2006). Pathogen effectors can directly or indirectly be identified by specific receptors encoded by host resistance (*R*) genes which then trigger an acute immune response that often results in HR, or cell death. Many fungal avirulence (*Avr*) genes encoding Avr proteins have gene-for-gene relationships with plant host *R* genes and typically act as effectors. This tertiary layer of defense is called effector-triggered immunity (ETI) (Macho & Zipfel, 2014; Presti *et al*., 2015). The *A. thaliana* ecotype Col-0 possesses approximately 165 NB-LRR genes, which is the largest class of *R* genes characterized by their nucleotide binding (NB) and leucine rich repeat (LRR) domains (Dangl & Jones, 2001; Wei *et al*., 2020). PTI and ETI make up the plant's inducible defense core.

Phytohormones

Three phytohormones are crucial in the orchestration of defense signaling - salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) (Bari & Jones, 2009; Glazebrook, 2005). SA accumulates in cells and triggers HR as a response to infection, and mediates defense against biotrophic and hemibiotrophic pathogens. It is also known to induce systemic acquired resistance (SAR), in which distal tissues acquire heightened resistance to secondary infection due to elevated expression of pathogenesis related (*PR*) genes (Durrant & Dong, 2004; Ross, 1961). JA and ET are associated with defense against necrotrophic pathogens (Bari & Jones, 2009). Similar to, but independent from SA, the phytohormones JA and ET are also known to trigger a systemic defense response, which is called

induced systemic resistance (ISR) (Pieterse *et al*., 1996). Although signaling pathways controlled by these plant hormones are tuned to defend against pathogens of distinct lifestyles, they are known to affect each other both synergistically and antagonistically (Clarke *et al*., 2000; Koornneef & Pieterse, 2008; Mur *et al*., 2006; Thomma *et al*., 2001). Cross-talk between phytohormone-signaling pathways allows the host plant to balance the energy cost of pathogen defense to that of its own growth and propagation (C. M. J. Pieterse *et al*., 2012).

Transcriptomic analysis in *A. thaliana* roots revealed that *M. phaseolina* induces JA-, ET-, and SA-responsive genes, which is consistent with the observation that this pathogen leads a hemibiotrophic lifestyle. Mutants compromised in JA and ET signaling exhibited susceptibility to *M. phaseolina* (Schroeder *et al*., 2019). *M. phaseolina* induced upregulation of JA- and ET-responsive genes have been reported in several species of hosts susceptible to this pathogen, both in the shoot and the root (Gaige *et al*., 2010). In *Sesamum indicum*, widely known as sesame, SA responsive differential gene expression in the root was in correlation with the early biotrophic phase of *M. phaseolina* infection both in resistant and susceptible varieties, although overall transcript levels were greater in the resistant variety (Chowdhury *et al*., 2017). Accumulation of JA- and ET- signaling gene transcripts were seen during the BNS and through the necrotrophic phase. There was also an interesting correlation between the expression pattern of JA-regulated gene *SiDef* in resistant and susceptible sesame, at 24 and 12 hours post-infection (hpi) respectively, with the timing of

BNS in the resistant (36-38 hpi) and susceptible (24 hpi) varieties (Chowdhury *et al*., 2017). Moreover, the authors conducted a priming assay with each of these phytohormones and found that pre-treatment of sesame with JA and ET significantly reduced *M. phaseolina*-associated disease symptoms, while JA and ET inhibitors significantly increased susceptibility to this pathogen. Taken together, the evidence suggests that the timing and amplitude of SA, JA and ET signaling are critical in determining the susceptibility of the host. While all three of these defense-associated phytohormones seem to cooperate in protecting host roots against *M. phaseolina*, it is important to consider that results based on gene expression studies in whole organs reflect responses that are averaged across different cell types. Cell-type specific regulation of phytohormone mediated defense has not been explored in *M. phaseolina* pathosystems.

ROS

As introduced earlier, plants produce ROS, primarily superoxide (O_2-) and hydrogen peroxide (H_2O_2), in response to pathogen invasion. In plants, H_2O_2 is a diffusible signal that activates defense-related genes, ultimately triggering hypersensitive cell death, or HR, in tissue neighboring the site of the oxidative burst (Levine *et al*., 1994). The level of ROS in tissue is enzymatically controlled by superoxide dismutases (SOD), which catalyze the conversion of O_2- to H_2O_2 and oxygen (Y. Wang *et al*., 2018). Sesame is a common oilseed crop species that frequently suffers *M. phaseolina* infection. When interactions of this host with *M. phaseolina* were studied, the susceptible cultivar VRI-1 showed consistently higher

accumulation of H_2O_2 in comparison to the resistant Nirmala variety, following the BNS (Chowdhury *et al*., 2017). Consistently, less lipid peroxidation and higher accumulation of *SiSOD* was observed during the necrotrophic phase in the resistant variety (Chowdhury *et al*., 2017).

Asai and Yoshioka (2009) found that necrotic lesions caused by *Botrytis cinerea* on *Nicotiana benthamiana* were reduced when the oxidative burst was suppressed, while disease lesions expanded when NO production was inhibited. Nevertheless, HR induction in the host plant is favorable for necrotrophic pathogens such as *M. phaseolina*, which feed on dead tissue, especially when the induction is uncontrolled, as is expected if the pathogen produces additional NO during infection (Sarkar *et al*., 2014). It has also been shown that changes in NO levels caused by *M. phaseolina* infection in sesame were dependent on the susceptibility of the cultivar, with resistant varieties exhibiting a rapid but moderate increase in NO, while susceptible varieties showed a delayed and robust decrease in NO (Acharya & Acharya, 2007). Similarly, in *A.thaliana-Sclerotinia sclerotiorum* interactions, rapid accumulation of NO was observed in resistant ecotypes, although accumulation of this ROS was also observed in susceptible ecotypes at later time points (Perchepied *et al*., 2010). In contrast, Chowdhury *et al.* reported that H_2O_2 accumulation caused by *M. phaseolina* was consistently higher in susceptible sesame genotypes between 24 and 72 hpi (Chowdhury *et al*., 2017). At later time points when the pathogen has presumably entered its necrotrophic phase, resistant genotypes showed signs of ROS detoxification. Current literature

on the crosstalk between NO and other ROS is summarized by Lindermayer and Durner (2015). The timing and amplitude of NO and other ROS production may be a factor contributing to host susceptibility against necrotrophic pathogens.

Secondary metabolites

Like *M. phaseolina*, plants produce a diverse array of secondary metabolites with roles in defense against pathogens. Secondary metabolites found in *A. thaliana* include phenylpropanoids, glucosinolates, terpenoids, and camalexin (Kliebenstein, 2004). Secondary metabolites synthesized in response to biotic stress are collectively called phytoalexins (Ahuja *et al*., 2012), while constitutively present compounds are called phytoanticipins (Tiku, 2020).

One of the major metabolic pathways often discussed in relation to plant defense is the phenylpropanoid pathway, which is required for the synthesis of diverse phenolic metabolites including lignols, flavonoids, isoflavonoids, and tannins (Fraser & Chapple, 2011; Rehman *et al*., 2012). Soybean (*Glycine max*) produces a repertoire of phytoalexins, including isoflavonoid derivative glyceollins, during both symbiotic and pathogenic microbial entry. Work by Lygin *et al.* (2013) showed that downregulation of isoflavonoid biosynthesis, and consequently glyceollins, significantly increases charcoal rot disease severity. In *Medicago truncatula*, genes involved in the phenylpropanoid pathway, as well as flavonoid and isoflavonoid biosynthesis were upregulated dramatically in the shoot but not in the root in response to *M. phaseolina* infection at 24 and 48 hpi (Gaige *et al*., 2010). As the authors point out, this could be the result of pathogen suppression

or tissue-specific gene regulation. Nonetheless this shows that *M. phaseolina*, despite being a soil-borne pathogen, strongly induces systemic upregulation of secondary metabolite synthesis genes. Accumulation of phenolic compounds synchronized with the induced activity of phenylalanine ammonia lyase (PAL), a key player phenylpropanoid pathway, was also seen in the root tissue of plate-grown sesame after infection (Chowdhury *et al*., 2017). Notably, this induction was stronger in the resistant Nirmala variety and it only occurred after the BNS. It is unknown according to this paper whether similar induction occurred in the shoot tissue. Radadiya *et al.* (2021) also observed upregulation of key enzymes in the phenylpropanoid pathway in sesame roots post inoculation with *M. phaseolina* in soil through transcriptome analysis. Both Chowdhury *et al.* (2017) and Radadiya *et al.* (2021) report earlier upregulation of these genes in different resistant varieties, and a delayed response in the susceptible varieties, suggesting that there is a correlation between timing of defense response activation and susceptibility to this pathogen.

Glucosinolates are another major class of secondary metabolites known for its abundance in plants of the Brassicaceae family, which includes the model plant *A. thaliana* and economically important crops like oilseed rape (*Brassica napa*). The glucosinolate-myrosinase system is a defense mechanism that releases toxic glucosinolate derivatives upon activation by physical damage to plant tissue (Wittstock & Halkier, 2002). It is commonly perceived as a primary defense against herbivores, but it is also known that some isothiocyanates, which are products of

the glucosinolate myrosinase reaction, have fungitoxic properties (Drobnica *et al*., 1967; Smolinska *et al*., 2003; Tierens *et al*., 2001). Cruciferous soil amendments have been used to successfully suppress generalist hemibiotrophic pathogens like *F. oxysporum* growth and protect crops from disease (Rosa & Rodrigues, 1999), however, it is still unclear whether this effect is the product of chemically active inputs or that of overall changes in the soil microbiome. Brassica seed meal amendment trials with strawberry have had limited success in protecting plants from *M. phaseolina* (Agostini, 2011; Mazzola *et al*., 2017). It remains to be seen whether glucosinolate activity in known cruciferae hosts like *A. thaliana*, *B. napa*, and *B. juncea* play a role in resistance against *M. phaseolina*.

Root-specific defense

M. phaseolina is a soil-borne pathogen, which means that the most accessible point of entry for hyphae will be in the root of host plants. While the currently accepted model describes phytohormone-mediated pathogen response as an antagonistic relationship between SA and JA with each pathway corresponding to a particular pathogen life cycle (Glazebrook, 2005), several studies specifically focusing on root responses paint a more complex picture. Inoculation of *Phytophthora parasitica* on *A. thaliana* caused a simultaneous upregulation of both SA and JA pathways in the root (Attard *et al*., 2010). *Fusarium oxysporum* infection repressed many known defense-associated genes in roots, and only three genes overlapped with previously reported microarrays with inoculated leaves (Chen *et al*., 2014).

It is therefore necessary to mention that generalizations on plant immune responses should be made with caution if data is derived from whole plants. For example, when JA is applied to *Brassica oleracea* to mimic wounding, the expression and regulation of known defense mechanisms like secondary metabolite synthesis and phytohormone responses varied between root and shoot tissue, and moreover dependent on the site of treatment (Tytgat *et al*., 2013). Root-specific pathogen-induced responses have been reviewed thoroughly by Chuberre *et al*. (2018), but it has yet to be confirmed whether defense responses to *M. phaseolina* are also tissue specific. In sorghum, *M. phaseolina* induced a greater induction of chitinase genes in the aerial tissue in comparison to roots (Sharma *et al*., 2014).

Research on *M. phaseolina*-host relationships and future perspectives

Despite the long history of growers losing crop yield to disease caused by *M. phaseolina*, sustainable and effective protection strategies against this pathogen are still lacking. Several factors contribute to the obstacles prohibiting the advancement of research in this field, including the high diversity of this pathogen, and susceptibility of field generated data to environmental variability. Recently, a high-throughput pathosystem using the model plant *A. thaliana* has been established, which will likely accelerate research in this field (Schroeder *et al*., 2019). In addressing pathogen variability, it seems unusual that a monotypic genus can represent the level of genetic and morphological diversity seen in *M.*

phaseolina without evidence of sexual reproduction. *F. oxysporum* also has not been documented to undergo sexual reproduction, but is suspected to preserve its diversity through horizontal gene transfer (Gordon, 2017). Research has led to the following hypothetical scenario: virulent genotypes emerged through significant selection pressures such as agricultural activity while recent relatives that are capable of outcrossing, possibly within populations existing today, sustain a "reservoir" gene pool (Gordon, 2017). Although there is no direct evidence that *M. phaseolina* fit this model, this analysis may provide critical insights to the evolution of *M. phaseolina*, considering its striking similarity to *F. oxysporum (Islam et al., 2012).*

There are decades of records documenting *M. phaseolina* pathogenicity on a wide range of crop species, including soybean (Coser *et al*., 2017; da Silva, Klepadlo, *et al*., 2019; Smith & Carvil, 1997), sesame (Chowdhury *et al*., 2017; Radadiya *et al*., 2021; Yan *et al*., 2021), strawberries (Burkhardt *et al*., 2019; Koike, 2008; Nelson *et al*., 2021), and more. The advancement of high-throughput sequencing and genome-wide analysis technologies have allowed for identification of many resistance-associated loci in cultivars of various host species, sampled from infected fields across the globe. Previously, Muchero *et al.* (2011) identified 9 QTL regions tied to *M. phaseolina* resistance in cowpea (*Vigna unguiculata*) through QTL mapping of a recombinant inbred line population. These regions include markers and genes associated with disease resistance homologous to genes in other species including soybean and medicago. Within candidate genes

identified, there was a noticeable presence of genes involved in pectin metabolism. A bimodal distribution of disease incidence was observed in castor (*Ricinus communis*), which suggests that a genetic variant with major effects on the disease resistance trait is present in the population (Tomar *et al*., 2017). The authors discovered three QTLs through linkage mapping with the most significant region predicted to explain 71.2% of the phenotypic variation, which combined with the phenotypic distribution could be an indication of major effect genes.

More recently, genome-wide association has been used to identify resistance loci in species with available reference genomes. Coser *et al.* (2017) conducted a disease screen using 459 soybean lines to identify *M. phaseolina* resistance loci through genome-wide association. In field and greenhouse experiments, a total of 19 SNPs were identified to have a significant association with the resistant phenotype. Orthologous gene regions to *Arabidopsis* were also identified, and these included genes involved in biotic and abiotic stress response, cell wall composition, and ethylene signaling. Surprisingly, there were no overlaps between these SNP regions and QTL regions identified by da Silva *et al.* (2019) using QTL-seq, which combines bulk segregant analysis with next generation sequencing to statistically link loci to a quantitative trait. This could be because the segregating population used for QTL-seq was derived from parental lines (PI567562A and PI567437) that were not included in the set tested by Coser *et al.* Nonetheless, it should be taken into consideration that factors such as treatment condition, sequencing methods, and genotypes of both host and pathogen could

yield variable QTL. Nelson *et al.* (2021) identified three loci linked to *M. phaseolina* resistance from a breeding population obtained from controlled crosses of commercial strawberry. Many gene regions that exhibit strong associations with disease phenotype have not been narrowed down to candidate genes nor have they been assessed for their function, and therefore evidence for a strong causal relationship between specific genes or molecular mechanisms and *M. phaseolina* resistance is still lacking. Cross-species synteny analysis may be a useful tool in identifying conserved trait loci.

Experimentation with *M. phaseolina* has primarily been focused in the field, however recently greenhouse studies have allowed for observation of disease progression in more controlled environments. Young sesame seedlings were inoculated in mycelium mixed soil and kept in artificial climate boxes in a study by Yan *et al*. (2021) and root tissue was collected for RNA-seq. Schroeder *et al.* (2019) introduced the use of agar plates for assessing *M. phaseolina* interaction with small hosts like *A. thaliana* seedlings. While many studies have used pre-infected plots to study the relationship between native *M. phaseolina* and host crops (Miklas *et al*., 1998; Tomar *et al*., 2017), various methods of pathogen inoculation have been employed, including mixing mycelium in soil (Bedawy & Moharm, n.d.; Mahmoud *et al*., 2018), dipping roots in mycelium (Nelson *et al*., 2021), cut-stem inoculation (Lygin *et al*., 2013; Pawlowski *et al*., 2015), and incubating seeds with mycelium (Coser *et al*., 2017). It has not been evaluated whether the method of pathogen introduction to the host has an effect on the

outcome of the infection. Commonly used metrics of disease susceptibility include necrotic lesion size, percent mortality, and overall disease symptom quantified by a numeric scoring scale. In order to evaluate the most suitable screening method for *M. phaseolina* resistance, Mengistu *et al.* (2007) tested 5 different disease metrics in soybean for their consistency across experimental years and environments. Colony-forming unit index (CFUI), calculated by counting colony forming units (CFU) derived from a set amount of ground tissue and dividing by the CFU count of the most susceptible genotype (highest CFU) within the experiment, was deemed to be the most consistent across all experimental conditions. This was followed by root and stem severity (RSS), determined by discoloration of stem and taproot tissue, which the authors noted was a less time and resource consuming alternative disease assessment method (Mengistu *et al*., 2007).

CONCLUSIONS

Movement towards reduced chemical use in agriculture is an imperative step towards sustainability. In order to develop resistant varieties of crops, it is necessary to first elucidate functions of specific genes/gene groups and quantify their contribution to host resistance and pathogen virulence. With modern sequencing technologies, high-throughput assay capabilities, and decades of research on *M. phaseolina* pathology, researchers are equipped to advance our understanding of generalist hemibiotrophic pathogens like *F. oxysporum* and *M.*

phaseolina, which are notoriously difficult to control. Slowly, we are beginning to understand why *M. phaseolina* is a successful pathogen. First, its wide host range and prevalence throughout the globe indicate high genomic plasticity that has allowed this fungus to adapt to local conditions and host plant species. This is supported by the presence of repetitive sequences and transposable elements within its genome. Moreover, no evidence for sexual reproduction has been documented for *M. phaseolina* thus far. This is striking considering the level of genomic variability seen across isolates even among the same host species and geographic origins, and evidence for host specificity seen in multiple examples. Several possible explanations including horizontal gene transfer, sporadic mutations mediated by TEs and repeats, and epigenetics could be hypothesized, but nonetheless it is likely that the instability of the *M. phaseolina* genome contributes to its extensive virulence capabilities. It is also important to note that the sampling of *M. phaseolina* in published studies are overwhelmingly biased towards those collected from agricultural fields and may not be an accurate representation of naturally occurring populations. Recently, more and more studies describing *M. phaseolina* pathosystems, using various isolates and host species, from various levels of genetic regulation are becoming available. Unfortunately, it seems that the likelihood of finding crop genotypes conferring complete immunity to this pathogen are very low. To date, no specific *R-Avr* gene interactions have been identified in any host interactions with *M. phaseolina*. Resistance, if seen at all, seems to be a quantitative trait. Several recurring themes can be identified in

the literature, such as: cell wall metabolism, oxidative stress and detoxification, and time-sensitive transition from biotrophic to necrotrophic response. These known plant immune responses potentially contribute partially to the overall disease phenotype. Through what mechanisms, and to what extent these biological processes impact the phenotype remains to be answered.

Literature Cited

Abawi, G. S. and Corrales (1990). Root Rots of Beans in Latin America and Africa: Diagnosis, Research Methodologies, and Management Strategies. CIAT.

Abbas, H. K., Bellaloui, N., Accinelli, C., Smith, J. R., & Shier, W. T. (2019). Toxin Production in Soybean (Glycine max L.) Plants with Charcoal Rot Disease and by *Macrophomina phaseolina*, the Fungus that Causes the Disease. *Toxins*, *11*(11). https://doi.org/10.3390/toxins11110645

Acharya, R., & Acharya, K. (2007). Evaluation of nitric oxide synthase status during disease progression in resistant and susceptible varieties of Sesamum indicum against *Macrophomina phaseolina*. *The Asian and Australasian Journal of Plant Science and Biotechnology*, *1*, 61–63.

Agostini, A. (2011). Effects of Brassicaceae Seed Meal Incorporation on Pre-plant Weed Emergence and Strawberry Productivity in *Macrophomina Phaseolina* infested and Non-infested Soil. http://sonoma-dspace.calstate.edu/handle/10211.1/1462

Ahmad, Y., Hameed, A., & Ghaffar, A. (2006). Enzymatic activity of fungal pathogens in corn. *Pakistan Journal of Botany*, *38*(4), 1305.

Ahuja, I., Kissen, R., & Bones, A. M. (2012). Phytoalexins in defense against pathogens. *Trends in Plant Science*, *17*(2), 73–90.

Almeida, Á. M. R., Amorim, L., Bergamin Filho, A., Torres, E., Farias, J. R. B., Benato, L. C., Pinto, M. C., & Valentim, N. (2003). Progress of soybean charcoal rot under tillage and no-tillage systems in Brazil. *Fitopatologia Brasileira*, *28*(2), 131–135.

Almeida, Á. M. R., Sosa-Gomez, D. R., Binneck, E., Marin, S. R. R., Zucchi, M. I., Abdelnoor, R. V., & Souto, E. R. (2008). Effect of crop rotation on specialization and genetic diversity of Macrophomina phaseolina. *Tropical Plant Pathology*, *33*(4), 257–264.

Alston, J. M., & Pardey, P. G. (2008). Public Funding for Research into Specialty Crops. *HortScience: A Publication of the American Society for Horticultural Science*, *43*(5), 1461–1470.

Ammon, V., Wyllie, T. D., & Brown, M. F. (1974). An ultrastructural investigation of pathological alterations induced by *Macrophomina phaseolina* (Tassi) Goid in seedlings of soybean, Glycine max (L.) Merrill. *Physiological Plant Pathology*, *4*(1), 1–4.

Arafat, M. Y., Narula, K., Nalwa, P., Sengupta, A., Chakraborty, N., & Chakraborty, S. (2022). Proteomic analysis of phytopathogenic fungus *Macrophomina phaseolina* identify known and novel mycelial proteins with roles in growth and virulence. *Journal of Proteins and Proteomics*, *13*(3), 149–157.

Arora, P., Dilbaghi, N., & Chaudhury, A. (2012). Opportunistic invasive fungal pathogen *Macrophomina phaseolina* prognosis from immunocompromised humans to potential mitogenic RBL with an exceptional and novel antitumor and cytotoxic effect. *European Journal of Clinical Microbiology & Infectious Diseases: Official Publication of the European Society of Clinical Microbiology*, *31*(2), 101–107.

Asai, S., Mase, K., & Yoshioka, H. (2010). Role of nitric oxide and reactive oxygen species in disease resistance to necrotrophic pathogens. *Plant Signaling & Behavior*, *5*(7), 872–874.

Asai, S., & Yoshioka, H. (2009). Nitric Oxide as a Partner of Reactive Oxygen Species Participates in Disease Resistance to Necrotrophic Pathogen Botrytis cinerea in *Nicotiana benthamiana*. *Molecular Plant-Microbe Interactions: MPMI*, *22*(6), 619–629.

Attard, A., Gourgues, M., Callemeyn-Torre, N., & Keller, H. (2010). The immediate activation of defense responses in *Arabidopsis* roots is not sufficient to prevent *Phytophthora parasitica* infection. *The New Phytologist*, *187*(2), 449–460.

Baird, R. E., Wadl, P. A., Allen, T., McNeill, D., Wang, X., Moulton, J. K., Rinehart, T. A., Abbas, H. K., Shier, T., & Trigiano, R. N. (2010). Variability of United States isolates of *Macrophomina phaseolina* based on simple sequence repeats and cross genus transferability to related genera within botryosphaeriaceae. *Mycopathologia*, *170*(3), 169–180.

Bandara, A. Y., Weerasooriya, D. K., Bradley, C. A., Allen, T. W., & Esker, P. D. (2020). Dissecting the economic impact of soybean diseases in the United States over two decades. *PloS One*, *15*(4), e0231141.

Bari, R., & Jones, J. D. G. (2009). Role of plant hormones in plant defense responses. *Plant Molecular Biology*, *69*(4), 473–488.

Bashir, M., & Malik, B. A. (1988). Diseases of major pulse crops in Pakistan—a review. *Tropical Pest Management*, *34*(3), 309–314.

Bedawy, I., & Moharm, M. (2019). Reaction and performance of some sesame genotypes for resistance to *Macrophomina phaseolina*, the incitant of charcoal rot disease. Alexandria Science Exchange Journal, 40(JANUARY-MARCH), 12–18.

Bhattacharya, D., Dhar, T. K., & Ali, E. (1992). An enzyme immunoassay of phaseolinone and its application in estimation of the amount of toxin in *Macrophomina phaseolina*-infected seeds. *Applied and Environmental Microbiology*, *58*(6), 1970–1974.

Bhattacharya, D., Dhar, T. K., Siddiqui, K. A. I., & Ali, E. (1994). Inhibition of seed germination by *Macrophomina phaseolina* is related to phaseolinone production. *The Journal of Applied Bacteriology*, *77*(2), 129–133.

Bigeard, J., Colcombet, J., & Hirt, H. (2015). Signaling mechanisms in pattern-triggered immunity (PTI). *Molecular Plant*, *8*(4), 521–539.

Bojórquez-Armenta, Y. de J., Mora-Romero, G. A., López-Meyer, M., Maldonado-Mendoza, I. E., Castro-Martínez, C., Romero-Urías, C. de L. A., Cordero-Ramírez, J. D., & Martínez-Álvarez, J. C. (2021). Evaluation of *Bacillus* spp. isolates as potential biocontrol agents against charcoal rot caused by *Macrophomina phaseolina* on common bean. *Journal of General Plant Pathology: JGPP*, *87*(6), 377–386.

Bokor, P. (2007). Macrophomina phaseolina causing a charcoal rot of sunflower through Slovakia. *Biologia* , *62*(2), 136–138.

Breitenbach, M., Weber, M., Rinnerthaler, M., Karl, T., & Breitenbach-Koller, L. (2015). Oxidative stress in fungi: its function in signal transduction, interaction with plant hosts, and lignocellulose degradation. *Biomolecules*, *5*(2), 318–342.

Brunner, F., Rosahl, S., Lee, J., Rudd, J. J., Geiler, C., Kauppinen, S., Rasmussen, G., Scheel, D., & Nürnberger, T. (2002). Pep-13, a plant defense-inducing pathogen-associated pattern from *Phytophthora transglutaminases*. *The EMBO Journal*, *21*(24), 6681–6688.

Burkhardt, A. K., Childs, K. L., Wang, J., Ramon, M. L., & Martin, F. N. (2019). Assembly, annotation, and comparison of *Macrophomina phaseolina* isolates from strawberry and other hosts. *BMC Genomics*, *20*(1), 802.

California Department of Food, & Agriculture. *CDFA > STATISTICS*. Retrieved September 10, 2018, from https://www.cdfa.ca.gov/statistics/?xid=PS_smithsonian

Caseys, C., Shi, G., Soltis, N., Gwinner, R., Corwin, J., Atwell, S., & Kliebenstein, D. (2019). A generalist pathogen view of diverse host evolutionary histories through polygenic virulence. In *bioRxiv* (p. 507491). https://doi.org/10.1101/507491

Chamorro, M., Domínguez, P., Medina, J. J., Miranda, L., Soria, C., Romero, F., López Aranda, J. M., Daugovish, O., Mertely, J., & De los Santos, B. (2015). Assessment of chemical and biosolarization treatments for the control of *Macrophomina phaseolina* in strawberries. *Scientia Horticulturae*, *192*, 361–368.

Chamorro, M., Seijo, T. E., Noling, J. C., De los Santos, B., & Peres, N. A. (2016). Efficacy of fumigant treatments and inoculum placement on control of *Macrophomina phaseolina* in strawberry beds. *Crop Protection* , *90*, 163–169.

Chan, Y.-H., & Sackston, W. E. (1969). Mechanisms of pathogenesis in *Sclerotium bataticola* on sunflowers. I. Production and translocation of a necrosis-inducing toxin. *Canadian Journal of Botany. Journal Canadien de Botanique*, *47*(7), 1147–1151.

Chan, Y.-H., & Sackston, W. E. (1970). Mechanisms of pathogenesis in *Sclerotium bataticola* on sunflowers. II. Pectolytic and cellulolytic enzyme production in vitro and in vivo. In *Canadian Journal of Botany* (Vol. 48, Issue 6, pp. 1073–1077). https://doi.org/10.1139/b70-155

Chen, Y. C., Wong, C. L., Muzzi, F., Vlaardingerbroek, I., Kidd, B. N., & Schenk, P. M. (2014). Root defense analysis against *Fusarium oxysporum* reveals new regulators to confer resistance. *Scientific Reports*, *4*(1), 1–10.

Chisholm, S. T., Coaker, G., Day, B., & Staskawicz, B. J. (2006). Host-microbe interactions: shaping the evolution of the plant immune response. *Cell*, *124*(4), 803–814.

Chooi, Y.-H., & Tang, Y. (2012). Navigating the fungal polyketide chemical space: from genes to molecules. *The Journal of Organic Chemistry*, *77*(22), 9933–9953.

Chowdhury, S., Basu, A., & Kundu, S. (2017). Biotrophy-necrotrophy switch in pathogen evoke differential response in resistant and susceptible sesame involving multiple signaling pathways at different phases. *Scientific Reports*, *7*(1), 17251.

Chuberre, C., Plancot, B., Driouich, A., Moore, J. P., Bardor, M., Gügi, B., & Vicré, M. (2018). Plant Immunity Is Compartmentalized and Specialized in Roots. *Frontiers in Plant Science*, *9*, 1692.

Clarke, J. D., Volko, S. M., Ledford, H., Ausubel, F. M., & Dong, X. (2000). Roles of salicylic acid, jasmonic acid, and ethylene in cpr-induced resistance in arabidopsis. *The Plant Cell*, *12*(11), 2175–2190.

Coser, S. M., Chowda Reddy, R. V., Zhang, J., Mueller, D. S., Mengistu, A., Wise, K. A., Allen, T. W., Singh, A., & Singh, A. K. (2017). Genetic Architecture of Charcoal Rot (*Macrophomina phaseolina*) Resistance in Soybean Revealed Using a Diverse Panel. *Frontiers in Plant Science*, *8*, 1626.

Dabi, M., Agarwal, P., & Agarwal, P. K. (2020). Overexpression of JcWRKY2 confers increased resistance towards *Macrophomina phaseolina* in transgenic tobacco. *3 Biotech*, *10*(11), 490.

Dangl, J. L., & Jones, J. D. (2001). Plant pathogens and integrated defence responses to infection. *Nature*, *411*(6839), 826–833.

Das, I. K., Indira, S., Annapurna, A., Prabhakar, & Seetharama, N. (2008). Biocontrol of charcoal rot in sorghum by fluorescent pseudomonads associated with the rhizosphere. *Crop Protection* , *27*(11), 1407–1414.

da Silva, M. P., Klepadlo, M., Gbur, E. E., Pereira, A., Mason, R. E., Rupe, J. C., Bluhm, B. H., Wood, L., Mozzoni, L. A., & Chen, P. (2019). QTL Mapping of Charcoal Rot Resistance in PI 567562A Soybean Accession. *Crop Science*, *59*(2). https://doi.org/10.2135/cropsci2018.02.0145

da Silva, M. P., Zaccaron, A. Z., Bluhm, B. H., Rupe, J. C., Wood, L., Mozzoni, L. A., Mason, R. E., Yingling, S., & Pereira, A. (2019). Bulked segregant analysis using next-generation sequencing for identification of genetic loci for charcoal rot resistance in soybean. *Physiological and Molecular Plant Pathology*, 101440.

Delledonne, M., Xia, Y., Dixon, R. A., & Lamb, C. (1998). Nitric oxide functions as a signal in plant disease resistance. *Nature*, *394*(6693), 585–588.

Delledonne, M., Zeier, J., Marocco, A., & Lamb, C. (2001). Signal interactions between nitric oxide and reactive oxygen intermediates in the plant hypersensitive disease resistance response. *Proceedings of the National Academy of Sciences of the United States of America*, *98*(23), 13454–13459.

de los Santos, B., Aguado, A., Borrero, C., Viejobueno, J., & Avilés, M. (2019). First Report of Charcoal Rot, Caused by *Macrophomina phaseolina*, on Blueberry in Southwestern Spain. *Plant Disease*, PDIS–04–19–0761–PDN.

Dhar, T. K., Siddiqui, K. A. I., & Ali, E. (1982). Structure of phaseolinone, a novel phytotoxin from. *Tetrahedron Letters*, *23*(51), 5459–5462.

Dhingra, O. D., Tenne, F. D., & Sinclair, J. B. (1976). Method for the determination of competitive saprophytic colonization of soil fungi. *Transactions of the British Mycological Society*, *66*(3), 447–456.

Doke, N. (1983). Involvement of superoxide anion generation in the hypersensitive response of potato tuber tissues to infection with an incompatible race of *Phytophthora infestans* and to the hyphal wall components. *Physiological Plant Pathology*, *23*(3), 345–357.

Drobnica, L., Zemanová, M., Nemec, P., Antos, K., Kristián, P., Stullerová, A., Knoppová, V., & Nemec, P., Jr. (1967). Antifungal activity of isothiocyanates and related compounds. I. Naturally occurring isothiocyanates and their analogues. *Applied Microbiology*, *15*(4), 701–709.

Durrant, W. E., & Dong, X. (2004). Systemic acquired resistance. *Annual Review of Phytopathology*, *42*, 185–209.

Fang, X., Phillips, D., Li, H., Sivasithamparam, K., & Barbetti, M. J. (2011). Comparisons of virulence of pathogens associated with crown and root diseases of strawberry in Western Australia with special reference to the effect of temperature. *Scientia Horticulturae*, *131*, 39–48.

Filho, E. S., & Dhingra, O. D. (1980). Effect of herbicides on survival of *Macrophomina phaseolina* in soil. *Transactions of the British Mycological Society*, *74*(1), 61–64.

Fraser, C. M., & Chapple, C. (2011). The phenylpropanoid pathway in *Arabidopsis*. *The Arabidopsis Book / American Society of Plant Biologists*, *9*, e0152.

Gaetán, S. A., Fernandez, L., & Madia, M. (2006). Occurrence of Charcoal Rot Caused by *Macrophomina phaseolina* on Canola in Argentina. *Plant Disease*, *90*(4), 524.

Gaige, A. R., Ayella, A., & Shuai, B. (2010). Methyl jasmonate and ethylene induce partial resistance in *Medicago truncatula* against the charcoal rot pathogen *Macrophomina phaseolina*. *Physiological and Molecular Plant Pathology*, *74*(5), 412–418.

Gao, Y., Xie, M., Yu, C., Zhang, M., Huang, J., Li, Q., Zhang, H., & Li, L. (2021). Heterologous expression of macrollins from phytopathogenic *Macrophomina phaseolina* revealed a cytochrome P450 mono-oxygenase in the biosynthesis of β-hydroxyl tetramic acid. *Journal of Agricultural and Food Chemistry*, *69*(50), 15175–15183.

Gaulin, E., Jauneau, A., Villalba, F., Rickauer, M., Esquerré-Tugayé, M.-T., & Bottin, A. (2002). The CBEL glycoprotein of *Phytophthora parasitica* var-*nicotianae* is involved in cell wall deposition and adhesion to cellulosic substrates. *Journal of Cell Science*, *115*(Pt 23), 4565–4575.

Glazebrook, J. (2005). Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annual Review of Phytopathology*, *43*, 205–227.

Gómez-Gómez, L., & Boller, T. (2000). FLS2: an LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in Arabidopsis. *Molecular Cell*, *5*(6), 1003–1011.

Gordon, T. R. (2017). *Fusarium oxysporum* and the Fusarium Wilt Syndrome. *Annual Review of Phytopathology*, *55*, 23–39.

Gupta, C., Dubey, R., & Maheshwari, D. (2002). Plant growth enhancement and suppression of *Macrophomina phaseolina* causing charcoal rot of peanut by fluorescent Pseudomonas. *Biology and Fertility of Soils*, *35*(6), 399–405.

Guthman, J., & Others. (2017). Land access and costs may drive strawberry growers' increased use of fumigation. *California Agriculture*, *71*(3), 184–191.

Hamann, T. (2012). Plant cell wall integrity maintenance as an essential component of biotic stress response mechanisms. *Frontiers in Plant Science*, *3*, 77.

Holmes, G. J., Mansouripour, S. M., & Hewavitharana, S. S. (2020). Strawberries at the Crossroads: Management of Soilborne Diseases in California Without Methyl Bromide. *Phytopathology*, *110*(5), 956–968.

Hossen, Q. M. M., Islam, M. S., & Emdad, E. M. (2019). Whole-genome optical mapping: Improving assembly of *Macrophomina phaseolina* MS6 through spanning of twelve blunt end chromosomes by obviating all errors …. *African Journal of*. https://academicjournals.org/journal/AJB/article-abstract/9D14A7362342

Hou, S., Liu, Z., Shen, H., & Wu, D. (2019). Damage-Associated Molecular Pattern-Triggered Immunity in Plants. *Frontiers in Plant Science*, *10*, 646.

Iqbal, U., & Mukhtar, T. (2020). Inhibitory Effects of some Fungicides against *Macrophomina phaseolina* Causing Charcoal Rot. *Pakistan Journal of Zoology*, *52*(2). https://doi.org/10.17582/journal.pjz/20181228101230

Islam, M. S., Haque, M. S., Islam, M. M., Emdad, E. M., Halim, A., Hossen, Q. M. M., Hossain, M. Z., Ahmed, B., Rahim, S., Rahman, M. S., Alam, M. M., Hou, S., Wan, X., Saito, J. A., & Alam, M. (2012). Tools to kill: genome of one of the most destructive plant pathogenic fungi *Macrophomina phaseolina*. *BMC Genomics*, *13*, 493.

Jackson, M. A., & Jaronski, S. T. (2009). Production of microsclerotia of the fungal entomopathogen *Metarhizium anisopliae* and their potential for use as a biocontrol agent for soil-inhabiting insects. *Mycological Research*, *113*(Pt 8), 842–850.

Jana, T. K., Singh, N. K., Koundal, K. R., & Sharma, T. R. (2005). Genetic differentiation of charcoal rot pathogen, *Macrophomina phaseolina*, into specific groups using URP-PCR. *Canadian Journal of Microbiology*, *51*(2), 159–164.

Jana, T., Sharma, T. R., & Singh, N. K. (2005). SSR-based detection of genetic variability in the charcoal root rot pathogen *Macrophomina phaseolina*. *Mycological Research*, *109*(Pt 1), 81–86.

Jones, J. D. G., & Dangl, J. L. (2006). The plant immune system. *Nature*, *444*(7117), 323–329.

Kaiser, S. A. K. M., & Das, S. N. (1988). Physical factors that influence the growth and spread of charcoal rot pathogen (*Macrophomina phaseolina*) infecting maize. *Phytopathologische Zeitschrift. Journal of Phytopathology*, *123*(1), 47–51.

Kaur, S., Dhillon, G. S., Brar, S. K., & Chauhan, V. B. (2012). Carbohydrate degrading enzyme production by plant pathogenic mycelia and microsclerotia isolates of *Macrophomina phaseolina* through koji fermentation. *Industrial Crops and Products*, *36*(1), 140–148.

Kaur, S., Dhillon, G. S., Brar, S. K., Vallad, G. E., Chand, R., & Chauhan, V. B. (2012). Emerging phytopathogen *Macrophomina phaseolina*: biology, economic importance and current diagnostic trends. *Critical Reviews in Microbiology*, *38*(2), 136–151.

Khaledi, N., & Taheri, P. (2016). Biocontrol mechanisms of *Trichoderma harzianum* against soybean charcoal rot caused by *Macrophomina phaseolina*. *Journal of Plant Protection Research*, *56*(1), 21–31.

Khalili, E., Javed, M. A., Huyop, F., Rayatpanah, S., Jamshidi, S., & Wahab, R. A. (2016). Evaluation of *Trichoderma* isolates as potential biological control agent against soybean charcoal rot disease caused by *Macrophomina phaseolina*. *Biotechnology, Biotechnological Equipment*, *30*(3), 479–488.

Khambhati, V. H., Abbas, H. K., Sulyok, M., Tomaso-Peterson, M., & Shier, W. T. (2020). First report of the production of mycotoxins and other secondary metabolites by *Macrophomina phaseolina* (Tassi) Goid. Isolates from soybeans (Glycine max L.) symptomatic with charcoal rot disease. *Journal of Fungi*, *6*(4), 332.

Kliebenstein, D. J. (2004). Secondary metabolites and plant/environment interactions: a view through *Arabidopsis thaliana* tinged glasses. *Plant, Cell & Environment*, *27*(6), 675–684.

Koike, S. T. (2008). Crown Rot of Strawberry Caused by *Macrophomina phaseolina* in California. *Plant Disease*, *92*(8), 1253–1253.

Koike, S. T., Arias, R. S., Hogan, C. S., Martin, F. N., & Gordon, T. R. (2016). Status of *Macrophomina phaseolina* on Strawberry in California and Preliminary Characterization of the Pathogen. *International Journal of Fruit Science*, *16*(sup1), 148–159.

Koornneef, A., & Pieterse, C. M. J. (2008). Cross Talk in Defense Signaling. *Plant Physiology*, *146*(3), 839–844.

Kubicek, C. P., Starr, T. L., & Glass, N. L. (2014). Plant Cell Wall–Degrading Enzymes and Their Secretion in Plant-Pathogenic Fungi. *Annual Review of Phytopathology*, *52*(1), 427–451.

Lakhran, L., Ahir, R. R., Choudhary, M., & Choudhary, S. (2018). Isolation, purification, identification and pathogenicity of *Macrophomina phaseolina* (Tassi) goid caused dry root rot of chickpea. *Journal of Pharmacognosy and Phytochemistry*, *7*(3), 3314–3317.

Levine, A., Tenhaken, R., Dixon, R., & Lamb, C. (1994). H2O2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. *Cell*, *79*(4), 583–593.

Lindermayr, C., & Durner, J. (2015). Interplay of reactive oxygen species and nitric oxide: nitric oxide coordinates reactive oxygen species homeostasis. *Plant Physiology*, *167*(4), 1209–1210. academic.oup.com.

Liu, H.-H., Huang, C.-C., Lin, Y.-H., Tseng, M.-N., & Chang, H.-X. (2022). Superoxide Initiates the Hyphal Differentiation to Microsclerotia Formation of *Macrophomina phaseolina*. *Microbiology Spectrum*, *10*(1), e0208421.

Lodha, S., & Mawar, R. (2020). Population dynamics of *Macrophomina phaseolina* in relation to disease management: A review. *Phytopathologische Zeitschrift. Journal of Phytopathology*, *168*(1), 1–17.

Lodha, S., Sharma, S. K., & Aggarwal, R. K. (1997). Solarization and natural heating of irrigated soil amended with cruciferous residues for improved control of *Macrophomina phaseolina*. *Plant Pathology*, *46*(2), 186–190.

Lorito, M., Woo, S. L., Harman, G. E., & Monte, E. (2010). Translational research on *Trichoderma*: from 'omics to the field. *Annual Review of Phytopathology*, *48*, 395–417.

Lygin, A. V., Zernova, O. V., Hill, C. B., Kholina, N. A., Widholm, J. M., Hartman, G. L., & Lozovaya, V. V. (2013). Glyceollin is an important component of soybean plant defense against *Phytophthora* sojae and *Macrophomina phaseolina*. *Phytopathology*, *103*(10), 984–994.

Macho, A. P., & Zipfel, C. (2014). Plant PRRs and the activation of innate immune signaling. *Molecular Cell*, *54*(2), 263–272.

Mahato, S. B., Siddiqui, K. A. I., Bhattacharya, G., Ghosal, T., Miyahara, K., Sholichin, M., & Kawasaki, T. (1987). Structure and Stereochemistry of Phaseolinic Acid: A New Acid from *Macrophomina phaseolina*. *Journal of Natural Products*, *50*(2), 245–247.

Mahmoud, A. F., Abou-Elwafa, S. F., & Shehzad, T. (2018). Identification of charcoal rot resistance QTLs in sorghum using association and in silico analyses. *Journal of Applied Genetics*, *59*(3), 243–251.

Ma, J., Hill, C. B., & Hartman, G. L. (2010). Production of *Macrophomina phaseolina* Conidia by Multiple Soybean Isolates in Culture. *Plant Disease*, *94*(9), 1088–1092.

Majumder, S., Datta, K., Sarkar, C., Saha, S. C., & Datta, S. K. (2018). The Development of *Macrophomina phaseolina* (Fungus) Resistant and Glufosinate (Herbicide) Tolerant Transgenic Jute. *Frontiers in Plant Science*, *9*, 920.

Marquez, N., Giachero, M. L., Declerck, S., & Ducasse, D. A. (2021). *Macrophomina phaseolina*: General Characteristics of Pathogenicity and Methods of Control. *Frontiers in Plant Science*, *12*, 634397.

Marquez, N., Giachero, M. L., Gallou, A., Debat, H. J., Cranenbrouck, S., Di Rienzo, J. A., Pozo, M. J., Ducasse, D. A., & Declerck, S. (2018). Transcriptional Changes in Mycorrhizal and Nonmycorrhizal Soybean Plants upon Infection with the Fungal Pathogen *Macrophomina phaseolina*. *Molecular Plant-Microbe Interactions: MPMI*, *31*(8), 842–855.

Masi, M., Sautua, F., Zatout, R., Castaldi, S., Arrico, L., Isticato, R., Pescitelli, G., Carmona, M. A., & Evidente, A. (2021). Phaseocyclopentenones A and B, Phytotoxic Penta- and Tetrasubstituted Cyclopentenones Produced by *Macrophomina phaseolina*, the Causal Agent of Charcoal Rot of Soybean in Argentina. *Journal of Natural Products*, *84*(2), 459–465.

Mayek-Pérez, N., GarcÍa-Espinosa, R., LÓpez-CastaÑeda, C., Acosta-Gallegos, J. A., & Simpson, J. (2002). Water relations, histopathology and growth of common bean (*Phaseolus vulgaris* L.) during pathogenesis of *Macrophomina phaseolina* under drought stress. *Physiological and Molecular Plant Pathology*, *60*(4), 185–195.

Mayék-Pérez, N., López-Castañeda, C., González-Chavira, M., Garcia-Espinosa, R., Acosta-Gallegos, J., de la Vega, O. M., & Simpson, J. (2001). Variability of Mexican isolates of *Macrophomina phaseolina* based on pathogenesis and AFLP genotype. *Physiological and Molecular Plant Pathology*, *59*(5), 257–264.

Mazzola, M., Agostini, A., & Cohen, M. F. (2017). Incorporation of *Brassica* seed meal soil amendment and wheat cultivation for control of *Macrophomina phaseolina* in strawberry. *European Journal of Plant Pathology / European Foundation for Plant Pathology*, *149*(1), 57–71.

Mengistu, A., Ray, J. D., Smith, J. R., & Paris, R. L. (2007). Charcoal Rot Disease Assessment of Soybean Genotypes Using a Colony-Forming Unit Index. *Crop Science*, *47*, 2453–2461.

Mengistu, A., Reddy, K. N., Zablotowicz, R. M., & Wrather, A. J. (2009). Propagule Densities of *Macrophomina phaseolina* in Soybean Tissue and Soil as Affected by Tillage, Cover Crop, and Herbicide. *Plant Health Progress*, *10*(1), 28.

Mertely, J., Seijo, T., & Peres, N. (2005, April). First report of *Macrophomina phaseolina* causing a crown rot of strawberry in Florida. *Plant Disease*, *89*(4), 434–434.

Mihail, J. D., & Taylor, S. J. (1995). Interpreting variability among isolates of *Macrophomina phaseolina* in pathogenicity, pycnidium production, and chlorate utilization. *Canadian Journal of Botany. Journal Canadien de Botanique*, *73*(10), 1596–1603.

Miklas, P. N., Stone, V., Urrea, C. A., Johnson, E., & Beaver, J. S. (1998). Inheritance and QTL Analysis of Field Resistance to Ashy Stem Blight in Common Bean. *Crop Science*, *38*(4), cropsci1998.0011183X003800040004x.

Msikita, W., James, B., Wilkinson, H. T., & Juba, J. H. (1998). First Report of *Macrophomina phaseolina* Causing Pre-harvest Cassava Root Rot in Benin and Nigeria. *Plant Disease*, *82*(12), 1402–1402.

Muchero, W., Ehlers, J. D., Close, T. J., & Roberts, P. A. (2011). Genic SNP markers and legume synteny reveal candidate genes underlying QTL for *Macrophomina phaseolina* resistance and maturity in cowpea [*Vigna* unguiculata (L) Walp.]. *BMC Genomics*, *12*, 8.

Mughogho, L K and Pande, S (1983) *Charcoal Rot of Sorghum.* In: Sorghum Root and Stalk Rots, Review: Proceedings of the Consultative Group Discussion on Research Needs and Strategies for Control of Sorghum Root and Stalk Rot Diseases, 27 Nov - 2 Dec 1983, Bellagio, Italy.

Mur, L. A. J., Kenton, P., Atzorn, R., Miersch, O., & Wasternack, C. (2006). The outcomes of concentration-specific interactions between salicylate and jasmonate signaling include synergy, antagonism, and oxidative stress leading to cell death. *Plant Physiology*, *140*(1), 249–262.

Neill, C. L., & Morgan, K. L. (2021). Beyond Scale and Scope: Exploring Economic Drivers of U.S. Specialty Crop Production With an Application to Edamame. *Frontiers in Sustainable Food Systems*, *4*. https://doi.org/10.3389/fsufs.2020.582834

Nelson, J. R., Verma, S., Bassil, N. V., Finn, C. E., Hancock, J. F., Cole, G. S., Knapp, S. J., & Whitaker, V. M. (2021). Discovery of three loci increasing resistance to charcoal rot caused by *Macrophomina phaseolina* in octoploid strawberry. *G3* . https://doi.org/10.1093/g3journal/jkab037

Nouri, M. T., Lawrence, D. P., Kallsen, C. E., & Trouillas, F. P. (2020). *Macrophomina* Crown and Root Rot of Pistachio in California. *Plants*, *9*(2). https://doi.org/10.3390/plants9020134

Nürnberger, T., Brunner, F., Kemmerling, B., & Piater, L. (2004). Innate immunity in plants and animals: striking similarities and obvious differences. *Immunological Reviews*, *198*, 249–266.

Orrego, A., Gavilán, M. C., Arévalos, A., Ortíz, B., Gaete Humada, B., Pineda-Fretez, A., Romero-Rodríguez, M. C., Flores Giubi, M. E., Kohli, M. M., & Iehisa, J. C. M. (2022). Identification of reference genes and their validation for gene expression analysis in phytopathogenic fungus *Macrophomina phaseolina*. *PloS One*, *17*(8), e0272603.

Pal, K. K., & McSpadden Gardener, B. (2006). Biological control of plant pathogens. *Plant Health Instructor*. https://doi.org/10.1094/phi-a-2006-1117-02

Pandey, A. K., & Basandrai, A. K. (2021). Will *Macrophomina phaseolina* spread in legumes due to climate change? A critical review of current knowledge. *Journal of Plant Diseases and Protection: Scientific Journal of the German Phytomedical Society* , *128*(1), 9–18.

Pawlowski, M. L., Hill, C. B., & Hartman, G. L. (2015). Resistance to Charcoal Rot Identified in Ancestral Soybean Germplasm. *Crop Science*, *55*, 1230–1235.

Pearson, C. A. S., Leslie, J. F., & Schwenk, F. W. (1986). Variable chlorate resistance in *Macrophomina phaseolina* from corn, soybean, and soil. *Phytopathology*, *76*(6), 646–649.

Pearson, C. A. S., Leslie, J. F., & Schwenk, F. W. (1987). Nitrogen source utilization by chlorate-resistant and chlorate-sensitive isolates of *Macrophomina phaseolina*. *Transactions of the British Mycological Society*, *88*(4), 497–502.

Perchepied, L., Balagué, C., Riou, C., Claudel-Renard, C., Rivière, N., Grezes-Besset, B., & Roby, D. (2010). Nitric oxide participates in the complex interplay of defense-related signaling pathways controlling disease resistance to *Sclerotinia sclerotiorum* in *Arabidopsis thaliana*. *Molecular Plant-Microbe Interactions: MPMI*, *23*(7), 846–860.

Perez-Brandán, C., Arzeno, J. L., Huidobro, J., Grümberg, B., Conforto, C., Hilton, S., Bending, G. D., Meriles, J. M., & Vargas-Gil, S. (2012). Long-term effect of tillage systems on soil microbiological, chemical and physical parameters and the incidence of charcoal rot by *Macrophomina phaseolina* (Tassi) Goid in soybean. *Crop Protection* , *40*, 73–82.

Pieterse, C. M. J., Van der Does, D., Zamioudis, C., Leon-Reyes, A., & Van Wees, S. C. M. (2012). Hormonal modulation of plant immunity. *Annual Review of Cell and Developmental Biology*, *28*, 489–521.

Pieterse, C. M., van Wees, S. C., Hoffland, E., van Pelt, J. A., & van Loon, L. C. (1996). Systemic resistance in *Arabidopsis* induced by biocontrol bacteria is independent of salicylic acid accumulation and pathogenesis-related gene expression. *The Plant Cell*, *8*(8), 1225–1237.

Poveda, J., Hermosa, R., Monte, E., & Nicolás, C. (2019). *Trichoderma* harzianum favours the access of arbuscular mycorrhizal fungi to non-host *Brassicaceae* roots and increases plant productivity. *Scientific Reports*, *9*(1), 11650.

Pratt, R. G., McLaughlin, M. R., Pederson, G. A., & Rowe, D. E. (1998). Pathogenicity of *Macrophomina phaseolina* to Mature Plant Tissues of Alfalfa and White Clover. *Plant Disease*, *82*(9), 1033–1038.

Lo Presti, L., Lanver, D., Schweizer, G., Tanaka, S., Liang, L., Tollot, M., Zuccaro, A., Reissmann, S., & Kahmann, R. (2015). Fungal effectors and plant susceptibility. *Annual Review of Plant Biology*, *66*, 513–545.

Purushotham, N., Jones, A., Poudel, B., Nasim, J., Adorada, D., Sparks, A., Schwessinger, B., & Vaghefi, N. (2020). Draft Genome Resource for *Macrophomina phaseolina* Associated With Charcoal Rot in Sorghum. *Molecular Plant-Microbe Interactions: MPMI*, *33*(5), 724–726.

Radadiya, N., Mangukia, N., Antala, V., Desai, H., Chaudhari, H., Dholaria, T. L., Dholaria, D., Tomar, R. S., Golakiya, B. A., & Mahatma, M. K. (2021). Transcriptome analysis of sesame-*Macrophomina phaseolina* interactions revealing the distinct genetic components for early defense responses. *Physiology and Molecular Biology of Plants: An International Journal of Functional Plant Biology*, *27*(8), 1675–1693.

Radha, K. (1953). The enzymic activity of *Macrophomina phaseoli,(*Maubl.), Ashby. *Proceedings: Plant Sciences*, *38*(6), 231–234.

Ramezani, M., Shier, W. T., Abbas, H. K., Tonos, J. L., Baird, R. E., & Sciumbato, G. L. (2007). Soybean Charcoal Rot Disease Fungus *Macrophomina phaseolina* in Mississippi Produces the Phytotoxin (−)-Botryodiplodin but No Detectable Phaseolinone. *Journal of Natural Products*, *70*(1), 128–129.

Ramos, A. M., Gally, M., Szapiro, G., Itzcovich, T., Carabajal, M., & Levin, L. (2016). In vitro growth and cell wall degrading enzyme production by Argentinean isolates of *Macrophomina phaseolina*, the causative agent of charcoal rot in corn. *Revista Argentina de Microbiologia*, *48*(4), 267–273.

Rangel-Montoya, E. A., Delgado-Ramírez, C. S., Sepulveda, E., & Hernández-Martínez, R. (2022). Biocontrol of *Macrophomina phaseolina* Using *Bacillus amyloliquefaciens* Strains in Cowpea (*Vigna unguiculata* L.). *Agronomy*, *12*(3), 676.

Regnault-Roger, C. (2020). Trends for Commercialization of Biocontrol Agents (Biopesticide). In J.-M. Mérillon & K. G. Ramawat (Eds.), *Plant Defence: Biological Control* (pp. 445–471). Springer International Publishing.

Rehman, F., Khan, F. A., & Badruddin, S. M. A. (2012). Role of Phenolics in Plant Defense Against Insect Herbivory. In L. D. Khemani, M. M. Srivastava, & S. Srivastava (Eds.), *Chemistry of Phytopotentials: Health, Energy and Environmental Perspectives* (pp. 309–313). Springer Berlin Heidelberg.

Reyes-Franco, M. C., Hernandez-Delgado, S., Beas-Fernandez, R., Medina-Fernandez, M., Simpson, J., & Mayek-Perez, N. (2006). Pathogenic and Genetic Variability within *Macrophomina phaseolina* from Mexico and Other Countries. *Phytopathologische Zeitschrift. Journal of Phytopathology*, *154*(7-8), 447–453.

Rosa, E. A. S., & Rodrigues, P. M. F. (1999). Towards a more sustainable agriculture system: The effect of glucosinolates on the control of soil-borne diseases. *The Journal of Horticultural Science & Biotechnology*, *74*(6), 667–674.

Ross, A. F. (1961). Systemic acquired resistance induced by localized virus infections in plants. *Virology*, *14*(3), 340–358.

Saleh, A. A., Ahmed, H. U., Todd, T. C., Travers, S. E., Zeller, K. A., Leslie, J. F., & Garrett, K. A. (2010). Relatedness of *Macrophomina phaseolina* isolates from tallgrass prairie, maize, soybean and sorghum. *Molecular Ecology*, *19*(1), 79–91.

Salvatore, M. M., Félix, C., Lima, F., Ferreira, V., Naviglio, D., Salvatore, F., Duarte, A. S., Alves, A., Andolfi, A., & Esteves, A. C. (2020). Secondary Metabolites Produced by *Macrophomina phaseolina* Isolated from Eucalyptus globulus. *Collection FAO: Agriculture*, *10*(3), 72.

Sankar, P., & Sharma, R. C. (2001). Management of charcoal rot of maize with *Trichoderma viride*. *Scientia Agricola*, *55*, 1–7.

Sarkar, T. S., Biswas, P., Ghosh, S. K., & Ghosh, S. (2014). Nitric oxide production by necrotrophic pathogen *Macrophomina phaseolina* and the host plant in charcoal rot disease of jute: complexity of the interplay between necrotroph-host plant interactions. *PloS One*, *9*(9), e107348.

Schroeder, M. M., Lai, Y., Shirai, M., Alsalek, N., Tsuchiya, T., Roberts, P., & Eulgem, T. (2019). A novel *Arabidopsis* pathosystem reveals cooperation of multiple hormonal response-pathways in host resistance against the global crop destroyer *Macrophomina phaseolina*. *Scientific Reports*, *9*(1), 20083.

Sharma, I., Kumari, N., & Sharma, V. (2014). Defense gene expression in *Sorghum bicolor* against *Macrophomina phaseolina* in leaves and roots of susceptible and resistant cultivars. *Journal of Plant Interactions*, *9*(1), 315–323.

Sheikh, A. H., & Ghaffar, A. (1984). Reduction in viability of sclerotia of *Macrophomina phaseolina* with polyethylene mulching of soil. *Soil Biology & Biochemistry*, *16*(1), 77–79.

Sheikh, A. H., & Ghaffar, A. (1987). Time-temperature relationships for the inactivation of sclerotia of *Macrophomina phaseolina*. *Soil Biology & Biochemistry*, *19*(3), 313–315.

Shier, W. T., Abbas, H. K., Baird, R. E., Ramezani, M., & Sciumbato, G. L. (2007). (-)-BOTRYODIPLODIN, A UNIQUE RIBOSE-ANALOG TOXIN. *Toxin Reviews*, *26*(4), 343–386.

Short, G. E., Wyllie, T. D., & Bristow, P. R. (1980). Survival of *Macrophomina phaseolina* in soil and in residue of soybean. *Survival*, *7*(13), 17.

Siddiqui, K. A., Gupta, A. K., Paul, A. K., & Banerjee, A. K. (1979). Purification and properties of heat-resistant exotoxin produced by *Macrophomina phaseolina* (Tassi) Goid in culture. *Experientia*, *35*(9), 1222–1223.

Singh, S. K., Nene, Y. L., & Reddy, M. V. (1990). Influence of cropping systems on *Macrophomina phaseolina* population of Soil. *Plant Disease, 74*(10), 812-814.

Smith, G. S., & Carvil, O. N. (1997). Field Screening of Commercial and Experimental Soybean Cultivars for Their Reaction to *Macrophomina phaseolina*. *Plant Disease*, *81*(4), 363–368.

Smolinska, U., Morra, M. J., Knudsen, G. R., & James, R. L. (2003). Isothiocyanates Produced by *Brassicaceae* Species as Inhibitors of *Fusarium oxysporum. Plant Disease*, *87*(4), 407–412.

Srivastava, S. K., & Dhawan, S. (1982). Phosphatidase Activity in *Brassica junce*a Plants Infected with Isolates of *Macrophomina phaseolina* and Its Role in Pathogenesis. *Bulletin of the Torrey Botanical Club*, *109*(4), 508–512.

Su, G., Suh, S. O., Schneider, R. W., & Russin, J. S. (2001). Host Specialization in the Charcoal Rot Fungus, *Macrophomina phaseolina. Phytopathology*, *91*(2), 120–126.

Tan, D. H. S., Sigler, L., Gibas, C. F. C., & Fong, I. W. (2008). Disseminated fungal infection in a renal transplant recipient involving *Macrophomina phaseolina* and *Scytalidium dimidiatum*: case report and review of taxonomic changes among medically important members of the *Botryosphaeriaceae*. *Medical Mycology: Official Publication of the International Society for Human and Animal Mycology*, *46*(3), 285–292.

Thomma, B. P., Penninckx, I. A., Broekaert, W. F., & Cammue, B. P. (2001). The complexity of disease signaling in *Arabidopsis*. *Current Opinion in Immunology*, *13*(1), 63–68.

Tierens, K. F., Thomma, B. P., Brouwer, M., Schmidt, J., Kistner, K., Porzel, A., Mauch-Mani, B., Cammue, B. P., & Broekaert, W. F. (2001). Study of the role of antimicrobial glucosinolate-derived isothiocyanates in resistance of *Arabidopsis* to microbial pathogens. *Plant Physiology*, *125*(4), 1688–1699.

Tiku, A. R. (2020). Antimicrobial Compounds (Phytoanticipins and Phytoalexins) and Their Role in Plant Defense. In J.-M. Mérillon & K. G. Ramawat (Eds.), *Co-Evolution of Secondary Metabolites* (pp. 845–868). Springer International Publishing.

Tomar, R. S., Parakhia, M. V., Rathod, V. M., Thakkar, J. R., Padhiyar, S. M., Thummar, V. D., Dalal, H., Kothari, V. V., Kheni, J., Dhingani, R. M., Sabara, P., & Golakiya, B. A. (2017). Molecular mapping and identification of QTLs responsible for charcoal rot resistance in Castor (*Ricinus communis* L.). *Industrial Crops and Products*, *95*, 184–190.

Tonin, R. F. B., Avozani, A., Danelli, A. L. D., & Reis, E. M. (2013). In vitro mycelial sensitivity of *Macrophomina phaseolina* to fungicides. *Pesquisa*. https://www.scielo.br/pdf/pat/v43n4/14.pdf

Tytgat, T. O. G., Verhoeven, K. J. F., Jansen, J. J., Raaijmakers, C. E., Bakx-Schotman, T., McIntyre, L. M., van der Putten, W. H., Biere, A., & van Dam, N. M. (2013). Plants know where it hurts: root and shoot jasmonic acid induction elicit differential responses in *Brassica oleracea*. *PloS One*, *8*(6), e65502.

VAN Baarlen, P., Staats, M., & VAN Kan, J. A. L. (2004). Induction of programmed cell death in lily by the fungal pathogen *Botrytis elliptica. Molecular Plant Pathology*, *5*(6), 559–574.

Verma, S., Tomar, R. S., Rathode, V., & Thakker, J. (2018). Genome sequencing analysis of Macrophomina Phaseolina resistant and susceptible castor genotype. *Biosciences Biotech Research Asia, 15*(1).

Wang, H., & Jones, R. W. (1995). A unique endoglucanase-encoding gene cloned from the phytopathogenic fungus *Macrophomina phaseolina*. *Applied and Environmental Microbiology*, *61*(5), 2004–2006.

Wang, Y., Branicky, R., Noë, A., & Hekimi, S. (2018). Superoxide dismutases: Dual roles in controlling ROS damage and regulating ROS signaling. *The Journal of Cell Biology*, *217*(6), 1915–1928.

Weems, J. D., Ebelhar, S. A., Chapara, V., Pedersen, D. K., Zhang, G. R., & Bradley, C. A. (2011). First Report of Charcoal Rot Caused by *Macrophomina phaseolina* on Sunflower in Illinois. *Plant Disease*, *95*(10), 1318–1318.

Wei, H., Liu, J., Guo, Q., Pan, L., Chai, S., Cheng, Y., Ruan, M., Ye, Q., Wang, R., Yao, Z., Zhou, G., & Wan, H. (2020). Genomic Organization and Comparative Phylogenic Analysis of NBS-LRR Resistance Gene Family in *Solanum pimpinellifolium* and *Arabidopsis thaliana*. *Evolutionary Bioinformatics Online*, *16*, 1176934320911055.

Wittstock, U., & Halkier, B. A. (2002). Glucosinolate research in the *Arabidopsis* era. *Trends in Plant Science*, *7*(6), 263–270.

Wrather, J. A., Anderson, T. R., Arsyad, D. M., Tan, Y., Ploper, L. D., Porta-Puglia, A., Ram, H. H., & Yorinori, J. T. (2001). Soybean disease loss estimates for the top ten soybean-producing countries in 1998. *Canadian Journal of Plant Pathology. Revue Canadienne de Phytopathologie*, *23*(2), 115–121.

Wrather, J. A., Kendig, S. R., & Tyler, D. D. (1998). Tillage Effects on *Macrophomina phaseolina* Population Density and Soybean Yield. *Plant Disease*, *82*(2), 247–250.

Wyllie, T. D., & Brown, M. F. (1970). Ultrastructural formation of sclerotia of *Macrophomina phaseoli*. *Phytopathology*, *60*(3), 524–528.

Yan, W., Ni, Y., Liu, X., Zhao, H., Chen, Y., Jia, M., Liu, M., Liu, H., & Tian, B. (2021). The mechanism of sesame resistance against *Macrophomina phaseolina* was revealed via a comparison of transcriptomes of resistant and susceptible sesame genotypes. *BMC Plant Biology*, *21*(1), 159.

Yasmin, H., Naz, R., Nosheen, A., Hassan, M. N., Ilyas, N., Sajjad, M., Anjum, S., Gao, X., & Geng, Z. (2020). Identification of New Biocontrol Agent against Charcoal Rot Disease Caused by *Macrophomina phaseolina* in Soybean (*Glycine max* L.). *Sustainability: Science Practice and Policy*, *12*(17), 6856.

Zaman, N. R., Kumar, B., Nasrin, Z., Islam, M. R., Maiti, T. K., & Khan, H. (2020). Proteome Analyses Reveal *Macrophomina phaseolina*'s Survival Tools When Challenged by *Burkholderia contaminans* NZ. *ACS Omega*, *5*(3), 1352–1362.

Chapter I: Exploring natural genetic variation within *Arabidopsis thaliana* for resistance against *Macrophomina phaseolina*

Abstract

Macrophomina phaseolina is a soil-borne pathogenic fungus, which causes the disease most commonly described as charcoal rot. Known for its wide host range and geographic distribution, it has caused detrimental effects on crop yields across the globe. Previous work on crop systems have shown varying tolerance to this pathogen within host plant genotypes. In order to harness the power of modern quantitative genetics approaches and molecular biology, a diverse panel of naturally occurring *Arabidopsis thaliana* accessions was tested for resistance against *M. phaseolina.* We first established an objective and reliable disease scoring method combining previously described agar plate-based infection assays with imaging analysis to quantify levels of host growth inhibition caused by *M. phaseolina*. Substantial variation in the levels of tolerance against this fungus was observed among the screened *A. thaliana* accessions. Based on the resulting quantitative data we estimated that more than half of the observed phenotypic variance could be explained by genetic effects. Our data further suggested that epistatic interactions play a major role in host responses against *M. phaseolina*. Genome-wide association studies identified several loci in the *A. thaliana* genome with statistical associations to the disease phenotype, each of which linked to multiple genes that could potentially play a role in *M. phaseolina* response.

Introduction

The soil-borne fungal pathogen *Macrophomina phaseolina* causes a disease most widely known as charcoal rot, which is characterized by the emergence of dark masses of hyphal cells in the host tissue called microsclerotia. Although charcoal rot has caused detrimental yield losses throughout the world in a wide range of crop species, effective long-term control methods have not been established. It has been speculated that this is in part due to the large genomic variability seen across populations, and the pronounced presence of genes coding for virulence-related enzymes within its genome (Islam *et al*., 2012). In several crop species, genomic loci, which exhibit high association with resistance phenotypes have been identified. However, the limitations associated with crop species, such as lack of assembled reference genomes, longer life cycles, and complex genome structures due to polyploidy, makes functional analyses of candidate genes within these loci difficult. Creating mutants to assess the functionality of genes or taking a marker-assisted breeding approach may not be practical in some host plants. It seems that there is little consensus among the loci that have been identified thus far, even between crop species where syntenic relationships are known and orthologous genes are identified (Coser *et al*. 2017; Adeyanju *et al*. 2015). This suggests that *M. phaseolina* resistance is a complex trait with multiple small contributions of interacting loci. In addition, this trait may be highly sensitive to environmental variability. There are also speculations that this pathogen employs host genotype specific virulence, although this has been

controversially debated throughout the years (Koike *et al*., 2016; Su *et al*., 2001). It has also been shown that the severity of charcoal rot symptoms correlate with more arid and hot climates (Kaur *et al*., 2012). Crop-based studies are often conducted in variable conditions, both in the field or in the greenhouse, which further adds a confounding factor when interpreting data from multiple studies.

Arabidopsis thaliana is widely used as a model plant and has historically contributed to the understanding of many traits with underlying complex genomic architectures. This is made possible in part by its self-pollinating, rapid life cycle and relatively small genome, as well as the massive public repository of data available for this organism. Studies in *A. thaliana* have led to the discovery of many resistance genes (*R*-genes) and corresponding pathogen avirulence (*avr*) genes from pathogens including *Peronospora parasitica* (Parker *et al*., 1996) and *Pseudomonas syringae (Dong et al., 1991). A. thaliana* has also been useful for understanding host-plant interactions with complex genetic interactions such as in *Fusarium oxysporum (Berrocal-Lobo & Molina, 2008)*, and *Botrytis cinerea* (Denby *et al*., 2004). For polygenic disease resistant traits associated with such pathogens, Mendelian segregation of major effect genes are rarely seen. Methods such as quantitative trait loci (QTL) mapping have been used to narrow down causal loci for such traits. QTL mapping involves the use of a segregating F_2, backcross, or recombinant inbred line (RIL) populations to narrow down possible regions in the genome harboring causal loci. Additional fine mapping and functional analysis may be necessary to confirm gene function in resistance (Collard *et al*., 2005). Such

methods can be very slow and require extremely large populations in order to increase the resolution, and thus may not be feasible in some host species.

In *A. thaliana*, an alternative or complementary approach has become popular in recent years, in which statistical models are applied to find associations between sequence variants and a particular phenotype. Genome-wide association is particularly useful in *A. thaliana* as it can maintain the inbred genotypes by self-fertilization, which allows for repeated phenotypic data collection. The narrow range in which linkage disequilibrium (LD) decays (50% within 5 kb) in *A. thaliana* provides additional confidence that variants identified through genome-wide association studies (GWAS) are highly linked to causal QTL (Gan *et al*., 2011; Korte & Farlow, 2013; Weigel & Nordborg, 2005). Moreover, complete genomic variant data of more than 1,000 naturally occurring inbred populations are available for use in GWAS (https://1001genomes.org/).

Association studies on continuous traits rely on the statistical predictability of linear regressions fitted across thousands of variant loci, and therefore are susceptible to confounding effects from population structure, or shared polymorphisms between related individuals (Korte & Farlow, 2013). This is often mitigated by the inclusion of a random effect in the mixed-model, which describes the known relationship between sampled individuals based on lineage or single nucleotide polymorphism (SNP) data similarity. Numerous examples of GWAS application on *A. thaliana* have been published, successfully identifying key genes underlying many quantitative phenotypes (Atwell *et al*., 2010).

Heritability is a metric used to describe how well a trait can be passed from parent to offspring in a specific population. In order to gain a comprehensive understanding of the genetic architecture shaping *A. thaliana* responses to *M. phaseolina,* we aim to dissect the genetic and environmental variables contributing to the phenotypic variance. Genetic effects on quantitative trait variation can either be additive or non-additive, and the ratio of genetic contribution to overall phenotypic variability in a population is called the broad sense heritability (H^2) of a trait. In plants, this is often estimated by comparing within- and between-genotype variability (Kruijer *et al*., 2015). Most applications of GWAS have focused on detection of additive genetic effects, although attempts to model variance effects from other sources such as the environment, gene-environment interactions, and epistasis have also been described (Heckerman *et al*., 2016; Korte *et al*., 2012; Lachowiec *et al*., 2015).

The portion of the phenotypic variance that can be attributed to additive genetic variance alone is called narrow-sense heritability (h^2). Traits associated with fitness, such as disease resistance, are generally said to have lower heritability (Yang, 2017). For example, Atwell *et al.* (2010) previously estimated broad-sense heritability in 107 traits, of which 14 were defense related. Disease-related broad-sense heritability estimates ranged from trichome density (H^2=0.88) to aphid propagation on leaves (H^2=0.42) (Atwell *et al*., 2010). By definition, heritability is a metric of genetic contributions to phenotypic variance, both of which is only relevant to the population from which data is obtained. Estimates of

heritability can be useful in assessing whether populations have sufficient genetic resources that will allow for improvements in host resistance through gene editing, transgenic approaches or breeding (Dudley & Moll, 1969; Visscher *et al*., 2008). To our knowledge, this is the first attempt to examine the proportion of genetic contributions to *M. phaseolina* resistance in the model plant *A. thaliana.*

Here, we focus on the genetic contributions to *M. phaseolina* response in *A. thaliana*, by introducing an objective phenotyping protocol based on the sterile agar-plate inoculation method described by Schroeder *et al.* (2019) (Schroeder *et al*., 2019). The percent growth inhibition relative to the control condition, normalized against a baseline accession, Columbia (Col-0), is used as a metric of *M. phaseolina* resistance in a total of 136 *A. thaliana* accessions. Data from this large-scale accession screen, performed with replicates, is then used to predict the heritability of the growth inhibition response and uncover potentiall causal loci.

Results

A. thaliana natural accessions show variability in responses to *M. phaseolina*

To screen for *M. phaseolina* resistance in *A. thaliana*, 97 natural accessions were tested using an agar plate-based assay previously introduced by Schroeder and colleagues (2019) (Schroeder *et al*., 2019). The accessions were chosen based on availability in the lab, but represent populations from a wide range of

geographic origins. All seeds were obtained from the Arabidopsis Biological Resource Centre (ABRC). Briefly, *A. thaliana* seedlings are grown in sterile ½ MS (Murashige and Skoog) agar medium for 10 days, then transferred to actively growing *M. phaseolina* mycelia on the same medium for inoculation. Seedlings are also transferred to uninoculated ½ MS plates as untreated controls. All seedlings on *M. phaseolina* treatment plates were scored individually at 14 days post-inoculation (dpi) based on comparisons with the control plates and visible disease characteristics including growth inhibition, chlorosis and necrosis, as shown in Figure 1.1.

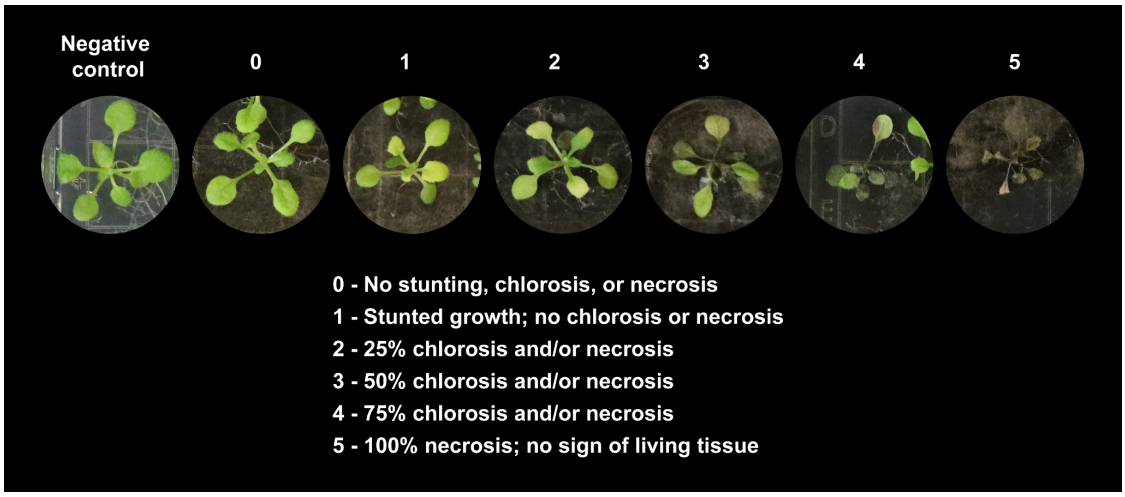

Figure 1.1. *M. phaseolina* disease severity is scored based on visible disease phenotypes with an arbitrary score ranging from 0 to 5. The left-most image shows a healthy Col-0 seeding on a control plate. Scores range from 0, where the seedling appears to have no symptoms compared to the control seedlings at that time-point, and 5, where complete necrosis of the tissue is observed.

While there were clear trends in phenotypic variability between accessions, we observed also significant variability between individuals of the same accession. As each accession tested in this assay are true breeding, or inbred lines derived from

natural populations, this variability in individual seedling responses are likely due to the infection efficiency of the pathogen or unknown environmental causes. Furthermore, screens were carried out in batches of seven to ten accessions due to capacity in the incubators. To reduce error from batch effects, scores were normalized to the average score of Col-0 seedlings, which was included in each batch of accessions. Average disease scores at 14 dpi relative to Col-0 are shown in Figure 1.2.

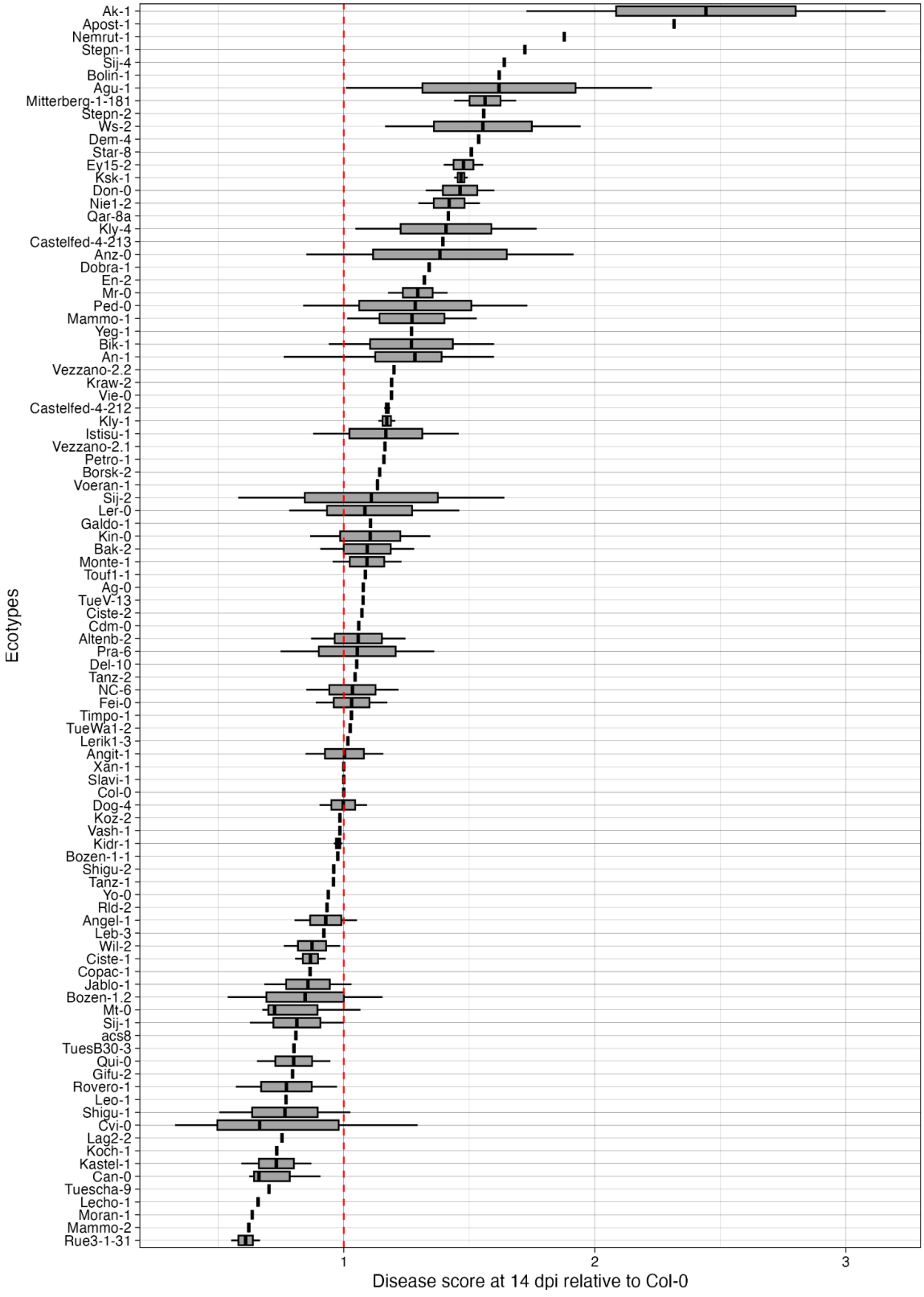
Ak-1
Apost-1
Nemrut-1
Stepn-1
Sij-4
Bolin-1
Agu-1
Mitterberg-1-181
Stepn-2
Ws-2
Dem-4
Star-8
Ey15-2
Ksk-1
Don-0
Nie1-2
Qar-8a
Kly-4
Castelfed-4-213
Anz-0
Dobra-1
En-2
Mr-0
Ped-0
Mammo-1
Yeg-1
Bik-1
An-1
Vezzano-2.2
Kraw-2
Vie-0
Castelfed-4-212
Kly-1
Istisu-1
Vezzano-2.1
Petro-1
Borsk-2
Voeran-1
Sij-2
Ler-0
Galdo-1
Kin-0
Bak-2
Monte-1
Touf1-1
Ag-0
TueV-13
Ciste-2
Cdm-0
Altenb-2
Pra-6
Del-10
Tanz-2
NC-6
Fei-0
Timpo-1
TueWa1-2
Lerik1-3
Angit-1
Xan-1
Slavi-1
Col-0
Dog-4
Koz-2
Vash-1
Kidr-1
Bozen-1-1
Shigu-2
Tanz-1
Yo-0
Rld-2
Angel-1
Leb-3
Wil-2
Ciste-1
Copac-1
Jablo-1
Bozen-1.2
Mt-0
Sij-1
acs8
TuesB30-3
Qui-0
Gifu-2
Rovero-1
Leo-1
Shigu-1
Cvi-0
Lag2-2
Koch-1
Kastel-1
Can-0
Tuescha-9
Lecho-1
Moran-1
Mammo-2
Rue3-1-31
Ecotypes
1
2
3
Disease score at 14 dpi relative to Col-0

Figure 1.2. *A. thaliana* natural accessions show a wide range in susceptibility to *M. phaseolina* infection. A box and whiskers plot of disease scores relative to Col-0 at 14 dpi for 97 *A. thaliana* accessions relative to Col-0. If box and whiskers are depicted, data is derived from multiple biological replicates (two to three replicates). Otherwise, only one biological replicate was performed. For each replicate, a total of 40 seedlings are scored and relative disease scores are calculated by dividing the average score for each accession with that of Col-0. Gray boxes represent the range between the first and third quartile, and notches indicate the median value. Whiskers extend 1.5 times the interquartile range (IQR) (Mcgill *et al*., 1978). Dashed line represents a score of 1, or equivalent to the score of Col-0. An one-way analysis of variance (ANOVA) was conducted using accessions as the factor, which showed that there are significant effects on relative disease scores due to the host genotypes selected in this screen (p=8.13E-3).

Significant variation (ANOVA; *p*=8.13E-3) in disease score relative to Col-0 at 14 dpi was seen among the tested accessions**.** Of the 97 tested accessions, 59 showed more severe disease symptoms (had a relative disease score greater than one), while 35 showed less severe disease symptoms (had a relative disease score less than one) relative to Col-0, and three had Col-0-like symptoms. The average disease score across all observed Col-0 seedlings at 14 dpi was 2.54, which was normalized to one in the relative disease score scale. This accession screen showed that significant variability in *M. phaseolina* responses can be observed within *A. thaliana* natural accessions. In evaluating disease scores, variability in multiple phenotypes were also observed, including rate of growth, chlorosis, and in some cases early flowering.

Quantification of growth inhibition in *M. phaseolina*-infected *A. thaliana* using digital image processing reveals significant variation in susceptibility

In order to objectively quantify severity of *M. phaseolina*-caused disease in *A. thaliana* and to streamline phenotyping under controlled conditions, the plate-based infection assay was combined with the digital image processing method *plant immunity and disease image-based quantification* (*PIDIQ*) (Laflamme *et al*., 2016). Seedlings were inoculated by placing them onto actively growing *M. phaseolina* hyphae, as described above. For digital image processing, photos of individual plates were taken on the second day post-inoculation, as well as at 7, 11, and 14 days under controlled light conditions and fixed camera settings. The distance from the plate to the camera was also fixed to ensure consistent

resolution. Images were processed on Fiji (2.1.0) software (Schindelin *et al*., 2012) using a modified version of *PIDIQ*, an ImageJ macro developed for the automated quantification of *A. thaliana* rosette size and chlorosis phenotypes, introduced by Laflamme *et al.* (2016). *PIDIQ* separates each image to its hue, saturation, and brightness using the HSB Track option and selects green pixels using set thresholds. This function was modified from the original macros, which was designed originally to process soil-grown *A. thaliana* on flats, to accurately select *A. thaliana* rosette pixels from the plate images (Figure 1.3).

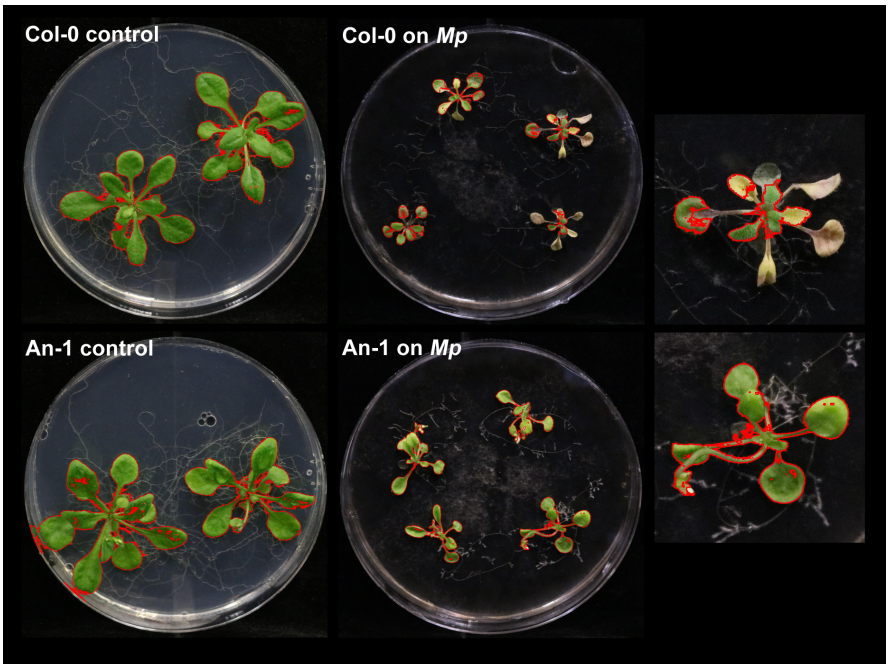

Figure 1.3. *M. phaseolina* disease severity is quantified on plate-grown *A. thaliana* using growth inhibition as a metric. ImageJ macro PIDIQ was used to quantify the healthy areas of the rosette for each plate photo. The area of the rosette quantified by PIDIQ is outlined by red pixels. The left two plates show controls with no *M. phaseolina*. The right two plates show seedlings transferred onto *M. phaseolina* (*Mp*) hyphae at 11 dpi.

A total of 88 *A. thaliana* natural accessions were tested against *M. phaseolina* to screen for resistant genotypes. Accessions were chosen from the

1001 Genomes database (https://1001genomes.org/index.html), which have been thoroughly genotyped with annotations for SNPs and structural variants (1001 Genomes Consortium, 2016). Accessions from the previous screen for which genomic variant data is available were included (49), and an additional 39 accessions were chosen to represent the genetic diversity in the 1001 Genomes accession set. In order to compare results across replicates while minimizing batch effects, Columbia (Col-0) was included in each experimental set as a reference accession. For each plate including treatment and control plates, percent growth was calculated from the pixel value obtained from *PIDIQ* at 1 and 11 dpi. Percent growth calculated from *M. phaseolina* treatment plates was divided by the average percent growth calculated from control plates. We call this value the growth inhibition index (GII). A GII of 1 indicates no growth inhibition caused by *M. phaseolina* treatment, while lower values indicate more severe growth inhibition. The mean GII calculated for replicate treatment plates for each genotype was calculated and divided by the mean GII of Col-0 calculated from the same experiment. The resulting value is referred to as “relative GII”. In cases where the necrosis is so severe that the pixel value of live tissue at 11 dpi is below that of 1 dpi, the GII and the corresponding value relative to Col-0 is negative.

Significant variation (ANOVA; $p<.001$) in GII relative to Col-0 at 11 dpi in response to *M. phaseolina* was seen among the 87 *A. thaliana* accessions tested (Figure 1.4). The average GII of Col-0 calculated across all biological replicates was 0.32. Of the 87 tested accessions, 40 showed more severe growth inhibition

(had a relative GII less than one), while 48 showed less severe growth inhibition (had a relative GII greater than one) relative to Col-0. Between the accession with the highest average relative GII (TuesB30-3) and the accession with the lowest relative GII (Mitterberg-1-181), statistically significant differences could be seen across biological replicates (student's t-test; p=.003). Accessions which exhibit consistently different GII in comparison to Col-0 were identified in this analysis; for example, Ak-1 and UKSE06-252 showed significantly lower average GII values in comparison to Col-0 (student's t-test; p=8.78E-03, p=3.10E-2, respectively). Relative GII values for Ak-1 and UKSE06-252 were significantly lower in comparison to relatively resistant accessions such as Copac-1 and Tuewa1-2 (student's t-test; Copac-1 and UKSE06-252, Copac-1 and Ak-1, Tuewa1-2 and UKSE06-252, Tuewa1-2 and Ak-1, all had p<0.001). These accessions were selected for transcriptomic analysis, described in Chapter II.

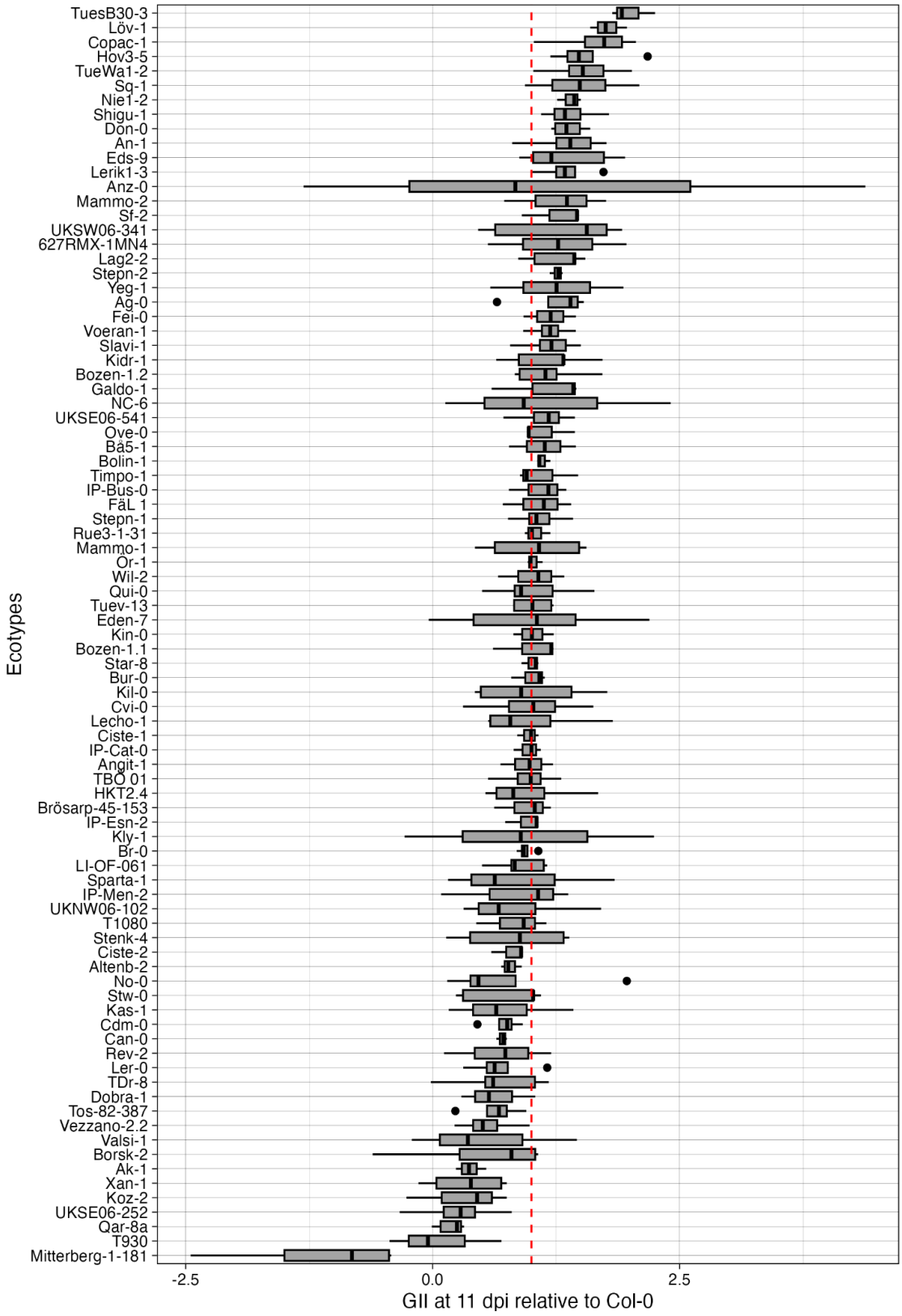
TuesB30-3
Löv-1
Copac-1
Hov3-5
TueWa1-2
Sq-1
Nie1-2
Shigu-1
Dön-0
An-1
Eds-9
Lerik1-3
Anz-0
Mammo-2
Sf-2
UKSW06-341
627RMX-1MN4
Lag2-2
Stepn-2
Yeg-1
Ag-0
Fei-0
Voeran-1
Slavi-1
Kidr-1
Bozen-1.2
Galdo-1
NC-6
UKSE06-541
Ove-0
Bä5-1
Bolin-1
Timpo-1
IP-Bus-0
FäL 1
Stepn-1
Rue3-1-31
Mammo-1
Or-1
Wil-2
Qui-0
Tuev-13
Eden-7
Kin-0
Bozen-1.1
Star-8
Bur-0
Kil-0
Cvi-0
Lecho-1
Ciste-1
IP-Cat-0
Angit-1
TBO 01
HKT2.4
Brösarp-45-153
IP-Esn-2
Kly-1
Br-0
LI-OF-061
Sparta-1
IP-Men-2
UKNW06-102
T1080
Stenk-4
Ciste-2
Altenb-2
No-0
Stw-0
Kas-1
Cdm-0
Can-0
Rev-2
Ler-0
TDr-8
Dobra-1
Tos-82-387
Vezzano-2.2
Valsi-1
Borsk-2
Ak-1
Xan-1
Koz-2
UKSE06-252
Qar-8a
T930
Mitterberg-1-181
Ecotypes
-2.5
0.0
2.5
GII at 11 dpi relative to Col-0

Figure 1.4. *A. thaliana* natural accessions exhibit a wide range in growth inhibition in response to *M. phaseolina* infection. A box and whiskers plot of growth inhibition index (GII) relative to Col-0 at 11 dpi for 87 *A. thaliana* accessions relative to Col-0. For each accession, 3 - 7 biological replicates are used for analysis. Gray boxes represent the range between the first and third quartile, and notches indicate the median value. Whiskers extend 1.5 times the interquartile range (IQR), and individual points represent outliers (Mcgill *et al*., 1978). Dashed line represents a GII of 1, or the GII value of Col-0. Each data point represents the mean GII is divided by the average GII of Col-0 for each experimental replicate. An one-way analysis of variance (ANOVA) was conducted using accessions as the factor, which showed that there are significant effects on relative GII due to the host genotypes selected in this assay (p<.001).

In order to determine whether growth inhibition is an appropriate phenotype to use as a measure of *A. thaliana* susceptibility against *M. phaseolina*, disease scores and GII data for 49 overlapping accessions were plotted against each other (Figure 1.5). There is a significant (p=3.2E-2) negative correlation between disease scoring and GII, which is expected as stronger inhibition of growth (or necrosis of tissue) is scored higher in the disease scoring scale (Figure 1.1). This indicates that GII would be an appropriate indicator for *M. phaseolina* resistance, but the presence of outliers also suggest that other observable phenotypes are subjectively described in the disease-scoring method. It is therefore important that individual phenotypes are parsed and assessed individually when studying *A. thaliana* response to *M. phaseolina*.

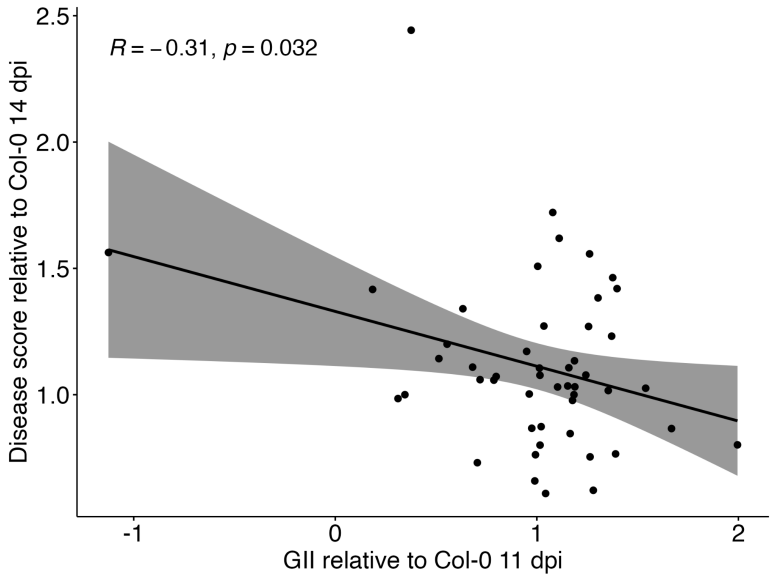

Figure 1.5. GII shows a significant negative correlation with comprehensive disease scoring. Forty-nine accessions scored using both the comprehensive scoring method and digital image analysis were compared. The x-axis represents the GII relative to Col-0, and the y-axis represents the average disease score (0-5) relative to Col-0. Pearson correlation between the two methods of susceptibility phenotyping examined for statistical significance. There is a significant (p=3.2E-2) negative relationship between disease scoring and GII, as expected. The solid line shows the regression line and 95% confidence intervals are represented by gray areas on the plot.

Genetic variance component of GII includes equal contribution of additive and epistatic effects

We sought to gain further understanding of the resistance phenotype as a heritable trait by crossing a relatively resistant accession with a relatively susceptible accession and observing phenotypes of F_2 progenies. Lerik1-3, which exhibits a higher average GII (1.35, SE=0.14) in comparison to Col-0 was chosen as the resistant representative accession, and Col-5 was chosen as the susceptible representative accession (Figure 1.4). At the time when the crosses were performed, Lerik1-3 was among the top resistant accessions, which exhibited

stable GII phenotypes across biological replicates. Col-5 is the glabrous derivative of Col-0 commonly used to generate recombinant inbred lines in *A. thaliana*. F_2 offspring from this cross were challenged with *M. phaseolina* to observe the rate of growth (percent change in pixel rosette size) calculated from 1 dpi to 11 dpi. Parent lines Col-5 and Lerik1-3 were also tested, and Lerik1-3 showed a significantly higher growth rate (p=2.72E-02) (Figure 1.6). The average percent growth of Col-5 seedlings treated with *M. phaseolina* was 153%, while that of Lerik1-3 was 267%. For simplicity, the threshold for Col-5-like or susceptible seedlings was set to the third quadrant of the Col-5 growth rate distribution, which was 179%, and the threshold for Lerik1-3-like or resistant seedlings was set to the first quadrant of the Lerik1-3 growth rate distribution, which was 232%. Any seedling which falls between 179% and 232% would not be considered; however no F_2 seedling out of the 72 examined had a growth rate that applied to this criteria. Out of the 72 seedlings examined, seven had a growth rate of -100%, meaning that they exhibited complete necrosis. Twenty-one out of 72 seedlings, including those that had completely necrosed, were considered “susceptible”, while 51 were considered “resistant”. The chi-squared goodness of fit test could not reject the null hypothesis, which assumes a 3:1 Mendelian ratio, or a monohybrid cross (χ^2 = 0.414; p = 5.20E-01).

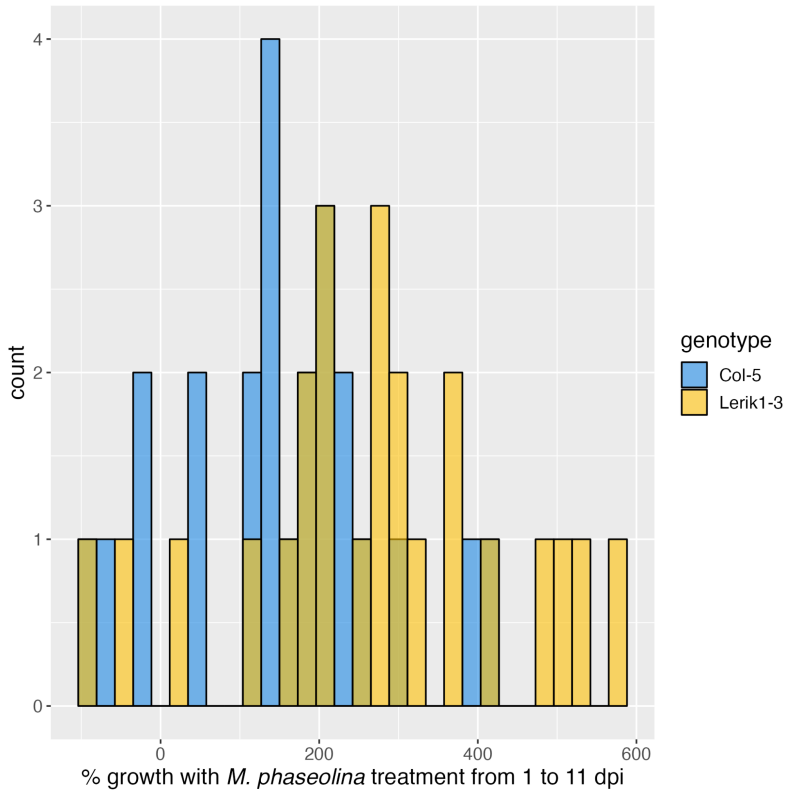

Figure 1.6. Parental accession Lerik1-3 shows significantly higher growth rates when challenged with *M. phaseolina* in comparison to parental accession Col-5. Histogram of growth rates, calculated by taking the difference in rosette size (pixel values) between 1 dpi and 11 dpi and dividing by the value at 1 dpi. A student's t-test was performed to show a significant difference between Col-5 (blue) and Lerik1-3 (yellow) seedlings (n=24; *p*=2.72E-02).

Despite these results, the normal distribution of GII values across accessions and analyses on F_2 progeny of crosses indicate that the chances of finding large effect loci contributing to resistance in *A. thaliana* are low. To evaluate the contribution of genetic variance to the GII phenotype, broad-sense heritability (H^2) was estimated with ANOVA (0.595; p = 3.108E-8) using data from all biological replicates and genotype as the fixed effect (Singh *et al*., 1993). A hierarchical generalized linear model was applied using the R package *hglm* (Rönnegård *et al*., 2010) to further investigate the effects of additive and epistatic

genetic components to variance. The combined fraction of additive and epistatic genetic effects was 0.673, which slightly exceeds the estimation of broad-sense heritability derived from ANOVA. This could be attributed to the use of average response variables, correction for kinship, and the simultaneous estimation of additive and epistatic variance components. Nevertheless, the effect of dominance on genetic variability seems to be quite small and thus will not be considered in future analysis. SNP-based kinship matrix for all accessions in the 1001 Genomes Project, adapted from (https://github.com/arthurkorte/GWAS) was used to estimate genetic variance components (*G*) (Togninalli *et al*., 2018). We borrow methods from Lachoweic *et al.* (2015) and fit a linear mixed model using polygenic additive (*a*) and epistatic (*b*) variance as random effects, as defined below,

$$y = \mu + a + b + e$$

$$a \sim N(0, G\sigma_a^2)$$

$$b \sim N(0, G \circ G\sigma_b^2)$$

where *G* is a genomic kinship matrix and *e* represents the residual. The contribution of the additive genetic effect alone, as described by the narrow-sense heritability (h^2), was 0.336 (p=2.81E-3), roughly half of the total genetic contribution. These results suggest that within the population represented in this screen, a significant portion of the phenotypic variance can be explained by genetic variability, and that the additive and additive-by-additive epistatic effects have nearly equal contributions to the GII phenotype. The selection of genotypes represented in this analysis is likely to contain allelic variations, which could

contribute to a significant effect on resistance, in terms of GII. However, it appears that at least some of these loci could be involved in epistasis, which cannot be fully dissected with the limited sample number in our study. Estimates of heritability and the contribution of variance components are summarized in Table 1.1.

Table 1.1. Estimates of heritability and genetic variance components based on the GII relative to Col-0 in a population of 87 *A. thaliana* natural accessions

Method	H^2	h^2	$V_{additive}$	$V_{epistatic}$
ANOVA	0.595[a] (p<0.001)	-	-	-
hglm	0.539[a] (p<0.001)	-	-	-
	0.673	0.336[b] (p=2.81E-3)	5.46E-2	5.46E-2

[a]H^2 was estimated using replicate measurements of GII relative to Col-0 and using genotype as the fixed effect.
[b]h^2 was estimated by factoring in additive and epistatic effects as random effects (see *Materials and methods*) and using the kinship matrix adapted from Togninalli *et al*. (Togninalli *et al*., 2018).

Genome-wide association identified putative resistance loci

Using the relative GII values measured for 87 accessions, GWAS was performed using the web-based platform easyGWAS (Grimm *et al*., 2017). Significance of associations between the observed phenotype and 1,799,622 SNPs, filtered for a minor-allele frequency >0.05 was tested implementing the efficient mixed-model association expedited (EMMAX) approach, which accounts for population structure (Kang *et al*., 2010). Genotype data are derived from multiple sources including the 1001 Genomes Project database (1001 Genomes Consortium, 2016). By default, easyGWAS corrects for multiple testing with the Bonferroni correction at a significance level α=0.05. No SNP was significant at

the genome-wide level (p<5.0E-8). The SNP with the lowest p-value throughout the genome resides in chromosome 4, but this locus is not within a genic region. Multiple significant SNPs were identified with p values under the suggestive significance threshold (p<1.0E-5), which assumes one false positive association per GWAS (Figure 1.7A) (Duggal *et al*., 2008). Although no strongly pronounced peaks were seen, several gene regions with concentrations of significant SNPs could be seen (Figure 1.7C). Top SNPs with significant p values, along with the predicted outcomes of the variants, are summarized in Table 1.2.

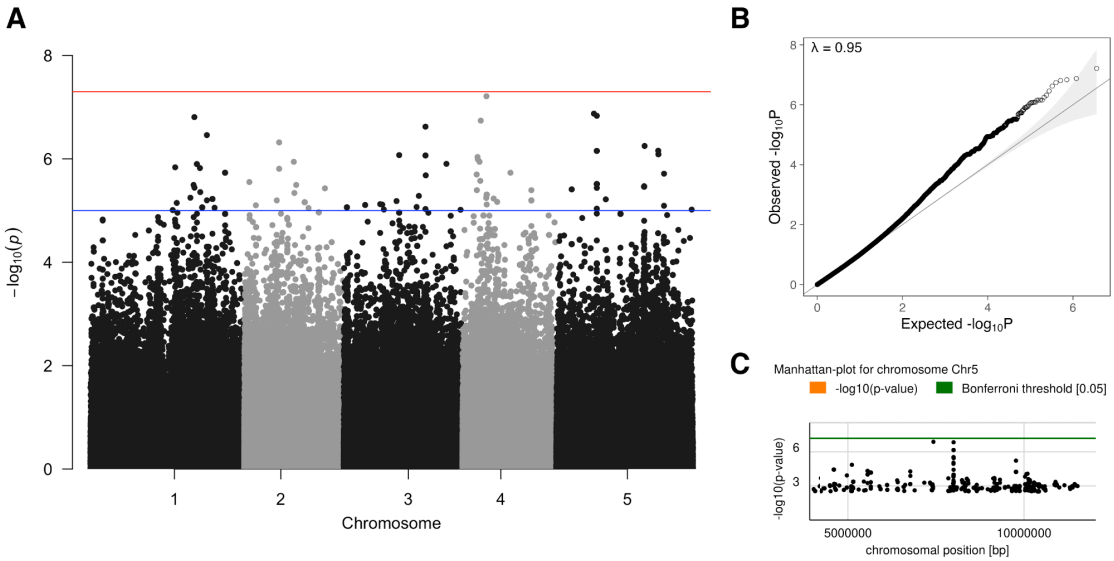

Figure 1.7. Manhattan plot of GWAS results showing statistically significant associations between GII relative to Col-0 phenotype and SNP loci. (A) Distribution of - $\log_{10}$ *p* values (Bonferroni correction, 0.05) across five *A. thaliana* chromosomes are depicted by individual data points. Minor alleles with frequency under 0.05 were filtered out, bringing the analyzed SNP count from 6,973,565 to 1,799,622. Genome-wide significance line (red) shows *p*=5E-8, and suggestive significance line (blue) shows *p*=1E-5. **(B)** Quantile-quantile (QQ) plot displaying observed *p* values vs. expected *p* values (- $\log_{10}$ *p*). The genomic inflation factor (***λ***) was 0.95, which is suggestive of some inflation of *p* values due to population structure. The straight line represents the distribution of *p* values under the null hypothesis. The gray area represents the 95% confidence interval. **(C)** Close up plot around region in chromosome 5 showing a loci of stacked SNPs with low *p* values at approximately 8,000,000 bps. The green horizontal line represents the genome-wide significance threshold with Bonferroni correction applied to correct for multiple testing at α=0.05.

Table 1.2. Top 20 SNPs with lowest *p* values from GWAS

Chr.	Position	*p*[a]	Allele frequency[b] Minor	Allele frequency[b] Major	Gene associated with SNP[c]	Most severe variant consequence	Gene name	Description
4	4758852	6.1E-08 *	5/T	82/C	-			
5	7427812	1.3E-07 *	5/T	82/A	**AT5G22410**	3 Prime UTR	RHS18	root hair specific 18
5	7999963	1.5E-07 *	22/T	65/A	**AT5G23720**	Intron	PHS1	tyrosine phosphatase Propyzamide-Hypersensitive 1 (PHS1).
1	20548277	1.6E-07 *	5/T	82/C	AT1G55060	Upstream	UBQ12	Ubiquitin-like gene, believed to be a pseudogene.
4	3627907	1.8E-07 *	5/G	82/C	-			
3	16149606	2.4E-07 *	6/C	81/A	AT3G44560	Upstream	FAR8	fatty acid reductase 8
1	23015707	3.5E-07 *	5/G	82/T	AT1G62290	Upstream	PASPA2	Saposin-like aspartyl protease family protein
2	6897531	4.8E-07 *	5/G	82/A	AT2G15830	Downstream		hypothetical protein
5	17452529	5.6E-07 *	5/C	82/T	**AT5G43420**	Missense	ATL16	RING/U-box superfamily protein
5	20146513	6.9E-07 *	7/C	80/G	AT5G49630	5 Prime UTR	AAP6	high affinity amino acid transporter.
5	7999880	7.0E-07 *	13/T	74/A	**AT5G23720**	Intron	PHS1	tyrosine phosphatase Propyzamide-Hypersensitive 1 (PHS1).
5	7999753	7.0E-07 *	13/A	74/G	**AT5G23720**	Intron	PHS1	tyrosine phosphatase Propyzamide-Hypersensitive 1 (PHS1).
5	8000013	7.0E-07 *	13/T	74/C	**AT5G23720**	Intron	PHS1	tyrosine phosphatase Propyzamide-Hypersensitive 1 (PHS1).
5	20189964	8.2E-07 *	6/A	81/G	**AT5G49690**	3 Prime UTR	UGT91C1	UDP-glycosyltransferase.
5	20175089	8.2E-07 *	6/G	81/T	AT5G49680	Upstream		similar to Arabidopsis SABRE.
3	10950622	8.5E-07 *	6/T	81/C	AT3G05605	Downstream		None
3	16150225	8.6E-07 *	6/A	81/T	AT3G44560	Upstream	FAR8	fatty acid reductase 8
3	16150220	8.6E-07 *	6/G	81/T	AT3G44560	Upstream	FAR8	fatty acid reductase 8
4	2988133	9.2E-07 *	5/T	82/C	AT4G05620	Upstream		Galactose oxidase/kelch repeat superfamily protein
4	3063920	1.0E-06 *	7/T	80/C	AT4G06479	Upstream		nucleic acid binding / zinc ion binding protein

[a]*p* values <1.0E-5 are indicated with *.
[b]Number of genotypes with allele/nucleotide of allele
[c]Polymorphisms within coding regions are shown in bold

The third most significant SNP resides in chromosome 5 at position 7,999,963, which is within the sixth intronic region of a gene encoding a tyrosine phosphatase *PROPYZAMIDE-HYPERSENSITIVE 1* (*PHS1*; AT5G23720). A cluster of SNPs associated with resistance is found around this region, forming a defined peak. Within the 50 kb window surrounding this SNP, 17 SNPs with *p* values indicating association (p<1.0E-5) were found. This region also encodes 33 genes, including genes associated with Gene Ontology (GO) terms "response to abiotic stimulus" (*DUF821*; AT5G23850), "defense response" (*MD2-RELATED LIPID RECOGNITION 3*; AT5G23820), and "cell death" (*LONG CHAIN BASE 2*; AT5G23670).

Discussion

Through systematic screening for *M. phaseolina* response phenotypes, we found that a wide range of disease symptom severity could be observed within *A. thaliana* natural accessions. In order to narrow down the genetic factors that contribute to *M. phaseolina* resistance or susceptibility, a quantitative and unbiased phenotyping method was assessed, based on a plate-based assay previously developed by the Eulgem lab (Schroeder *et al*., 2019). Statistically significant correlation between growth inhibition and overall disease progress was found, and therefore the growth inhibition index (GII) was used in subsequent experiments as a measurable phenotype representing *M. phaseolina* resistance.

We were then able to apply statistics to infer heritability of the trait and parse different genetic components, which may be contributing to the phenotype. The plate-based infection assay controls for environmental factors leading to phenotypic variability, and therefore allows us to focus our analysis on the genetic components affecting the phenotype within *A. thaliana* variance. These estimates also rely on the large-scale genomic data and knowledge on relatedness between tested accessions of *A. thaliana.* Additionally, phenotypic data obtained from these screens were used to search for significant associations with genomic SNP data. The GWAS identified several loci where clusters of SNPs with suggestive significance form peaks, which potentially have high linkage to causal resistance alleles. Ultimately, we aim to demonstrate the utility of this pathosystem to isolate and study purely genetic contributions to variations in *M. phaseolina* resistance.

Quantitative assessment of *M. phaseolina* responses in *A. thaliana*

A. thaliana natural variability has been exploited for decades in plant genetics and these studies have led to discoveries of causal loci for numerous phenotypes (Alonso-Blanco & Koornneef, 2000). This has also been shown in *A. thaliana* interactions with pathogens that cause disease phenotypes of quantitative nature, such as *Fusarium oxysporum* and *Botrytis cinerea (Denby et al., 2004; Diener & Ausubel, 2005)*. The lack of genotypes exhibiting complete resistance in previously studied crop hosts suggests that variations in *M. phaseolina* responses are caused by complex genetic interactions between QTL and the environment. Resistance gene (*R*-gene) expression has been shown to be mediated by, in part,

environmental cues as a part of the fitness-immunity balance maintained by the host plant (MacQueen & Bergelson, 2016). Several studies searching for *M. phaseolina*-resistance in crop hosts have been published, but conflicting results and variability in assay conditions have made cross-comparisons difficult (Coser *et al*., 2017; da Silva, Klepadlo, *et al*., 2019; da Silva, Zaccaron, *et al*., 2019). In our analysis, we verified whether the natural allelic variation in the model plant *A. thaliana* displays variability in disease phenotypes similarly to crop hosts, while reducing the environmental factors that can contribute to phenotypic variability.

M. phaseolina infection is commonly diagnosed by plant collapse and presence of necrotic lesions or black specs in vascular tissue, which is characteristic of charcoal rot. Evaluations of crop genotype susceptibility in field conditions are often costly and require an extensive time frame. The assay used in our analysis with *A. thaliana* has the advantage of a rapid data turnover, made possible by the use of 10- to 14-day old seedlings. However due to the young stage in which assays are conducted, typical metrics of susceptibility (size of necrotic lesions, yield loss, etc.) are impractical for the plate assay. Since disease scoring is the first step of genetic analysis, whether it be QTL mapping or GWAS, it is critical that non-genetic factors, which may cause noise in the data, are reduced. *M. phaseolina* causes various disease symptoms on young *A. thaliana* seedlings, including chlorosis, necrosis, growth inhibition, and early flowering for some accessions that survive for a longer period. Some of these symptoms were unsuitable for quantifying responses against *M. phaseolina*. For example,

chlorosis, which can also be quantified using PIDIQ, was not considered for our analysis due to the fact that it is only observed for a short period of time before leaf tissue becomes necrotic. Highly susceptible seedlings succumb to the virulent hyphae before chlorosis phenotypes appear. The limited number of time points from which data was collected could not accurately capture the rate of seedling chlorosis.

Inhibition of growth, or more precisely the area of live tissue quantified by image analysis, is a suitable parameter for simultaneously capturing both the reduction of growth rates and emergence of necrosis in this regard. GII represents the average percent growth of *M. phaseolina* treatment seedlings from 1 to 11 dpi, divided by the average percent growth of control or non-*M. phaseolina* treated seedlings. This method allows us to control accession-based native variations in growth rates. Comprehensive disease scores and GII, both of which are normalized with Col-0 as the internal control, were significantly correlated (p=3.2E-2), which indicates that the latter phenotyping method is a good alternative to the former, more subjective disease-scoring method. Additionally, since *M. phaseolina* susceptibility is often associated with drought stress, we investigated whether resistance to *M. phaseolina* is partially a consequence of adaptations to certain climate zones. Thus we examined if there was a correlation between the latitude or longitude of the geographical origin for each accession and GII; however no significant association was found (latitude: R=0.05, p=0.65, longitude: R=-7.8E-2, p=0.47. Data acquired from https://1001genomes.org/accessions.html).

Implications of heritability estimates

To the best of our knowledge, work described here are the first attempts to extensively characterize *A. thaliana* responses to *M. phaseolina* in multiple genomic backgrounds. While in previously studied crop pathosystems susceptibility to *M. phaseolina* has been shown to be a quantitative trait, there is also evidence to suggest that certain isolates of *M. phaseolina* are adapted to infecting specific host species (Koike *et al*., 2016). Through our *A. thaliana* accession screen, several highly resistant accessions were identified, which exhibited higher rates of survival at 14 dpi compared to more susceptible accessions. Regardless of the average relative disease score or relative GII value obtained across plates from multiple replicates, there were variations in seedling resistance in each genotype, which varied from minimal apparent disease symptoms to complete necrosis. Surviving seedlings were observed as late as 21 dpi, at which point, it was decided that the natural decline in nutrient and water content, as well as the capacity in the plate was no longer suitable for seedling growth. It is therefore possible that these individuals were completely resistant to *M. phaseolina*, and if so, the observed resistance could be due to spontaneous mutations, or other external factors.

To examine whether any characteristics of monogenic Mendelian inheritance patterns can be observed, a cross between the relatively resistant Lerik1-3 and Col-5, a derivative of the susceptible reference accession Col-0, were generated. Col-5 has a recessive polymorphism in the *GLABRA1* (*GL1*) gene,

which makes it impaired in trichome development, and thus useful for selecting successful crosses. Mean percent growth between parent genotypes were significant (Figure 1.6. *p*=2.72E-02), but large overlap was seen between the two histograms which plotted parental distributions of percent growth. Thresholds of percent growth values were determined based on the interquartile range of each parent phenotype distribution. Individuals which were Col-5-like were considered to be “susceptible”, while individuals which were Lerik1-3-like were considered to be “resistant”. To our surprise, the F_2 individuals, which were examined exhibited a simple 3:1 phenotypic ratio, with the majority being “resistant”. Our results obtained from the Col-5 × Lerik1-3 cross imply that the resistance trait is a monogenic trait segregating according to Mendelian ratios. However, this contradicts previous observations in other crop species, as well as the heritability estimation conducted based on the accession screen with 87 accessions. *M. phaseolina* resistance has been previously described as a highly complex, quantitative trait controlled by many genes. The phenotypic gradient seen in parental genotypes, which are inbred homozygous lines, would not be seen if this is truly a monogenic trait. The 3:1 ratio observed in the F_2 population may be a false positive outcome that could potentially be attributed to low sample size and phenotypic classification thresholds that were too permissive.

We sought to parse additive and epistatic components contributing to genetic variability, to further gain insight to the genetic architecture of *M. phaseolina* resistance. Statistical analysis on the 87−accession screen data

estimated that roughly half of the genetic variance was due to epistatic interactions, which indicates that the underlying cause of phenotypic variability is much more complex than expected. Dominant genetic effects were not considered in this analysis because all genotypes tested in this analysis are inbred homozygous lines of naturally occurring ecotypes. The estimated broad-sense heritability ranged from 0.539 to 0.673, depending on the method of calculation and the inclusion of a kinship matrix. This implies that there are additional sources of variance contributing to the phenotypic variance, which is unexplained. Despite our best efforts to minimize effects from environmental variability, small fluctuations in experimental conditions and interactions between genes and the environment could have impacted the phenotypic outcomes.

Association analysis

Using GII (relative to Col-0) data from the 87 *A. thaliana* accessions tested against *M. phaseolina,* a GWAS was conducted using the online platform easyGWAS (Grimm *et al*., 2017). The most significant SNP (p=6.14E-08), which lies at 4,758,852 bp near the centromeric region on chromosome 4, is more than 20 kb downstream from the nearest protein-coding gene. It lies in the approximately 1 kb region between two transposable elements (TE), AT4G07936 and AT4G07937. TE can affect nearby gene regulation by altering the local methylation state and chromatin structure, or by generating trans-acting small RNAs (Le *et al*., 2014; McCue & Slotkin, 2012). Biotic stress can also drive methylation of TE and alter their expression (Dowen *et al*., 2012). Whether or not

polymorphisms in this region have an effect on the expression of AT4G07936 or AT4G07937, and if these TE are expressed during *M. phaseolina* infection, remains to be answered. However, considering the location of this SNP and that no other significant SNPs were found in this region, it seems unlikely that this locus has a significant influence on *M. phaseolina* resistance.

The second most significant SNP (*p*=1.33E-07) is found within the 3'-untranslated region (3'-UTR) of the protein-encoding gene *ROOT HAIR SPECIFIC 18* (AT5G22410; *RHS 18*). Polymorphisms in this region may induce post-transcriptional events that alter expression of the gene. *RHS18* works downstream of important root hair development genes, and has associations to gene ontology (GO) terms "response to oxidative stress", and "response to stress" (Bruex *et al*., 2012). Although there are no other significant SNPs nearby, it is relatively close to the cluster of SNPs which resemble a peak, at approximately 8,000,000 bp on chromosome 5. The strongest association observed at this peak (*p*=1.47E-07) is found in the intron region of the gene encoding *PROPYZAMIDE-HYPERSENSITIVE 1* (AT5G23720; *PHS1*), at 7,999,963 bp. This ABA-responsive tyrosine phosphatase is involved in microtubule organization, stomatal opening, and flowering (Tang *et al*., 2016). *PHS1* is known to interact with mitogen-activated protein kinases (MAPKs) *MPK12* and *MPK18* and induces rapid microtubule depolymerization in response to osmotic stress (Fujita *et al*., 2013; Walia *et al*., 2009). Cortical microtubule reorganization determines the cell shape, defines the

cell division planes, and has important roles in root hair development (Bibikova *et al*., 1999).

There are multiple examples of genes that are involved in both pathogen defense and root hair development, such as *BOTRYTIS-INDUCED KINASE1* (*BIK1*) and *OXIDATIVE SIGNAL-INDUCIBLE1* (*OXI1*), both of which processes involve the production of reactive oxygen species (ROS) (Rentel *et al*., 2004; Veronese *et al*., 2006). Root hair development is also regulated by the jasmonic acid (JA) response pathway via *ETHYLENE INSENSITIVE 3* (*EIN3*) and *EIN3-LIKE 1* (*EIL1*) (Zhu *et al*., 2011). Although in our analysis, we could not observe differences in root hair development during *M. phaseolina* infection due to high density of microsclerotia and hyphae, it is likely that phytohormone responses and production of ROS due to infection have an impact on root cell differentiation and growth. Whether genotype-specific differences in root hair development conversely affects defense remains to be answered. Three additional SNPs with *p*-values of suggestive significance are found close by within the same intron of *RHS18*. The SNPs at 7,427,812 and 4,758,852 bp in chromosome 5 have a high linkage disequilibrium (r^2=0.74), and therefore are likely to be linked to the same causal loci.

Another region where a suspected peak can be seen in chromosome 3, where the most significant SNP has a *p*-value of 2.38E-07. This SNP is 1,517 bp upstream of the gene encoding *FATTY ACID REDUCTASE 8* (AT3G44560; *FAR8*). Genes of the same fatty acid reductase family, *FAR4* and *FAR5*, are also found

within 30 kb upstream of *FAR8*. These enzymes are involved in the reduction of fatty acyl-coenzyme A to primary alcohols. Cuticular wax, which coats the surface of aerial tissue to protect the plant from biotic and abiotic stresses, is mainly composed of long-chain fatty acids (Kunst & Samuels, 2003). During biotic interactions, monomers of cuticle components can act as molecular signals for both the host itself and invading pathogens. While cuticle breakdown products can act as defense-signaling elicitors in host plants, they can also be an indicator for pathogens to prepare cutinolytic enzymes for host cell penetration (Serrano *et al*., 2014). Two additional SNPs are found nearby in the same upstream region of *FAR8*.

It is important to keep in mind that according to the narrow-sense heritability estimates, approximately half of the genetic contributions to phenotypic variance are predicted to be derived from epistatic interactions, which is not considered in this GWAS analysis. This is likely a large source of the missing heritability, a term which refers to the lack of contributions from SNP associations identified through GWAS. Contributions to the genetic variance caused by epistatic interactions between two loci may not have been captured in this analysis, due to low statistical power. Genes identified in the GWAS discussed above showed no significant differential expression in response to *M. phaseolina.* Significant variability in basal expression was also not observed between the tested accessions (Ak-1, UKSE06-252, Copac-1, Tuewa1-2, and Col-0; Chapter II). Additional bioinformatics analysis would be necessary to identify epistatic interactions between SNPs.

Several association studies on crops have been published previously, including soybean (Coser *et al*., 2017), sorghum (Adeyanju *et al*., 2015), and strawberry (Nelson *et al*., 2021). Coser *et al.* (2017) previously conducted two association studies based on phenotypic data obtained from greenhouse and field conditions in soybean. In each analysis, multiple loci with suggestive significance were identified, and within these loci several defense-related *A. thaliana* orthologs of genes associated with suggestive polymorphisms were reported (Coser *et al*., 2017). Although none of the genes identified by Coser *et al.* (2017) were found in our analysis, *TERPENE SYNTHASE 21* (*TPS21*; AT5G23960), the *A. thaliana* ortholog of *Glyma.12g216200*, is just downstream of the peak in chromosome 5 where *PHS1* was identified. Significant SNP regions containing genes with defense-related annotations were also identified in sorghum and strawberry, but it is not certain whether these candidate genes are orthologous to each other, or if similar biological processes are involved.

In this investigation, we identified several loci in the *A. thaliana* genome with significant associations to the GII phenotype. Estimation of narrow-sense heritability indicated that additive genetic variance could explain at least a third of the phenotypic variability, and thus we attempted to identify the genetic factors that make up this quantitative trait. Genes directly encompassing, or within a 50 kb range upstream or downstream of, these SNPs were not necessarily explicitly associated with fungal defense but were involved in biological processes that could potentially play a role in biotic stress response and immunity. GWA is a test of

genomic association between loci and a trait, so causal factors may lie outside of a gene region. Moreover, since significant epistatic interactions are also expected, polymorphisms in these identified regions could be interacting with other genes contributing to *M. phaseolina* response phenotypes. In which case, *p*-values estimated in this study may be underestimated.

Our findings in *A. thaliana* are consistent with what has been described in other host species, in that *M. phaseolina* resistance is a complex trait involving interactions between multiple genes. It should also be noted that while our aim was to reduce the effect of variables which are not genetic, the results obtained in this analysis only describe interactions between *A. thaliana* and the specific isolate that was available to us. *M. phaseolina* is also known to exhibit great genomic variability among isolates, which is likely to influence its virulence. Candidate genes should be strategically tested against a diverse panel of pathogen isolates to evaluate the modularity of defense loci identified in model species like *A. thaliana*.

Materials and methods

Plant materials and growth conditions

A total of 136 *A. thaliana* accessions obtained from Arabidopsis Biological Resource Centre (ABRC) were used to screen for *M. phaseolina* resistance phenotypes. Accessions were chosen with the help of Dr. Christopher Fiscus, a former graduate student from Dr. Daniel Koenig's lab. Seeds were surface

sterilized by soaking five minutes in 70% ethanol, then 10 minutes in 6% sodium hypochlorite, and rinsing five times with sterile water. Seeds (30 to 40 per plate) were sown on 100 x 15 mm polystyrene plates with solid 0.87% agar media containing ½ MS (Murashige and Skoog), 0.25% sucrose and 0.05% 2-(N-morpholino)ethanesulfonic acid (MES). pH was adjusted to 5.7 with potassium hydroxide. Plates were wrapped in micropore tape and stratified in the dark at 4°C for four days. Next, plates were transferred to a growth room set to long-day conditions (16 h of light/8 h of dark, 23°C, 100 μE m^{-2} s^{-1}) and kept for 10 days. Plates were set up vertically so that roots would grow in a uniform direction towards one side of the plate.

A cross between *A. thaliana* accessions Col-5 × Lerik1-3 was generated to assess phenotypic variance in F_2 progeny. Emasculated Col-5 flowers were pollinated with Lerik1-3 flowers. Resulting seeds from crossed siliques (F_1) were assessed for presence of trichomes indicating successful cross pollination. Three F_1 plants with trichomes were grown to maturity and F_2 seeds were collected for phenotyping.

M. phaseolina-response screen

Dried disks of *M. phaseolina* culture on potato dextrose agar (PDA) were used as inoculum. The original propagule was isolated by Phil Roberts at the UC Riverside Agricultural Operations Field 11 (Muchero *et al*., 2011), and propagated by members of the lab of Thomas Eulgem. Disks were cut into small pieces and

placed in sterilized mortar and pestle with a small amount of sterile water. *M. phaseolina* is ground until a black slurry forms. This was then placed in ½ MS agar media that has been autoclaved to a liquid state and cooled. The concentration of distinguishable microsclerotium was adjusted to approximately 70 per 100 µL under the microscope. Three mL of inoculum ½ MS media mix was pipetted onto one side of solid agar ½ MS media so that the inoculum solidifies on one side of the square plate. Once the *M. phaseolina* containing media solidified, an additional 3 mL was pipetted onto the opposing side of the plate and solidified in a tilted orientation. Plates were wrapped in micropore tape and aluminum foil to limit UV exposure. *M. phaseolina* culture plates were placed in an incubator at 34°C for four days, then transferred to the growth room for two days. After ten days of growth under light for *A. thaliana* and six days after transfer of *M. phaseolina* to ½ MS media, seedlings were transferred to each *M. phaseolina* plate with actively growing hyphae.

Disease symptom scoring

For the first screen with 97 accessions, 10 seedlings were placed on each plate for both control- and *M. phaseolina*-treatment plates. For each biological replicate, one control plate and two *M. phaseolina* plates were prepared for each accession. Columbia (Col-0, CS1092) is included in each batch as an internal control to mitigate effects on the phenotype by batch effects. At 1 dpi and 11 dpi, each seedling was compared to those on the control plates, and given a score from 0-5 based on the following criteria: 0 - seedlings which appear to have no

symptoms; 1 - seedlings show stunted growth in comparison to those on control plates but no chlorosis or necrosis is observed; 2 - stunted growth is observed and up to 25% of the seedling is chlorotic or necrotic; 3 - stunted growth is observed and up to 50% of the seedling is chlorotic or necrotic; 4 - stunted growth is observed and up to 75% of the seedling is chlorotic or necrotic; and 5 - seedlings are completely necrosed (Figure 1.1). Photos of individual plates were also taken at each time point to keep as a record. A portion of the data was collected and scored by Ayat Riahi, a fellow Ph.D. candidate in the Eulgem lab.

Plant immunity and disease image-based quantification (PIDIQ)

In the second screen with 88 accessions, six seedlings were transferred to each *M. phaseolina* plate, and only three seedlings were placed on each control plate to ensure that leaves from neighboring plants did not overlap with each other. Seedlings on *M. phaseolina* are typically severely stunted in growth, which allows for more seedlings to be placed per plate. In total, for every batch of experiments, at least two control plates and four treatment plates were prepared per accession. Up to nine accessions were tested for each experimental batch, and the number of technical replicates conducted for each accession ranged from three to seven. Columbia (Col-0, CS1092) was included as an internal control.

All photographs used for analysis were taken on a Canon EOS Rebel T6i with a EF-S 18-55mm f/3.5-5.6 IS II lens, on the manual setting with fixed exposure settings (ISO 6400, 1/30, F5.6) and light conditions to ensure consistency across data collection. The position of the camera and the plates, placed on top of a black

backdrop, were also fixed so that a pixel represents roughly the same area of the subject. Control plates and treatment plates were imaged with the lid off. Images were collected at 1 dpi and 11 dpi. 1 dpi was preferred over 0 dpi as the initial data point since freshly transferred seedlings were often tilted, which would not allow the camera to capture the full seedling area.

Images were loaded and processed with Fiji (2.1.0) (Schindelin et al., 2012), using the macro *plant immunity and disease image-based quantification* (*PIDIQ*) developed by Laflamme *et al.* (2016). The parameters set for the hue, saturation, and brightness (HSB) track were modified from the original program to optimize detection of rosette size in our photos. Based on optimization, pixels that resemble live and healthy green tissue within a plate are selected, and the total rosette area is calculated per plate. The parameters were kept constant for control plates as well. *PIDIQ* by default also selects yellowing tissue which resembles chlorosis, but this option was disabled in our analysis. The results table was imported for calculation of the GII, which is defined as the ratio between percent growth of seedlings on *M. phaseolina* infected plates and the percent growth of seedlings grown on control plates. First, percent growth is calculated from each plate, regardless of *M. phaseolina* treatment or control, by taking the difference in pixel value from 11 dpi and 1 dpi, and dividing the difference by the 1 dpi value. Each percent growth from the *M. phaseolina* treatment plates were divided by the average percent growth from the control plates to obtain the GII index. The GII

values for each plate is averaged by accession to obtain the average GII, and that average is divided by the average GII of Col-0 plates.

Heritability estimation

Broad-sense heritability (H^2) of GII relative to Col-0 in a population of 87 tested accessions was estimated using a one-way ANOVA and the genotype as a fixed effect. For this, the function *anova* from the R package *stats* (4.2.2) was used, which takes outputs from the *lm* function which fits a linear regression model on the data points. H^2 is estimated as $V_{Genetic}/V_{total}$, or

$$H^2 = \frac{\sigma_G^2}{\sigma_G^2 + \sigma_e^2/r}$$

where σ^2_e is the residual mean square (MS) within groups and $\sigma^2_G=(MS_{between}-MS_{within})/r$, when r is the degrees of freedom. Additionally, broad-sense heritability was also estimated with a generalized hierarchical linear model (HLM) using the R package *hglm* (2.2-1) with genotype as the fixed effect. ANOVA emphasizes significance of differences between fixed groups of a population, while HLM is often used in situations where groups are randomly sampled (Huta, 2014). Broad-sense heritability estimates excluding any population structure data, were compared to assess how modeling methods affect variability based on different assumptions.

In order to distinguish additive and non-additive contributions of genetic variance to phenotypic variance, additive and epistatic variance was included in a mixed model regression as random effects using *hglm*. Additive and epistatic

genetic effects were defined as described in the work by Lachowiec *et al*. (2015). The genomic kinship matrix generated from SNP data in 2029 accessions used to correct for population structure was adapted from the work of Togninalli *et al.* (2018). Contributions of dominance genetic variance was not considered for this estimation because the tested groups in our experiment are natural accessions assumed to be highly inbred and homozygous. Narrow-sense heritability (h^2) was estimated as $V_{additive}/V_{total}$.

Genome-wide association analysis

Genome wide association (GWA) analysis on the GII values relative to Col-0 for 87 accessions was performed on the online platform easyGWAS (https://easygwas.ethz.ch) (Grimm *et al*., 2017). SNPs integrated within easyGWAS were filtered for a minor allele frequency threshold of 0.05 and tested for association with the phenotype (GII relative to Col-0) using the additive model. The efficient mixed-model association expedited (EMMAX) algorithm was selected for the association test (Kang *et al*., 2010). All other parameters were set to default. After filtering, 1,799,622 loci were tested for significant association. Annotations of candidate genes were obtained from Araport11 (Cheng *et al*., 2017).

Literature Cited

1001 Genomes Consortium. (2016). 1,135 Genomes Reveal the Global Pattern of Polymorphism in *Arabidopsis thaliana*. *Cell*, *166*(2), 481–491.

Adeyanju, A., Little, C., Yu, J., & Tesso, T. (2015). Genome-Wide Association Study on Resistance to Stalk Rot Diseases in Grain Sorghum. *G3* , *5*(6), 1165–1175.

Alonso-Blanco, C., & Koornneef, M. (2000). Naturally occurring variation in *Arabidopsis*: an underexploited resource for plant genetics. *Trends in Plant Science*, *5*(1), 22–29.

Atwell, S., Huang, Y. S., Vilhjálmsson, B. J., Willems, G., Horton, M., Li, Y., Meng, D., Platt, A., Tarone, A. M., Hu, T. T., Jiang, R., Muliyati, N. W., Zhang, X., Amer, M. A., Baxter, I., Brachi, B., Chory, J., Dean, C., Debieu, M., … Nordborg, M. (2010). Genome-wide association study of 107 phenotypes in *Arabidopsis thaliana* inbred lines. *Nature*, *465*(7298), 627–631.

Berrocal-Lobo, M., & Molina, A. (2008). *Arabidopsis* defense response against Fusarium oxysporum. *Trends in Plant Science*, *13*(3), 145–150.

Bibikova, T. N., Blancaflor, E. B., & Gilroy, S. (1999). Microtubules regulate tip growth and orientation in root hairs of *Arabidopsis thaliana*. *The Plant Journal: For Cell and Molecular Biology*, *17*(6), 657–665.

Bruex, A., Kainkaryam, R. M., Wieckowski, Y., Kang, Y. H., Bernhardt, C., Xia, Y., Zheng, X., Wang, J. Y., Lee, M. M., Benfey, P., Woolf, P. J., & Schiefelbein, J. (2012). A gene regulatory network for root epidermis cell differentiation in *Arabidopsis*. *PLoS Genetics*, *8*(1), e1002446.

Cheng, C.-Y., Krishnakumar, V., Chan, A. P., Thibaud-Nissen, F., Schobel, S., & Town, C. D. (2017). Araport11: a complete reannotation of the *Arabidopsis thaliana* reference genome. *The Plant Journal: For Cell and Molecular Biology*, *89*(4), 789–804.

Collard, B. C. Y., Jahufer, M. Z. Z., Brouwer, J. B., & Pang, E. C. K. (2005). An introduction to markers, quantitative trait loci (QTL) mapping and marker-assisted selection for crop improvement: The basic concepts. *Euphytica/ Netherlands Journal of Plant Breeding*, *142*(1), 169–196.

Coser, S. M., Chowda Reddy, R. V., Zhang, J., Mueller, D. S., Mengistu, A., Wise, K. A., Allen, T. W., Singh, A., & Singh, A. K. (2017). Genetic Architecture of Charcoal Rot (*Macrophomina phaseolina*) Resistance in Soybean Revealed Using a Diverse Panel. *Frontiers in Plant Science*, *8*, 1626.

da Silva, M. P., Klepadlo, M., Gbur, E. E., Pereira, A., Mason, R. E., Rupe, J. C., Bluhm, B. H., Wood, L., Mozzoni, L. A., & Chen, P. (2019). QTL mapping of charcoal rot resistance in PI 567562A soybean accession. *Crop Science*, *59*(2), 474–479.

da Silva, M. P., Zaccaron, A. Z., Bluhm, B. H., Rupe, J. C., Wood, L., Mozzoni, L. A., Mason, R. E., Yingling, S., & Pereira, A. (2020). Bulked segregant analysis using next-generation sequencing for identification of genetic loci for charcoal rot resistance in soybean. *Physiological and Molecular Plant Pathology*, *109*, 101440.

Denby, K. J., Kumar, P., & Kliebenstein, D. J. (2004). Identification of *Botrytis cinerea susceptibility* loci in *Arabidopsis thaliana. The Plant Journal: For Cell and Molecular Biology*, *38*(3), 473–486.

Diener, A. C., & Ausubel, F. M. (2005). *RESISTANCE TO FUSARIUM OXYSPORUM 1*, a dominant *Arabidopsis* disease-resistance gene, is not race specific. *Genetics*, *171*(1), 305–321.

Dong, X., Mindrinos, M., Davis, K. R., & Ausubel, F. M. (1991). Induction of Arabidopsis defense genes by virulent and avirulent *Pseudomonas syringae* strains and by a cloned avirulence gene. *The Plant Cell*, *3*(1), 61–72.

Dowen, R. H., Pelizzola, M., Schmitz, R. J., Lister, R., Dowen, J. M., Nery, J. R., Dixon, J. E., & Ecker, J. R. (2012). Widespread dynamic DNA methylation in response to biotic stress. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(32), E2183–E2191.

Dudley, J. W., & Moll, R. H. (1969). Interpretation and use of estimates of heritability and genetic variances in plant breeding 1. *Crop Science*, *9*(3), 257–262.

Duggal, P., Gillanders, E. M., Holmes, T. N., & Bailey-Wilson, J. E. (2008). Establishing an adjusted *p*-value threshold to control the family-wide type 1 error in genome wide association studies. *BMC Genomics*, *9*, 516.

Fujita, S., Pytela, J., Hotta, T., Kato, T., Hamada, T., Akamatsu, R., Ishida, Y., Kutsuna, N., Hasezawa, S., Nomura, Y., Nakagami, H., & Hashimoto, T. (2013). An atypical tubulin kinase mediates stress-induced microtubule depolymerization in *Arabidopsi*s. *Current Biology: CB*, *23*(20), 1969–1978.

Gan, X., Stegle, O., Behr, J., Steffen, J. G., Drewe, P., Hildebrand, K. L., Lyngsoe, R., Schultheiss, S. J., Osborne, E. J., Sreedharan, V. T., Kahles, A., Bohnert, R., Jean, G., Derwent, P., Kersey, P., Belfield, E. J., Harberd, N. P., Kemen, E., Toomajian, C., … Mott, R. (2011). Multiple reference genomes and transcriptomes for *Arabidopsis thaliana*. *Nature*, *477*(7365), 419–423.

Grimm, D. G., Roqueiro, D., Salomé, P. A., Kleeberger, S., Greshake, B., Zhu, W., Liu, C., Lippert, C., Stegle, O., Schölkopf, B., Weigel, D., & Borgwardt, K. M. (2017). easyGWAS: A Cloud-Based Platform for Comparing the Results of Genome-Wide Association Studies. *The Plant Cell*, *29*(1), 5–19.

Heckerman, D., Gurdasani, D., Kadie, C., Pomilla, C., Carstensen, T., Martin, H., Ekoru, K., Nsubuga, R. N., Ssenyomo, G., Kamali, A., Kaleebu, P., Widmer, C., & Sandhu, M. S. (2016). Linear mixed model for heritability estimation that explicitly addresses environmental variation. *Proceedings of the National Academy of Sciences of the United States of America*, *113*(27), 7377–7382.

Huta, V. (2014). When to use hierarchical linear modeling. *The Quantitative Methods for Psychology*, *10*(1), 13–28.

Islam, M. S., Haque, M. S., Islam, M. M., Emdad, E. M., Halim, A., Hossen, Q. M. M., Hossain, M. Z., Ahmed, B., Rahim, S., Rahman, M. S., Alam, M. M., Hou, S., Wan, X., Saito, J. A., & Alam, M. (2012). Tools to kill: genome of one of the most destructive plant pathogenic fungi *Macrophomina phaseolina*. *BMC Genomics*, *13*, 493.

Kang, H. M., Sul, J. H., Service, S. K., Zaitlen, N. A., Kong, S.-Y., Freimer, N. B., Sabatti, C., & Eskin, E. (2010). Variance component model to account for sample structure in genome-wide association studies. *Nature Genetics*, *42*(4), 348–354.

Kaur, S., Dhillon, G. S., Brar, S. K., Vallad, G. E., Chand, R., & Chauhan, V. B. (2012). Emerging phytopathogen *Macrophomina phaseolina*: biology, economic importance and current diagnostic trends. *Critical Reviews in Microbiology*, *38*(2), 136–151.

Koike, S. T., Arias, R. S., Hogan, C. S., Martin, F. N., & Gordon, T. R. (2016). Status of *Macrophomina phaseolina* on Strawberry in California and Preliminary Characterization of the Pathogen. *International Journal of Fruit Science*, *16*(sup1), 148–159.

Korte, A., & Farlow, A. (2013). The advantages and limitations of trait analysis with GWAS: a review. *Plant Methods*, *9*, 29.

Korte, A., Vilhjálmsson, B. J., Segura, V., Platt, A., Long, Q., & Nordborg, M. (2012). A mixed-model approach for genome-wide association studies of correlated traits in structured populations. *Nature Genetics*, *44*(9), 1066–1071.

Kruijer, W., Boer, M. P., Malosetti, M., Flood, P. J., Engel, B., Kooke, R., Keurentjes, J. J. B., & van Eeuwijk, F. A. (2015). Marker-based estimation of heritability in immortal populations. *Genetics*, *199*(2), 379–398.

Kunst, L., & Samuels, A. L. (2003). Biosynthesis and secretion of plant cuticular wax. *Progress in Lipid Research*, *42*(1), 51–80.

Lachowiec, J., Shen, X., Queitsch, C., & Carlborg, Ö. (2015). A Genome-Wide Association Analysis Reveals Epistatic Cancellation of Additive Genetic Variance for Root Length in *Arabidopsis thaliana*. *PLoS Genetics*, *11*(9), e1005541.

Laflamme, B., Middleton, M., Lo, T., Desveaux, D., & Guttman, D. S. (2016). Image-Based Quantification of Plant Immunity and Disease. *Molecular Plant-Microbe Interactions: MPMI*, *29*(12), 919–924.

Le, T.-N., Schumann, U., Smith, N. A., Tiwari, S., Au, P. C. K., Zhu, Q.-H., Taylor, J. M., Kazan, K., Llewellyn, D. J., Zhang, R., Dennis, E. S., & Wang, M.-B. (2014). DNA demethylases target promoter transposable elements to positively regulate stress responsive genes in *Arabidopsis*. *Genome Biology*, *15*(9), 458.

MacQueen, A., & Bergelson, J. (2016). Modulation of *R*-gene expression across environments. *Journal of Experimental Botany*, *67*(7), 2093–2105.

McCue, A. D., & Slotkin, R. K. (2012). Transposable element small RNAs as regulators of gene expression. *Trends in Genetics: TIG*, *28*(12), 616–623.

Mcgill, R., Tukey, J. W., & Larsen, W. A. (1978). Variations of Box Plots. *The American Statistician*, *32*(1), 12–16.

Muchero, W., Ehlers, J. D., Close, T. J., & Roberts, P. A. (2011). Genic SNP markers and legume synteny reveal candidate genes underlying QTL for *Macrophomina phaseolina* resistance and maturity in cowpea [*Vigna unguiculata* (L) Walp.]. *BMC Genomics*, *12*, 8.

Nelson, J. R., Verma, S., Bassil, N. V., Finn, C. E., Hancock, J. F., Cole, G. S., Knapp, S. J., & Whitaker, V. M. (2021). Discovery of three loci increasing resistance to charcoal rot caused by *Macrophomina phaseolina* in octoploid strawberry. *G3*. https://doi.org/10.1093/g3journal/jkab037

Parker, J. E., Holub, E. B., Frost, L. N., Falk, A., Gunn, N. D., & Daniels, M. J. (1996). Characterization of eds1, a mutation in *Arabidopsis* suppressing resistance to *Peronospora parasitica* specified by several different RPP genes. *The Plant Cell*, *8*(11), 2033–2046.

Rentel, M. C., Lecourieux, D., Ouaked, F., Usher, S. L., Petersen, L., Okamoto, H., Knight, H., Peck, S. C., Grierson, C. S., Hirt, H., & Knight, M. R. (2004). OXI1 kinase is necessary for oxidative burst-mediated signaling in *Arabidopsis*. *Nature*, *427*(6977), 858–861.

Rönnegård, L., Shen, X., & Alam, M. (2010). Hglm: A package for fitting hierarchical generalized linear models. *The R Journal*, *2*(2), 20–28.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J.-Y., White, D. J., Hartenstein, V., Eliceiri, K., Tomancak, P., & Cardona, A. (2012). Fiji: an open-source platform for biological-image analysis. *Nature Methods*, *9*(7), 676–682.

Schroeder, M. M., Lai, Y., Shirai, M., Alsalek, N., Tsuchiya, T., Roberts, P., & Eulgem, T. (2019). A novel *Arabidopsis* pathosystem reveals cooperation of multiple hormonal response-pathways in host resistance against the global crop destroyer *Macrophomina phaseolina. Scientific Reports*, *9*(1), 20083.

Serrano, M., Coluccia, F., Torres, M., L'Haridon, F., & Métraux, J.-P. (2014). The cuticle and plant defense to pathogens. *Frontiers in Plant Science*, *5*, 274.

Singh, M., Ceccarelli, S., & Hamblin, J. (1993). Estimation of heritability from varietal trials data. *TAG. Theoretical and Applied Genetics. Theoretische Und Angewandte Genetik*, *86*(4), 437–441.

Su, G., Suh, S. O., Schneider, R. W., & Russin, J. S. (2001). Host Specialization in the Charcoal Rot Fungus, *Macrophomina phaseolina*. *Phytopathology*, *91*(2), 120–126.

Tang, Q., Guittard-Crilat, E., Maldiney, R., Habricot, Y., Miginiac, E., Bouly, J.-P., & Lebreton, S. (2016). The mitogen-activated protein kinase phosphatase PHS1 regulates flowering in *Arabidopsis thaliana*. *Planta*, *243*(4), 909–923.

Togninalli, M., Seren, Ü., Meng, D., Fitz, J., Nordborg, M., Weigel, D., Borgwardt, K., Korte, A., & Grimm, D. G. (2018). The AraGWAS Catalog: a curated and standardized *Arabidopsis thaliana* GWAS catalog. *Nucleic Acids Research*, *46*(D1), D1150–D1156.

Veronese, P., Nakagami, H., Bluhm, B., Abuqamar, S., Chen, X., Salmeron, J., Dietrich, R. A., Hirt, H., & Mengiste, T. (2006). The membrane-anchored BOTRYTIS-INDUCED KINASE1 plays distinct roles in *Arabidopsis* resistance to necrotrophic and biotrophic pathogens. *The Plant Cell*, *18*(1), 257–273.

Visscher, P. M., Hill, W. G., & Wray, N. R. (2008). Heritability in the genomics era-concepts and misconceptions. *Nature Reviews. Genetics*, *9*(4), 255–266.

Walia, A., Lee, J. S., Wasteneys, G., & Ellis, B. (2009). *Arabidopsis* mitogen-activated protein kinase MPK18 mediates cortical microtubule functions in plant cells. *The Plant Journal: For Cell and Molecular Biology*, *59*(4), 565–575.

Weigel, D., & Nordborg, M. (2005). Natural variation in *Arabidopsis*. How do we find the causal genes? *Plant Physiology*, *138*(2), 567–568.

Yang, R.-C. (2017). Genome-wide estimation of heritability and its functional components for flowering, defense, ionomics, and developmental traits in a geographically diverse population of *Arabidopsis thaliana*. *Genome / National Research Council Canada = Genome / Conseil National de Recherches Canada*, *60*(7), 572–580.

Zhu, Z., An, F., Feng, Y., Li, P., Xue, L., A, M., Jiang, Z., Kim, J.-M., To, T. K., Li, W., Zhang, X., Yu, Q., Dong, Z., Chen, W.-Q., Seki, M., Zhou, J.-M., & Guo, H. (2011). Derepression of ethylene-stabilized transcription factors (EIN3/EIL1) mediates jasmonate and ethylene signaling synergy in *Arabidopsis*. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(30), 12539–12544.

Chapter II: Transcriptomic analysis of *Arabidopsis thaliana* roots reveals resistance-correlated gene expression patterns in response to *Macrophomina phaseolina*

Abstract

The soil-borne fungal pathogen *Macrophomina phaseolina* is known for its wide host range and persistence in agricultural fields despite years of global efforts to control populations. Fundamentally, the mechanism of virulence is not well known, but genotypes with elevated resistance have been found in several crop species. Here, we attempt to identify key defense pathways that are induced in model plant *Arabidopsis thaliana* to gain a multi-faceted understanding of plant response to *M. phaseolina*. Transcript profiling of five natural *A. thaliana* accessions with varying levels of resistance to this pathogen, across three infection time frames, revealed a delay in defense-related gene expression in susceptible genotypes. It was also shown that susceptibility to *M. phaseolina* is not necessarily correlated with similarity of the transcript profiles in the chosen accessions, Copac-1, Tuewa1-2, Col-0, Ak-1, and UKSE06-252, suggesting complex interplay of genes leading to *M. phaseolina* tolerance. Moreover, gene-enrichment analysis identified previously unrecognized biological processes that were affected by *M. phaseolina* infection. These findings provide new insights to genotype-dependent host transcriptional responses to *M. phaseolina*.

Introduction

The high genetic variability seen in marker-based analyses of the soil-borne fungus *Macrophomina phaseolina* implies a complex virulence strategy with high modularity that contributes to the ability of the pathogen to infect a wide range of hosts. In the previous chapter, we saw that in *Arabidopsis thaliana*, it is unlikely that a single polymorphism dictates whether the genotype survives *M. phaseolina* infection. However, it was also observed that a wide spectrum of susceptibility exists within natural accessions of *A. thaliana*. Higher statistical power may be necessary to identify key contributors to defense at the genomic level, but perhaps differential regulation is more pronounced at the transcript level. Here, we sought to investigate *M. phaseolina* response at a higher order of gene regulation, using lines we determined to be significantly resistant and susceptible to the pathogen in the accession screen.

Published work on *M. phaseolina* thus far highlights the ability of this pathogen to cause severe disease on a wide spectrum of host species. Many attempts to identify genes in crop plants that contribute to resistance have been hindered due to the lack of pre-existing data and means of rapidly creating reliable knockout mutations to investigate individual gene functions. The introduction of the *A. thaliana* - *M. phaseolina* pathosystem by Schroeder *et al*. (2019) provides access to these resources by using a thoroughly studied model. The agar plate-based *M. phaseolina* infection assay that was developed for *A. thaliana* allows the experimenter to have a high level of control over the conditions in which

quantitative analyses and reliable replicates can be produced in high throughput studies.

Marker-based analyses of *M. phaseolina* isolates have shown high genetic diversity across samples collected from various geographical locations and host species (Baird *et al*., 2010). Moreover, growth conditions also seem to impact virulence, as *M. phaseolina*-resistance QTLs identified from greenhouse and field experimentation in soybean did not overlap (Coser *et al*., 2017). For these reasons, it is difficult to extract coherent conclusions regarding *M. phaseolina* host resistance from comparisons between studies with varying growth conditions, pathogen and host genotypes. Our work aims to mediate these issues using a model plant and controlled growth conditions to monitor the default *A. thaliana* transcriptomic response with the only differential factor being the host genotype.

Because *M. phaseolina* is known to be a hemibiotroph with distinct biotrophic and necrotrophic phases, we decided to collect samples at three different time points. Chowdhury *et al.* (2017) previously demonstrated in sesame (*Sesamum indicum L.*) that a biotrophy to necrotrophy switch (BNS) occurs during infection of *M. phaseolina*, at approximately 24 hours post infection (hpi) in the susceptible host variety, and at 36-38 hpi in the resistant variety. This switch was characterized by the development of intercellular secondary hyphae, necrosis of host tissue, and the expression of necrotrophy marker gene *NECROSIS INDUCING PROTEIN 1* (*NPP1*; MPH_06622). Schroeder *et al.* (2019) analyzed gene expression in *M. phaseolina*-infected *A. thaliana* samples at 24 and 48 hpi,

in which genes inducible by the hormones jasmonic acid (JA) and ethylene (ET) were robustly up-regulated, indicating responses to necrotrophic pathogens. This suggests that BNS in *A. thaliana* seedlings occurs earlier than 24 hpi. In this analysis, 12 hpi was selected as an additional time point in an attempt to capture the onset of the switch.

Two recent studies by independent research groups have been published in which transcriptome profiles of sesame in response to *M. phaseolina* were analyzed. Radadiya *et al.* (2021) investigated responses in more mature plants (60 days) grown in the soil, while Yan *et al.* (2021) inoculated seedlings at a younger stage (three pairs true leaves). Both studies compared transcriptomic responses to *M. phaseolina* in susceptible and resistant genotypes. Yan *et al.* (2021) saw that overall, the susceptible genotype responded more robustly in terms of gene transcription. Within the *M. phaseolina*-inducible genes, an enrichment of ribosome-related processes was seen in resistant genotypes, suggesting that activation of defense partially occurs at the translational level. Yan *et al.* (2021) also noted regulation of ROS levels via peroxidases and rapid recognition of the pathogen by receptor-like kinases (RLKs) as possible contributors to the resistance phenotype observed. Radadiya *et al.* (2021) also noted the early up regulation of peroxidases in resistant genotypes, while overall number of DEGs were higher in the susceptible genotype. If transcriptomic responses to *M. phaseolina* are conserved across plant families, we would expect to see similar results in *A. thaliana*. It is however also interesting to explore the possibility of host-

species specificity which has been debated in numerous studies (Burkhardt *et al*., 2019; Koike *et al*., 2016; Su *et al*., 2001).

In this study, we report the root-transcriptomic response to *M. phaseolina* in five natural *A. thaliana* accessions, ranging in levels of resistance, across three different timepoints. Accessions were chosen based on results from a previous screening performed on agar plates (see Chapter I). These included: Columbia (Col-0; CS1092) as a reference line; two accessions considered to be "susceptible", Achkarren (Ak-1; CS6602), UKSE06-252 (CS78800), based on significantly lower growth inhibition indexes (GII) in comparison to Col-0, and two accessions considered to be "resistant", Copac (Copac-1; CS76420); and Tubingen - Wanne (Tuewa1-2; CS76405), based on significantly higher GIIs in comparison to Col-0, (https://1001genomes.org/accessions.html). We observed in all tested accessions a robust induction of defense-related genes involving multiple genes responsive to the stress-inducible phytohormones salicylic acid (SA), JA, and ET, as early as 12 hpi. Induction of defense responses seem to dampen during the 12- to 48- hpi period for "resistant" genotypes Copac-1 and Tuewa1-2, while the amplitude of gene induction increases in "susceptible" genotypes Ak-1 and UKSE06-252. Furthermore, there were significant differences in basal gene expression level between these genotypes, many of which are annotated as defense-related. Major similarities between *A. thaliana* defense induction in response to *M. phaseolina* to that of other crop species were seen in the differential

expression (DE) analysis, as well as genes and biological processes that have not been previously associated with *M. phaseolina* response.

Results

Cross-accession transcriptomic analysis of *A. thaliana* roots in response to *M. phaseolina*

Based on the Growth Inhibition Index (GII) phenotypes that were determined for 88 natural *A. thaliana* accessions (Chapter I), two highly resistant (Copac-1 and TueWa1-2), two highly susceptible (UKSE06-252 and Ak-1) and Col-0 were chosen for further analysis to profile their transcriptomic response to *M. phaseolina* over the course of time. Col-0 was used as a reference accession with intermediate tolerance to *M. phaseolina*, in order to control for variation between experimental batches. Accessions were chosen based on statistical significance between average relative (to Col-0) GII values, according to student's t-test results (Copac-1 and UKSE06-252, Copac-1 and Ak-1, Tuewa1-2 and UKSE06-252, Tuewa1-2 and Ak-1, all combinations showed $p<0.001$) in Chapter I. Additionally, morphological similarity to Col-0 was also considered as a criterion for choosing accessions.

For each accession, mRNA isolated from roots challenged with *M. phaseolina* using the plate-assay method was collected at 12, 24 and 48 hpi. Time-matched control samples without *M. phaseolina* inoculation were also collected.

For each of the 30 experimental conditions, three independent biological replicates were conducted. Total RNA isolated from root tissue was processed to purify mRNA using the NEBNext Poly(A) mRNA Magnetic Isolation Module. Next, libraries were generated using the NEBNext Ultra II Directional RNA Library Prep Kit. Libraries were pooled and sequenced at the UC Berkeley QB3 facility on the Novaseq 6000 platform. Unique reads from Col-0 samples were mapped to the *A. thaliana* TAIR10 genome. For the other accession samples, a reference genome was constructed by applying genotype-specific variant calls, including SNPs and indels, to the Col-0 reference genome using GATK (4.3.3.0.0; Van der Auwera & O'Connor, 2020). Annotations from the Col-0 reference were then transferred over to the generated accession genomes using Liftoff (1.6.3, Shumate & Salzberg, 2020) to which reads from Ak-1, UKSE06-252, Copac-1, and TueWa1-2 sample reads were mapped. Reads from treatment samples that could not be mapped to *A. thaliana* were aligned to the *M. phaseolina MS6* (GCA_000302655; Ensemble Fungi release 56) genome (Cunningham *et al*., 2022). Pairwise correlation between transcriptomes from each library was calculated and hierarchically clustered (Figure 2.1). The smallest correlation coefficient among biological replicates was 0.98, indicating high correlation. Principal component analysis (PCA) on all 90 libraries showed clear clustering of *M. phaseolina* treatment and control samples in correspondence with PC1, which represents the greatest variance (61%) in the data. Secondly, the next largest source of variance (9%) represented by PC2 distinguishes samples by accessions; clustering Ak-1,

TueWa1-2 and Copac-1 samples, while Col-0 and UKSE06-252 were distinguished in a separate cluster (Figure 2.2). This highlights the importance of genotype in assessing transcriptomic response to *M. phaseolina*. Both hierarchical clustering and PCA show high correlation between triplicate experimental samples.

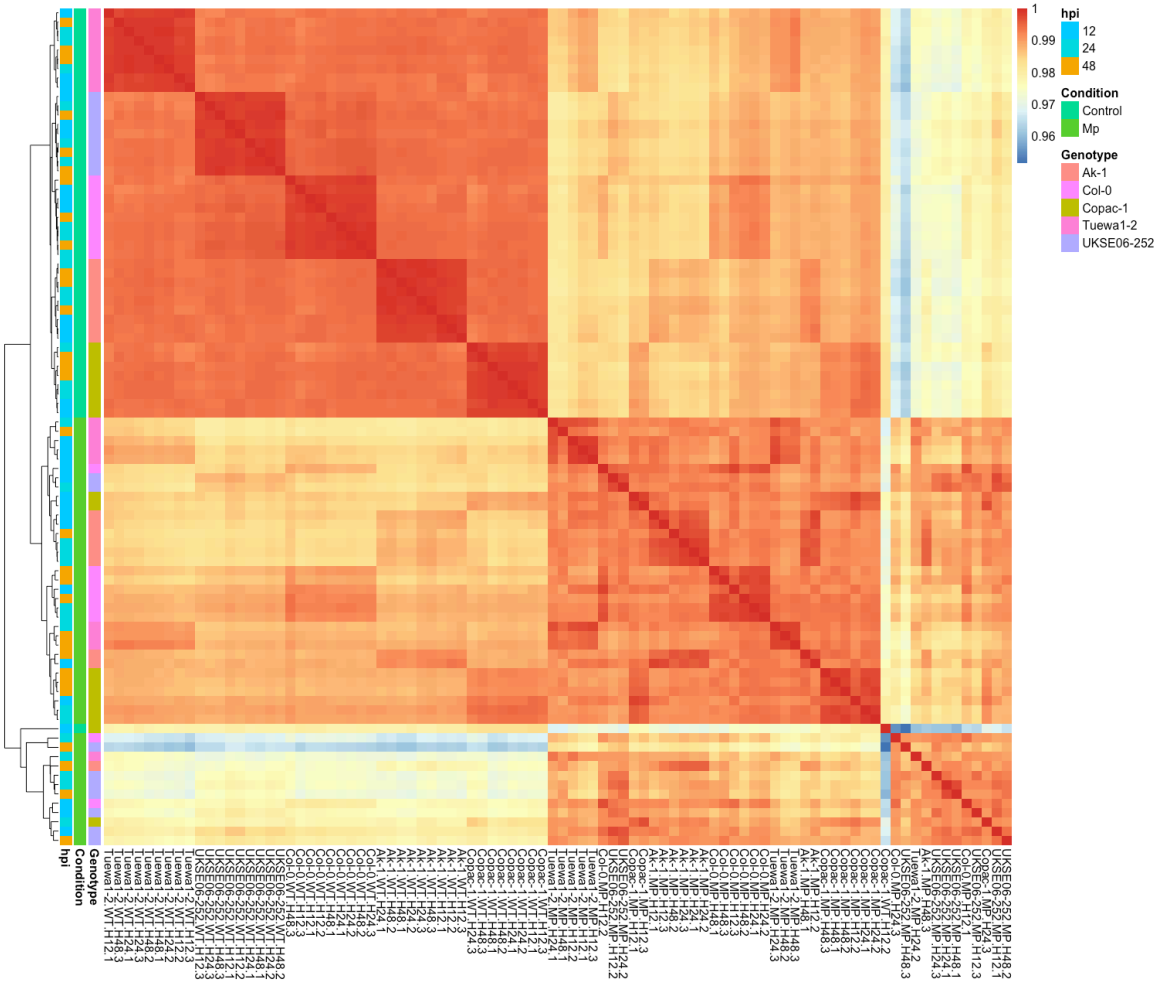

Figure 2.1. Pairwise comparison of normalized read counts for all RNA-seq libraries. Pearson correlation between all samples including biological replicates were calculated based on read counts normalized by the *variance stabilizing transformations* (*VST*) function in DESeq2 and clustered by similarity (Anders & Huber, 2010).

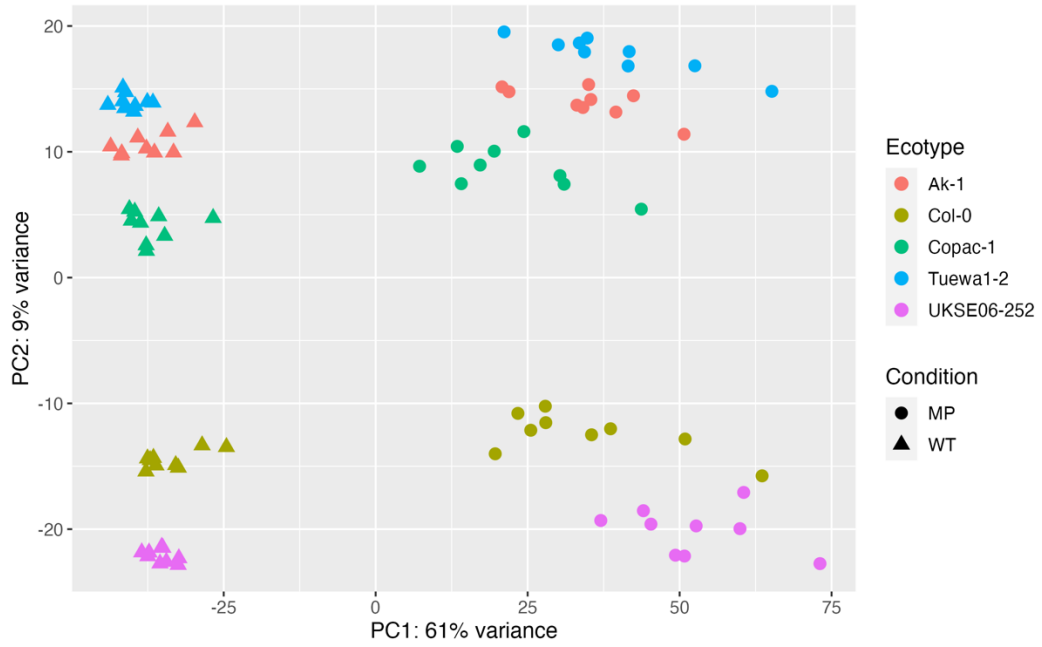

Figure 2.2. Principal component analysis (PCA) of normalized read counts for all RNA-seq libraries. Individual points represent samples including biological replicates. Shapes represent experimental conditions (MP = *M. phaseolina* treatment, WT = control), and colors represent *A. thaliana* genotypes.

The ratio of housekeeping gene expression between *M. phaseolina* ubiquitin carrier protein MPH_07192, the closest homolog of *Talaromyces versatilis* ubiquitin carrier protein *ubcB (Llanos et al., 2015)*, and *A. thaliana GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE C2,* or *AtGAPDH* (AT1G13440) was calculated by converting reads to transcripts per million (TPM) (Figure 2.3). While the *MPH_07192/AtGAPDH* ratio remained relatively constant in Col-0 and resistant genotypes, this ratio increases in correlation with time in susceptible genotypes. UKSE06-252 samples have a significantly larger *MPH_07192/GAPDH* ratio compared to Ak-1, TueWa1-2, and Copac-1 at 12 hpi. This observation correlates with the susceptibility of Ak-1 and UKSE06-252, in that

more genetic material from the pathogen was found in samples from susceptible genotypes relative to the host genetic material.

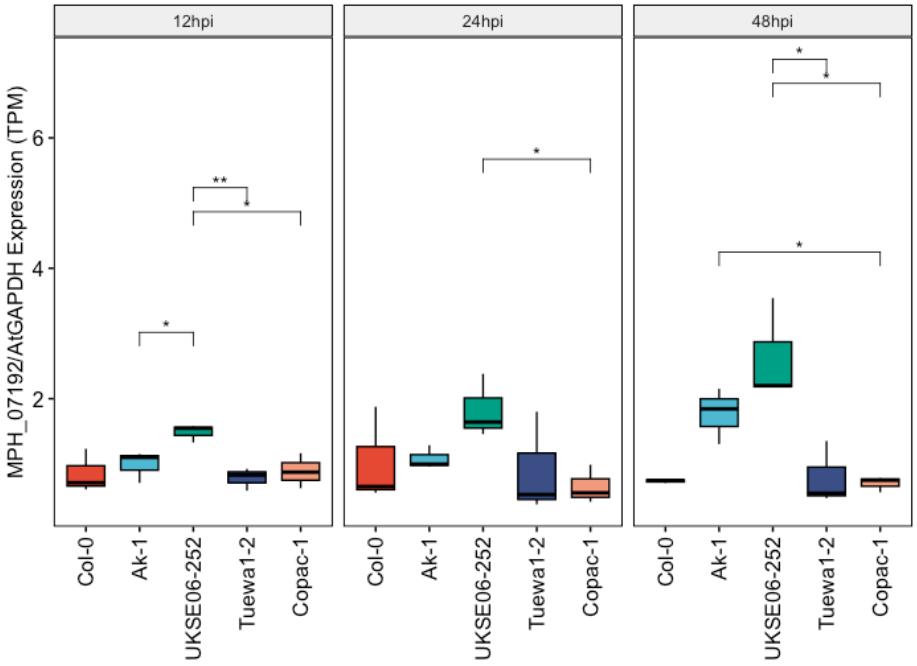

Figure 2.3. Ratio of *MPH_07192/AtGAPDH* expression. Read counts of *M. phaseolina* MPH_07192 and *A. thaliana AtGAPDH* were converted to transcripts per million (TPM) and ratios of *MPH_07192/AtGAPDH* were calculated for each sample including three biological replicates. Multiple *t*-tests were performed to identify significant differences between genotype groups at each time point. ** $p < .01$, * $p < .05$.

A. thaliana accessions express varying levels of defense-related genes in roots

Each genotype was compared to Col-0 for the corresponding time point in a pairwise differential expression analysis to assess basal gene expression variability. Genes with $\log_2$ fold change greater than 1 or less than -1 and adjusted *p* value threshold of 0.01 was considered differentially expressed. In total, 4,959 genes were found to be differentially expressed in at least one comparison against Col-0 (Figure 2.4A). To our surprise, 3,818 out of 4,959, or 76% of these genes were also found in the *M. phaseolina*-response differentially expressed gene

(DEG) set, which includes all genes differentially expressed in at least one comparison among the 15 comparisons between *M. phaseolina*-treatment and control conditions (Figure 2.4C). This suggests that a vast majority of genes with variability in basal expression level across different accessions are responsive to, and potentially involved in, *M. phaseolina*-induced biological processes. Hierarchical clustering of samples based on differential gene expression shows that UKSE06-252 clusters distinctly from the other genotypes. This was statistically supported by bootstrap resampling (1,000 iterations) using the R package *pvclust* (Suzuki & Shimodaira, 2006) (Figure 2.4B). As of the remaining genotypes, the expression profiles of Copac-1 and Tuewa1-2, which are relatively resistant to *M. phaseolina*, were more similar to each other than they were to that of Ak-1, although compared to the distance between the cluster which includes the UKSE06-252 libraries, the distance is much smaller between Ak-1 and the resistant genotypes.

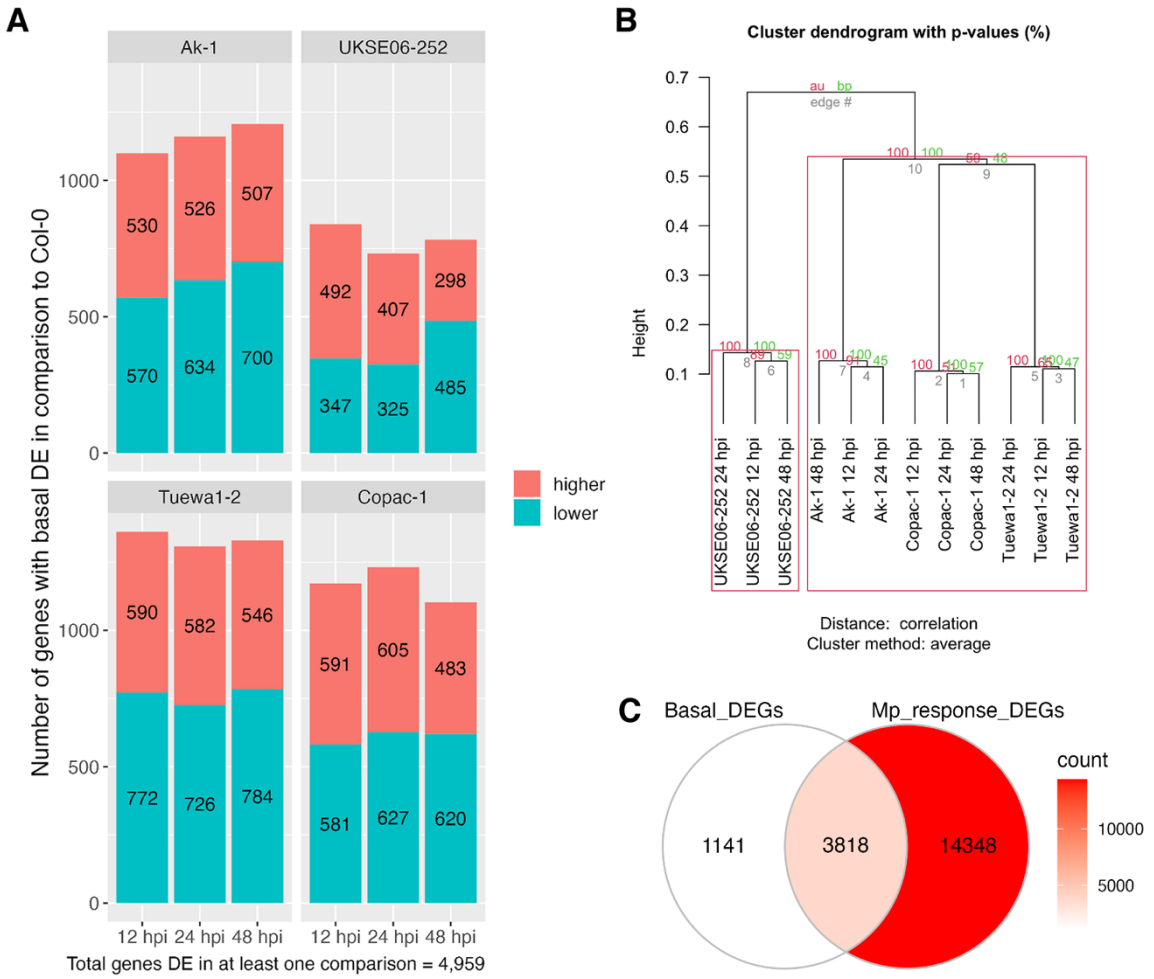

Figure 2.4. Genotypic variance is seen in basal differential gene expression between *A. thaliana* accessions. (A) Basal gene expression comparisons between accession control samples. All comparisons are made with the control Col-0 transcriptome profile of the same time point. Genes labeled "higher" have significantly higher expression ($p < .01$, $\log_2$ fold change > 1) in the corresponding genotype than in Col-0, and genes labeled "lower" have significantly lower expression ($p < .01$, $\log_2$ fold change < -1) in the corresponding genotype than in Col-0. **(B)** Clustered dendrogram of basal gene differential expression profiles in comparison to Col-0. Hierarchical clustering by correlation was used with the average linkage method. To assess the stability of the clustering, bootstrap resampling was applied (1,000 iterations) using the R package *pvclust* (Suzuki and Shimodaira 2006). Average linkage is used for clustering and vertical distances represent the Euclidean distance between clusters. Significance between clusters was quantified through bootstrap resampling represented by two *p*-values: au (red), Approximately Unbiased and bp (green), Bootstrap Probability. Highly supported clusters are indicated with a red frame. **(C)** Comparison of basal expression DEGs and *M. phaseolina*-response DEGs.

From this total basal DEG pool, genes with distinct expression patterns in correlation with their *M. phaseolina*-tolerance phenotype were categorized: (1) there were 232 genes with relatively high basal expression in both resistant genotypes, and (2) 202 genes with relatively high basal expression in both susceptible genotypes (Figure 2.5). Furthermore, genes in these categories responsive to *M. phaseolina* were also identified by comparing to the DEG pool generated from control/treatment comparisons. For each gene, the average log_2-fold change across the three time points was calculated per genotype to determine whether overall DE was up- or down-regulated in response to *M. phaseolina*. A total of 93 out of 232 genes that were expressed higher in basal levels in resistant genotypes were also up-regulated in response to *M. phaseolina* in resistant genotypes, while only 56 of them were down-regulated. Similarly, 103 out of these 232 genes were upregulated in response to *M. phaseolina* in susceptible genotypes.

Between basal DEGs highly expressed in susceptible genotypes, and those highly expressed in resistant phenotypes, the difference in the number of DEGs transcriptionally responsive to *M. phaseolina* was statistically significant in resistant genotypes but not in susceptible genotypes (Figure 2.5). This suggests a non-random relationship between basal-expression patterns and *M. phaseolina* inducibility. Furthermore, genes which show high basal expression in resistant genotypes are significantly more likely to be up-regulated in response to *M. phaseolina* than suppressed.

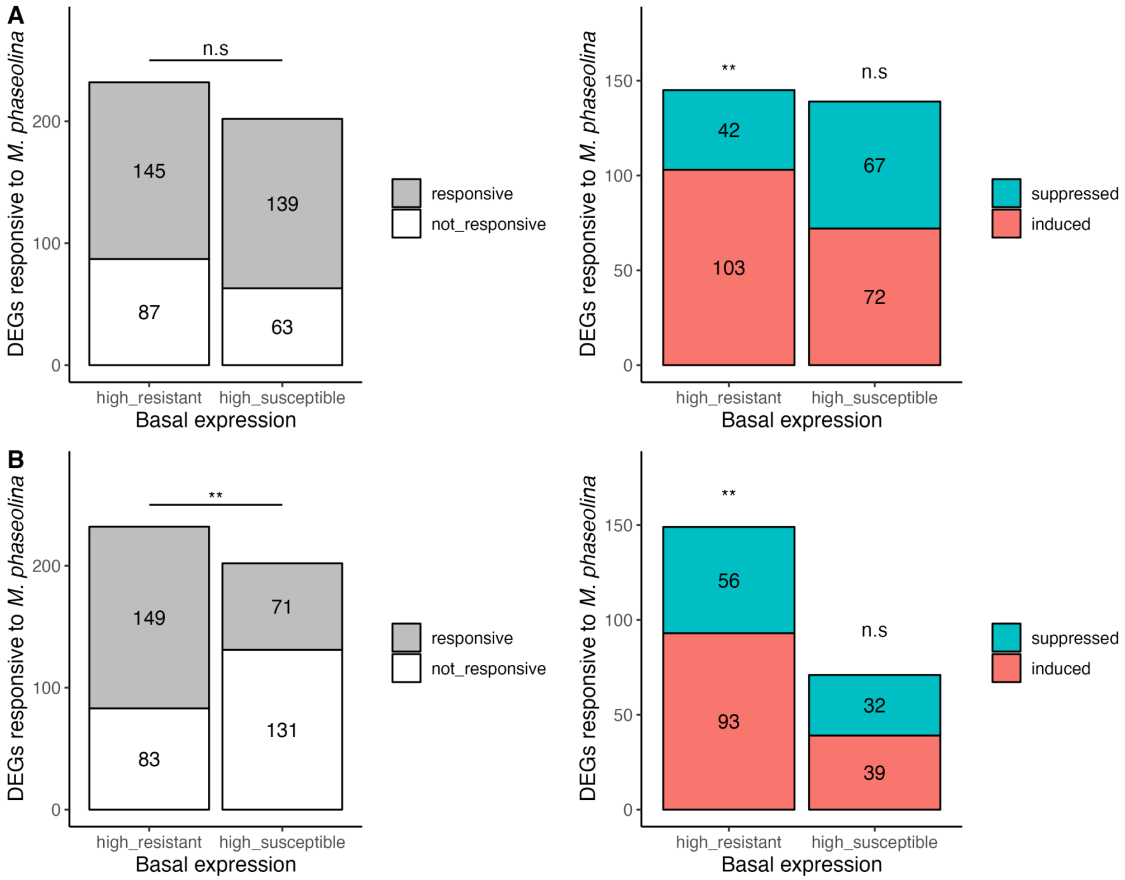

Figure 2.5. Many genes exhibiting basal gene expression patterns correlated with *M. phaseolina*-tolerance phenotype are also responsive to *M. phaseolina*. Stacked bar plots show (A) basal DEG response to *M. phaseolina* in susceptible genotypes, and **(B)** basal DEG response to *M. phaseolina* in resistant genotypes. The grayscale bar plots on the left show the number of basal DEGs in each class (highly expressed in resistant genotypes or highly expressed in susceptible genotypes), and whether they are responsive (gray) or not responsive (white) to *M. phaseolina*. A X^2 test of independence was performed on the data for each group of genotypes (resistant and susceptible), to evaluate whether there is a significant relationship between basal expression (highly expressed in resistant or highly expressed in susceptible accessions) and *M. phaseolina* responsiveness. (** - p<.01, n.s - no significance). The colored bar plots on the right show exclusively *M. phaseolina*-responsive basal DEGs, and whether they are induced (pink) or suppressed (blue). X^2 goodness of fit tests were performed to see if there were significant differences between the expected ratio of induced:suppressed DEGs (50:50) and the actual distribution (** - p<.01, n.s - no significance).

Only 39 out of 202 genes that had higher basal expression in susceptible genotypes were up-regulated in response to *M. phaseolina* in resistant genotypes, while 72 of these genes were up-regulated by *M. phaseolina* in susceptible genotypes. This suggests that many genes with higher basal expression in susceptible genotypes are dispensable to *M. phaseolina* defense. Of the genes that had high basal expression in susceptible genotypes, a greater proportion of genes were responsive to *M. phaseolina* in susceptible samples in comparison to resistant samples. This also means that many genes that were relatively suppressed at basal levels in resistant samples were not responsive to pathogen interaction. This suggests that fewer genes that were highly expressed in susceptible genotypes and are related to defense. Alternatively, this could be an indication that defense activation in susceptible and resistant genotypes are mediated by accumulation of different gene transcripts.

Gene Ontology (GO) term analysis on both basal DEG groups revealed that the group representing genes with higher basal expression in resistant genotypes was enriched in GO terms including “defense response” (p=9.59E-07), “defense response to other organism” (p=9.18E-06) and “response to external biotic stimulus” (p=1.09E-04). There was no significant GO Biological Process term enrichment in the group representing genes with higher basal expression in susceptible genotypes. Furthermore, genes with high basal expression in resistant genotypes and were induced in response to *M. phaseolina* (93 genes) were enriched for similar defense-related GO terms including “defense response”

(*p*=1.42E-10), “defense response to bacterium” (*p*=9.36E-05), “response to fungus” (*p*=1.14E-02), “toxin metabolic process” (*p*=9.08E-03), and “response to chemical” (*p*=3.32E-02). No GO term enrichment was found for genes with low basal expression in susceptible genotypes and were suppressed in response to *M. phaseolina* (42 genes). This indicates that transcripts with uniquely high abundance in resistant genotypes function in defense and abiotic stress response.

Although their significance towards *M. phaseolina* requires further functional analysis, expression patterns of these defense-related genes are notable as they may be factors in determining resistance against *M. phaseolina*. For example, *WRKY66* (AT1G80590) is a transcription factor responsive to SA, abscisic acid (ABA), and salt. Other notable transcription factors in this category include *JUNGBRUNNEN 1* or *JUB1* (AT2G43000), a NAC transcription factor responsive to hydrogen peroxide and involved in senescence (Shahnejat-Bushehri *et al*., 2016), and *MYB15* (AT3G23250), which plays a key role in lignin biosynthesis during ETI (Chezem *et al*., 2017). Genes involved in cell wall modification (*MODIFYING WALL LIGNIN-2*; AT4G19370) and chitinases (*CHITINASE*; AT2G43570), genes in the *recognition of Peronospora parasitica 5* or *RPP5* gene cluster (AT4G16940, AT4G16960, and AT4G16990) (Yi & Richards, 2007), *EF-TU RECEPTOR* (*EFR*; AT5G20480), which recognizes the potent bacterial PAMP EF-Tu and triggers PTI, were also a part of this gene set.

Genes with high basal expression in susceptible genotypes and induced in response to *M. phaseolina* (72 genes) were enriched for the GO terms signal

transduction (*p*=3.38E-02) and “signaling” (*p*=4.80E-02). No GO term enrichment was observed for genes with low basal expression in resistant genotypes and suppressed in response to *M. phaseolina* (32 genes). Although there was no significant enrichment of defense-related GO terms, genes with known functions in biotic-stress response can be found in this gene set. For example, several genes in the *RPP5* cluster including *SUPPRESSOR OF NPR1-1* or *SNC1* (AT4G16890) and AT4G16920 were expressed at higher levels in susceptible genotypes. In addition, several NLR genes (AT1G15890, AT1G27180, AT4G16890, AT4G19520, AT4G19530, AT4G36150) were basally repressed in resistant genotypes and induced by *M. phaseolina* only in susceptible genotypes. GO term enrichment seen in each basal DEG category is summarized in Figure 2.6.

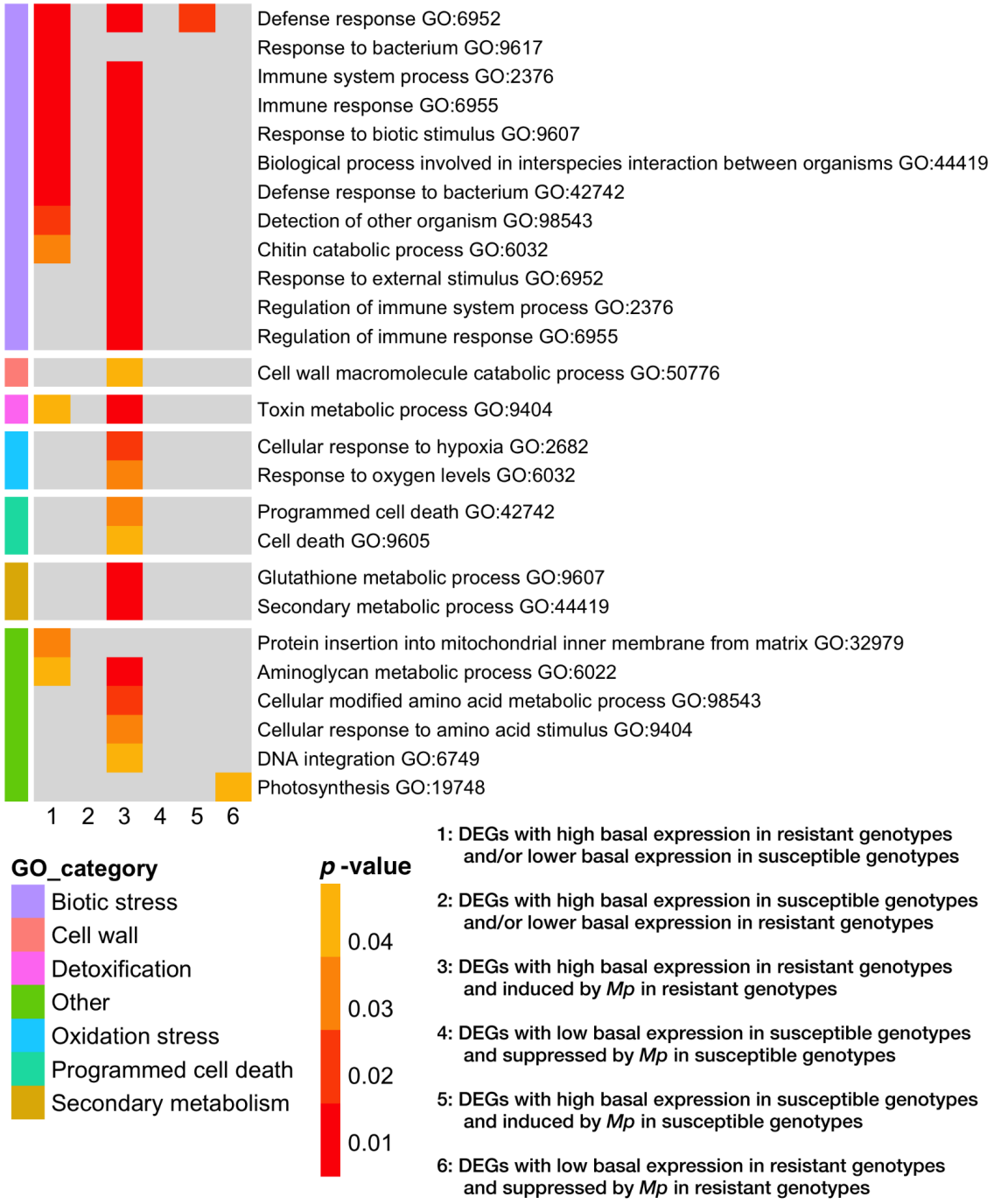

Figure 2.6. GO term enrichment on basal DEGs. Full DEG lists obtained from *ShinyGO* (v0.741; http://bioinformatics.sdstate.edu/go/) were simplified and reduced with *REVIGO* (http://revigo.irb.hr/), then further categorized by larger groups of similar biological functions. The numbers on the x axis denote each DEG group for which analysis was conducted. *Mp* = *M. phaseolina*.

Transcriptional response to *M. phaseolina* varies across accessions and in correlation with susceptibility-related growth suppression

To elucidate *A. thaliana* transcriptional responses to *M. phaseolina*, each sample generated from *M. phaseolina* treatment was compared to the corresponding untreated control sample. For three time points and five tested accessions, a total of 15 comparisons were performed. On average, the susceptible genotypes Ak-1 and UKSE06-252 had more *M. phaseolina*-induced DEGs than the resistant genotypes TueWa1-2 and Copac-1, in all three timepoints. Moreover, for susceptible genotypes, the number of DEGs steadily increased with time, while the number of resistant-genotype DEGs had a negative correlation with time. Col-0 differential gene expression resembles that of the resistant genotypes at 48 hpi. In terms of transcriptional activation, susceptible genotypes show a more robust response to *M. phaseolina* than resistant genotypes, and the amplitude of the response shows an increasing trend at 48 hpi. As shown in Figure 2.7C-E, the proportion of DEGs shared exclusively among susceptible genotypes increases with time, while the proportion of DEGs shared among resistant genotypes remains relatively low.

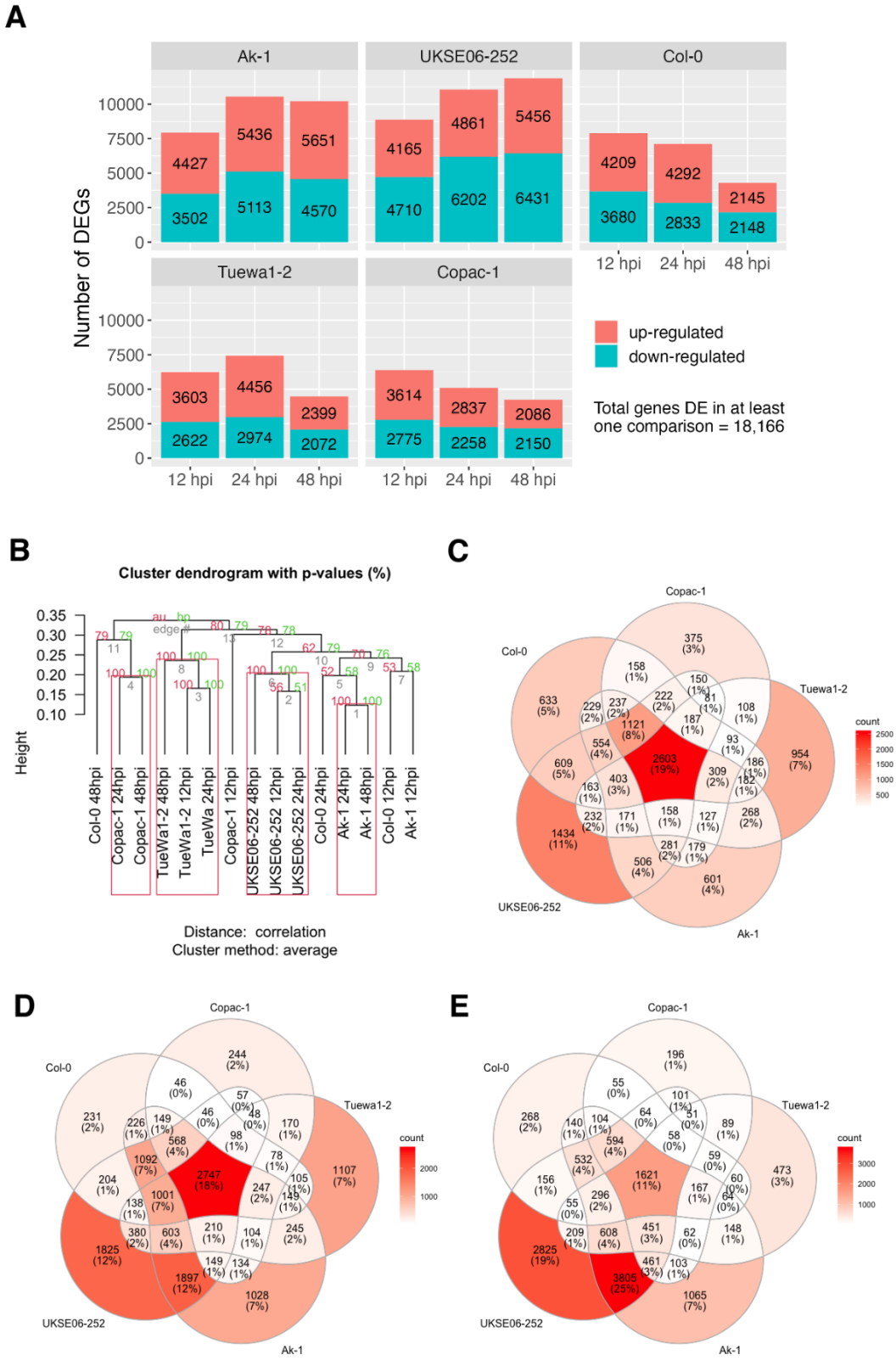
A
Ak-1
UKSE06-252
Col-0
Tuewa1-2
Copac-1
Number of DEGs
12 hpi
24 hpi
48 hpi
up-regulated
down-regulated
Total genes DE in at least one comparison = 18,166
B
Cluster dendrogram with p-values (%)
Height
Distance: correlation
Cluster method: average
C
D
E
count

Figure 2.7. DEGs in response to *M. phaseolina* show resistance-correlated similarities and progression of disease with time post-infection. (A) The number of DEGs identified in the RNA-seq in response to *M. phaseolina.* **(B)** Clustered dendrogram of *M. phaseolina* induced gene expression profiles. Hierarchical clustering by correlation with the average linkage method. Significance between clusters was quantified through bootstrap resampling represented by two *p*-values: au (red), Approximately Unbiased and bp (green), Bootstrap Probability. Highly supported clusters are shown with a red frame. **(C) ~ (D)** Comparison of *M. phaseolina* induced DEGs across five accessions show shifting trends in correspondence to hpi. Overlapping DEGs at **(C)** 12 hpi, **(D)** 24 hpi, and **(E)** 48 hpi.

Hierarchical clustering based on *M. phaseolina*-response DEGs show significant similarity between 24 hpi and 48 hpi samples for all genotypes tested except Col-0. Significance is supported by bootstrap resampling (Figure 2.7B). Susceptible genotypes form a clade that includes Col-0 12 and 24 hpi samples and is most similar to the Copac-1 12 hpi sample. Interestingly, the Col-0 sample at 48 hpi forms a clade with Copac-1 24 and 48 hpi sample. Given that Copac-1 is phenotypically considered the most resistant genotype tested, perhaps the *M. phaseolina* transcriptional response in Col-0 resembles that of susceptible genotypes at earlier time points and resistant genotypes at later time points.

Previously, Schroeder *et al*. (2019) conducted RNA-seq on Col-0 roots collected at 24 and 48 hpi after *M. phaseolina* treatment. In this previous transcriptomic study, 335 genes were identified as differentially expressed in a comparison between control samples and *M. phaseolina* treated samples at 24 hpi, while 6,227 DEGs were identified in a comparison between control and treatment samples at 48 hpi (Schroeder *et al*., 2019). While the majority of DEGs found in our new dataset were shared with that of Schroeder *et al.* (2019), many unique DEGs were also identified in both datasets (Figure 2.8). This may be due to the difference in the adjusted *p*-value cutoff used, variability in experimental conditions and the choice of an additional time point at 12 hpi in our current study.

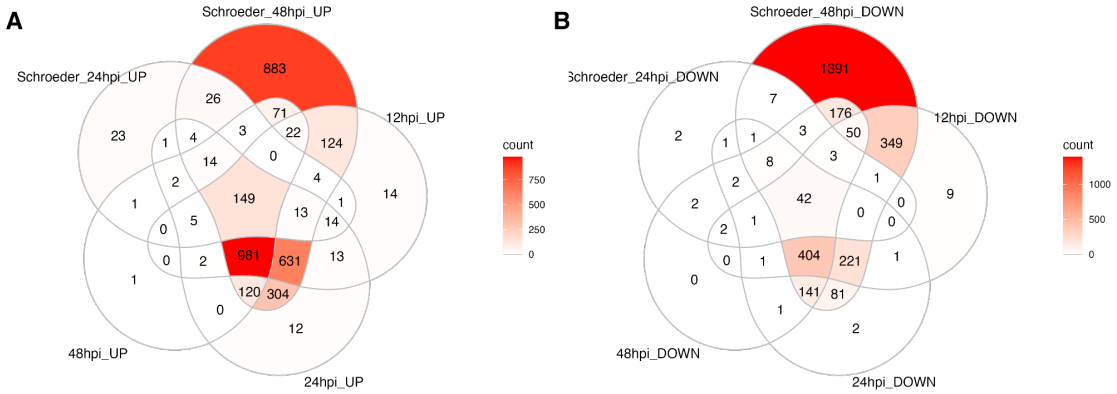

Figure 2.8. DEGs in common between RNA-seq analysis from (Schroeder *et al.* 2019) **(A)** Up-regulated DEGs and **(B)** down-regulated DEGs show considerable overlap with data described by Schroeder *et al*. (2019). Genes unique to the 12 hpi time point have also been identified. The darkness of the color represents enrichment of DEGs in the category.

As *M. phaseolina* is considered a hemibiotrophic pathogen, activation of phytohormone-dependent defense response pathways was assessed by looking at the expression pattern of seven salicylic acid- (*PAD4, NPR1, WRKY70, WRKY54, SID2, PR1,* and *PR2*), five jasmonic acid- (*JAR1, JIN1, COI1, JAZ2, PDF1.2,* and *LOX1*), and five ethylene- (*EIN2, EIN3, EIL1, ERF1, ERF2,* and *ERF3*) marker genes associated with each of these plant defense-hormone pathways, as well as *TYROSINE AMINOTRANSFERASE 3* or *TAT3*, which had the highest $\log_2$ fold change in the 24 hpi comparison from Schroeder *et al*. (2019). Of these genes, *PR1, JIN1, COI1, PDF1.2* showed no significant differential expression in any sample. Up regulation was observed for most genes in most samples, except *SID2*, *JAR1*, and *EIN2* which show slight down regulation in less than half of the sample classes. Several genes, including *PAD4, NPR1, WRKY70*, and *EIN3,* generally show higher up regulation at 48 hpi. Based on these

transcriptomics data, out of the three phytohormone-response pathways, ethylene seems to be the most robustly induced (Figure 2.9).

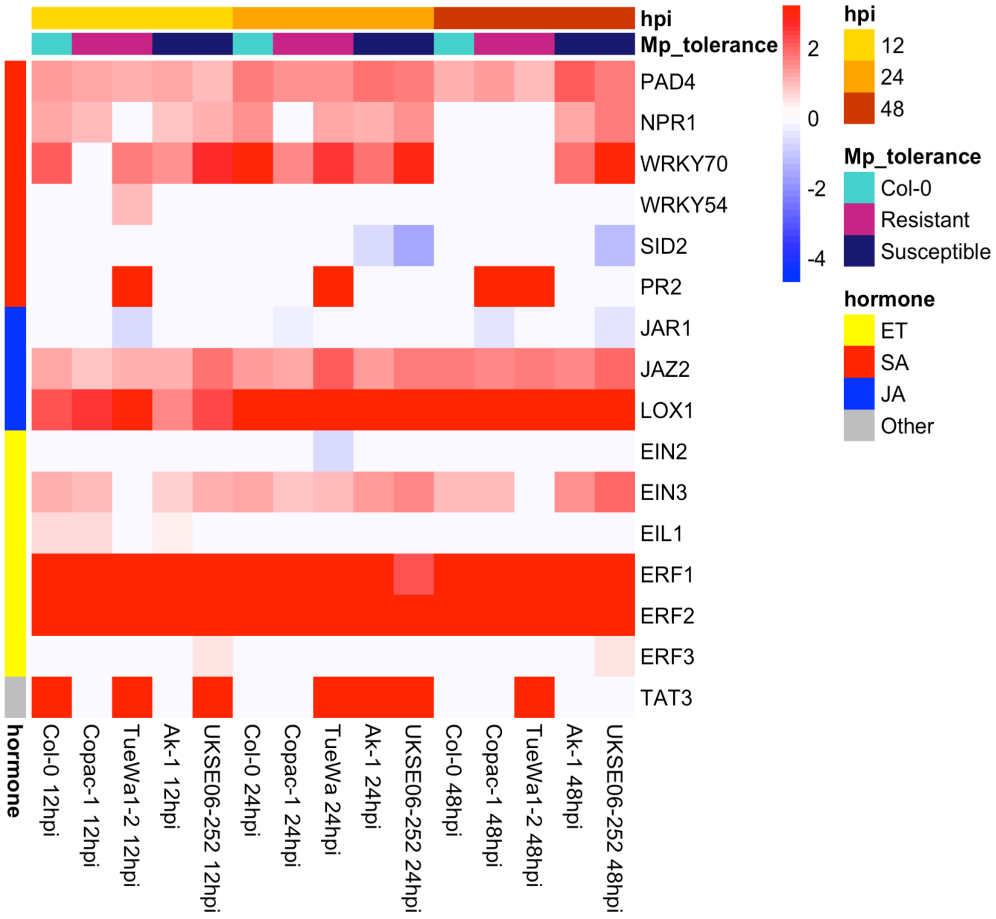

Figure 2.9. Key phytohormone response genes are activated in response to *M. phaseolina*. Heatmap showing $\log_2$ fold expression change data on select phytohormone response genes. Genes known to be involved in SA response are labeled red, genes known to be involved in JA response are labeled blue, and genes known to be involved in ET response are labeled yellow. *TAT3* is included since it has shown consistent robust responses to *M. phaseolina* in the shoot (unpublished data). *PR1* (AT2G14610), *JIN1* (AT1G32640), *COI1* (AT2G39940), and *PDF1.2* (AT5G44420) were also checked but no differential expression was seen in any of the tested samples (data not shown).

In order to gain a more comprehensive view of JA- and SA-mediated transcript responses to *M. phaseolina*, genes identified to be responsive to each of these phytohormones through meta-analysis by Zhang and colleagues (2020) were represented in a heatmap (Figure 2.10) (Zhang *et al*., 2020).

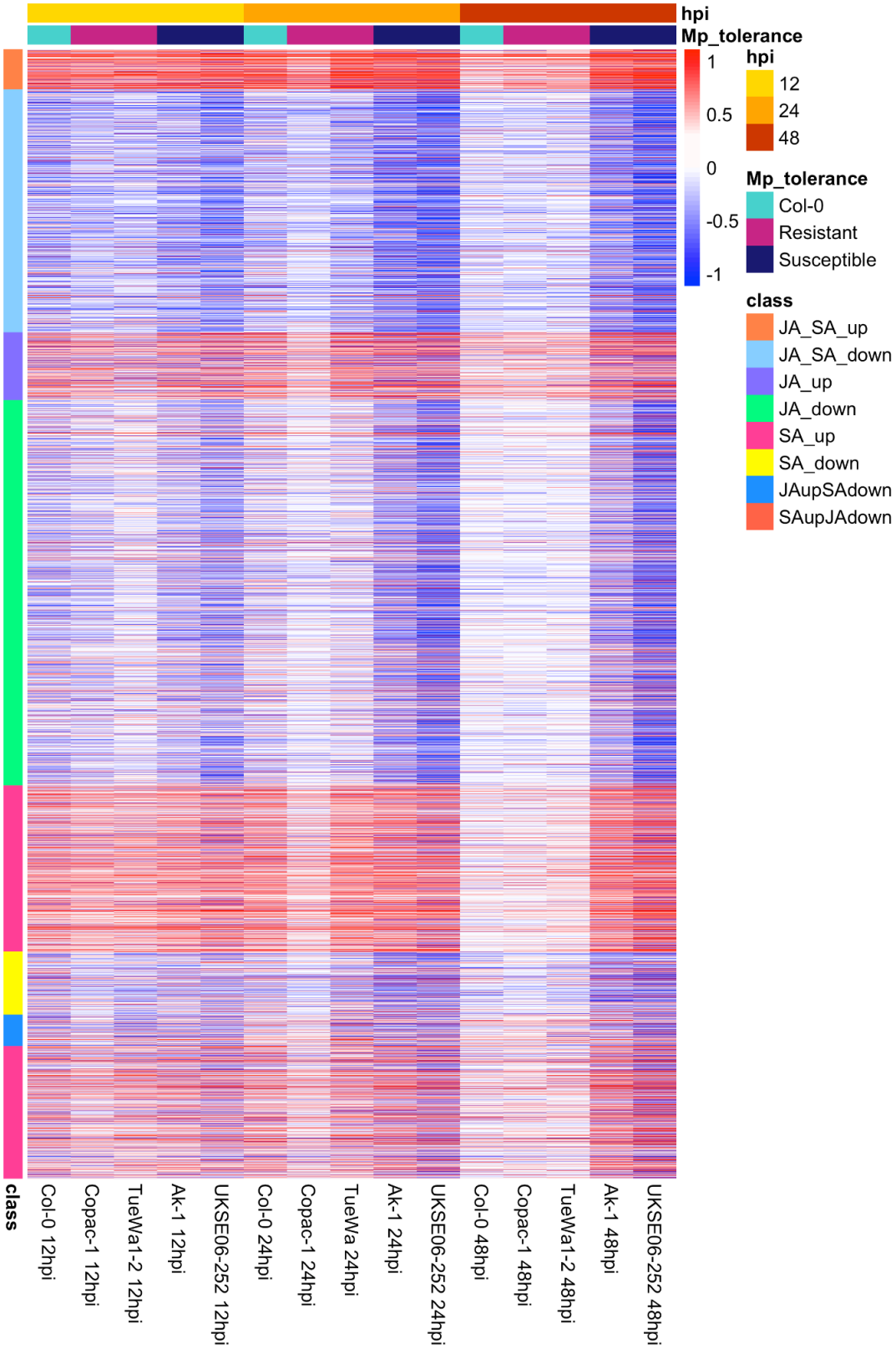
hpi
Mp_tolerance
1
0.5
0
-0.5
-1
hpi
12
24
48
Mp_tolerance
Col-0
Resistant
Susceptible
class
JA_SA_up
JA_SA_down
JA_up
JA_down
SA_up
SA_down
JAupSAdown
SAupJAdown
class
Col-0 12hpi
Copac-1 12hpi
TueWa1-2 12hpi
Ak-1 12hpi
UKSE06-252 12hpi
Col-0 24hpi
Copac-1 24hpi
TueWa 24hpi
Ak-1 24hpi
UKSE06-252 24hpi
Col-0 48hpi
Copac-1 48hpi
TueWa1-2 48hpi
Ak-1 48hpi
UKSE06-252 48hpi

Figure 2.10. SA- and JA-responsive genes show genotype dependent response to *M. phaseolina*. SA and JA genes classified by Zhang *et al.* (2020) were selected to visualize differential expression (log_2-fold change) induced by *M. phaseolina* in a heatmap. Genes are sorted based on the eight classifications described in Zhang *et al*.: JA_SA_up = induced by JA and SA, JA_SA_down = suppressed by JA and SA, JA_up = induced by JA, JA_down = suppressed by JA, SA_up = induced by SA, SA_down = suppressed by SA, JAupSAdown = induced by JA and suppressed by SA, SAupJAdown = induced by SA and suppressed by JA. Expression profiles are grouped by hpi and *M. phaseolina*-tolerance level of the accession tested (resistant, susceptible, or Col-0).

In general, gene expression in response to *M. phaseolina* corresponds with the descriptions from the work of Zhang *et al.* (2020) (Figure 2.10). Notably, regardless of the phytohormone pathway the gene is regulated by, differential expression decreases with time for resistant genotypes Copac-1 and Tuewa1-2, as well as Col-0. On the other hand, the amplitude of differential expression, both up and down regulation, increases for susceptible genotypes UKSE06-252 and Ak-1, potentially indicating ongoing induction of active defense responses. At 12 hpi, the amplitude of transcriptional activation and repression seems to be similar across all genotypes tested.

Out of the 330 genes described as JA induced and SA suppressed (JA↑SA↓) by Zhang *et al.* (2020), 9% were up-regulated (log_2 fold change >1) and 9% were down-regulated (log_2 fold change <-1) in Copac-1 at 12 hpi. At 48 hpi, 11% were up-regulated and 3 % were down-regulated in this accession. This shift potentially indicates repression of SA-induced responses in Copac-1. In contrast, at 12 hpi, 13% were up-regulated and 19% were down-regulated in UKSE06-252. At 48 hpi, 19% were up-regulated and 24% were down-regulated. Proportionally, more genes in this set were down-regulated in UKSE06-252 suggesting dominance of SA-dependent responses. Out of the 1,316 genes described as SA-induced and JA-suppressed (SA↑JA↓) by Zhang *et al.* (2020), 13% were up-regulated and 3% were down-regulated in Copac-1 at 12 hpi. At 48 hpi, 10% were up-regulated and 2 % were down-regulated. Moreover, at 12 hpi 21% were up-

regulated and 9% were down-regulated in UKSE06-252. At 48 hpi, 31% were up-regulated and 15% were down-regulated.

Core *M. phaseolina* response genes are shared among tested accessions

Out of the 18,166 genes differentially expressed in at least one experimental condition (genotype and time point) in response to *M. phaseolina*, 904 genes were differentially expressed in all 15 samples. Out of these 904 genes, 688 were commonly up-regulated, 215 were commonly down-regulated, and one was up-regulated in all genotypes except UKSE06-252 in which it was down-regulated in response to *M. phaseolina*. Collectively, these genes represent a core set of *M. phaseolina* responsive genes that were differentially expressed in all *A. thaliana* genotypes tested.

Up-regulated genes were in enriched in GO terms indicating biotic stress response, such as “defense response to fungus” (*p*=2.21E-29), “response to chitin” (*p*=1.81E-29), “defense response to bacterium” (*p*=6.70E-47), “response to wounding” (*p*=8.64E-30), and “regulation of defense response” (p=1.43E-35). Other terms related to defense-associated hormone pathways were also enriched, such as “regulation of jasmonic acid mediated signaling pathway” (*p*=2.90E-07), “salicylic acid-mediated signaling pathway” (3.68E-02), “systemic acquired resistance” (*p*=4.97E-08), “ethylene-activated signaling pathway” (*p*=1.92E-05), and “response to abscisic acid” (*p*=2.72E-31). Enrichment of terms which suggest induction of secondary metabolites, including “camalexin biosynthetic process”

(*p*=1.74E-04), “aromatic amino acid family catabolic process” (*p*=1.37E-02), and “branched-chain amino acid catabolic process” (*p*=4.40E-03) were also seen. Terms related to ROS response, such as “response to ozone” (*p*=1.57E-02) and “response to hydrogen peroxide” (*p*=2.38E-02), xenobiotic detoxification, and programmed cell death were also significantly enriched. Other enriched terms suggested responses to abiotic-stress factors, such as water deprivation, starvation, salt stress, and temperature.

Down-regulated genes were enriched in GO terms primarily involving growth and development, including “root morphogenesis” (*p*=2.31E-09), “developmental growth” (*p*=4.81E-08), “cell differentiation” (*p*=2.75E-05) , “plant epidermis development” (*p*=5.34E-04), “cell wall polysaccharide biosynthetic process” (*p*=4.90E-02), and “plant-type cell wall biogenesis” (*p*=3.40E-02).

To see if there were significant differences in $\log_2$-fold change between susceptible and resistant genotypes, student’s t-tests were applied to compare values from three time points across pairs of accessions for each DEG group (up- or down-regulated) described above. Genes with significant *p*-values ($p<0.05$) in Copac-1 vs. Ak-1, Copac-1 vs. UKSE06-252, Tuewa1-2 vs. Ak-1, and Tuewa1-2 vs. UKSE06-252 were identified. Additionally, genes with significant *p*-values in Copac-1 vs. Tuewa1-2 and/or Ak-1 vs. UKSE06-252 comparisons were removed, since these are genotype specific DEGs and without DE correlated to *M. phaseolina* tolerance. This left 9 genes, 6 up-regulated and 3 down-regulated. Up-regulated genes included U-box type E3 ubiquitin ligase *PUB21* (AT5G37490),

PATTERN-TRIGGERED IMMUNITY (PTI) COMPROMISED RECEPTOR-LIKE CYTOPLASMIC KINASE 1 (*PCRK1*; AT3G09830), while down-regulated genes included *EARLY NODULIN-LIKE PROTEIN 8* (*ENODL*8; AT1G64640), and *XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE* 8 (*XTH8*; AT1G11545).

M. phaseolina responsive genes in resistant genotypes have unique differential expression patterns

To understand what is causing the loss in differential expression correlated with time in resistant genotypes, DEGs that apply to both of the following criteria were isolated: (1) increasing in amplitude of up or down regulation with time in resistant genotypes; and (2) differential expression stayed constant or decreased in amplitude of up or down regulation with time in susceptible genotypes. This was calculated by first taking the absolute value of the $\log_2$-fold change, then calculating the rate of increase or decrease in respect to hpi. Genes were kept if the rate was positive for resistant genotypes and negative or zero for susceptible genotypes. Genes for which all samples showed a $\log_2$-fold change between <1 and >-1 were also filtered out. In total, 120 DEGs fit this criterion, which is referred to as *resistant increasing DEGs*. The average $\log_2$-fold change across samples was positive for 66 genes and negative for 54 genes. GO term enrichment revealed a single term for up-regulated genes in this set: “Response to oxygen-containing compound” (*p*=3.50E-02). Down-regulated genes were enriched in GO terms

including “External encapsulating structure organization” (*p*=4.40E-04), “Cell wall organization” (*p*=7.70E-04), “Polysaccharide metabolic proc.” (*p*=3.10E-03), “Regulation of cell fate specification” (*p*=3.20E-03), and “Regulation of root morphogenesis” (*p*=3.60E-02).

Furthermore, the following question was proposed: is the decreasing differential expression in resistant genotypes a result of active suppression via gene transcript levels due to biological processes antagonistic to defense, or passive, in that lack of immunity-triggered signals at later time points allows for natural decline of defense-related processes? To gain insight to this, resistant genotype unique DEGs with decreasing differential expression, or *resistant decreasing DEGs,* were identified. First, the rate of change in the absolute value of $\log_2$-fold change across 12, 24, and 48 hpi was calculated for each DEG in resistant-genotype samples. A total of 3,364 genes had negative values in both resistant genotypes. From this, genes with $\log_2$-fold change between -1 and 1 in all resistant samples and genes that also had a decreasing DE trend in susceptible genotypes were filtered out. In total, 714 genes, 548 up-regulated and 166 down-regulated, were identified. Up-regulated genes were enriched in biotic stress-related GO terms including “Response to chitin” (*p*=5.40E-16), “Cellular response to hypoxia” (*p*=1.20E-21), “Response to oxygen-containing compound” (*p*=6.80E-16), “Cellular response to chemical stimulus” (*p*=1.40E-14), “Response to external biotic stimulus” (*p*=2.00E-08), and “Response to hormone” (*p*=1.10E-07). GO term analysis on the down-regulated genes showed enrichment in terms related to the

cell wall metabolism, including “Xyloglucan metabolic proc.” (*p*=1.60E-04), “Cell wall polysaccharide metabolic proc.” (*p*=2.60E-04), “Glucan metabolic proc.” (*p*=2.20E-06), “Cell wall biogenesis” (*p*=3.20E-04), and “Cell wall organization” (*p*=9.40E-05). Major biological processes enriched in each DEG group are summarized in Figure 2.11.


Response to absence of light GO:9646
Response to salt GO:1902074
Defense response to insect GO:2213
Immune system process GO:2376
Regulation of immune system process GO:2682
Defense response GO:6952
Immune response GO:6955
Response to biotic stimulus GO:9607
Response to bacterium GO:9617
Response to chitin GO:10200
Regulation of defense response GO:31347
Defense response to bacterium GO:42742
Biological process involved in interspecies interaction between organisms GO:44419
Regulation of stomatal closure GO:90333
Stomatal movement GO:10118
Cell wall macromolecule catabolic process GO:16998
Cytoskeleton-dependent intracellular transport GO:22402
External encapsulating structure organization GO:45229
Carboxylic acid catabolic process GO:46395
Defense response by callose deposition GO:52542
Callose localization GO:52545
Cell wall organization or biogenesis GO:71554/GO:71555/GO:42546
Carbohydrate related process GO:5975/GO:1901136/GO:16051
Polysaccharide related process GO:5976/GO:33037
Process utilizing autophagic mechanism GO:61919
Autophagy GO:6914
Proteasomal ubiquitin-independent protein catabolic process GO:10499
Protein ubiquitination GO:16567
Regulation of protein ubiquitination GO:31145
Toxin metabolic process GO:9404
Response to toxic substance GO:9636
Detoxification GO:98754
Root epidermal cell differentiation GO:10053
Regulation of cell fate GO:10453/GO:42659
Maintenance of shoot apical meristem identity GO:10492
Photosynthesis GO:15979
Cell growth GO:16049
Cell cycle process GO:22402
Cell cycle phase transition GO:44380
Positive regulation of meiotic nuclear division GO:45836
Positive regulation of growth GO:45927
Post-embryonic root development GO:48528
Cell division GO:51301
Specification of plant organ axis polarity GO:90708
Response to reactive oxygen species GO:302
Response to oxidative stress GO:6979
Hydrogen peroxide metabolic process GO:42743
Cellular response to hypoxia GO:71456
Reactive oxygen species metabolic process GO:72593
Peroxisome organization and transportation GO:7031/GO:15919/GO:43574
Response to oxygen levels GO:70482
Response to abscisic acid GO:9737
Auxin metabolic process GO:9850
Regulation of salicylic acid metabolic process GO:10337
Indole-containing compound related process GO:42430/GO:42435/GO:42436
S-adenosylmethionine metabolic process GO:46500
Leaf senescence GO:10150
Regulation of cell death GO:10941
Programmed cell death GO:12501
Regulation of leaf senescence GO:1900055
Positive regulation of leaf senescence GO:1900057
Cell death GO:8219
Glycosinolate metabolic process GO:16554
Secondary metabolic process GO:19748
Glutathione metabolic process GO:6749
Phenol-containing compound metabolic process GO:18958
Regulation of isoprenoid metabolic process GO:19747
Acylglycerol transport GO:34196
Organonitrogen compound related process GO:1901565/GO:1901566
Organic hydroxy compound related process GO:1901615/GO:1901617
7 8 9 10 11 12 13 14 15 16 17 18
GO_category
Abiotic stress
Biotic stress
Cell wall
Degradation
Detoxification
Growth/differentiation
Oxidation stress
Phytohormone
Programmed cell death
Secondary metabolism
p -value
0.04
0.03
0.02
0.01
7: DEGs up regulated in all genotypes/time points
8: DEGs down regulated in all genotypes/time points
9: DEGs with increasing up regulation/time in resistant genotypes
10: DEGs with increasing down regulation/time in resistant genotypes
11: DEGs with decreasing up regulation/time in resistant genotypes
12: DEGs with decreasing down regulation/ime in resistant genotypes
13: DEGs with increasing up regulation/time in susceptible genotypes
14: DEGs with increasing down regulation/time in susceptible genotypes
15: DEGs with decreasing up regulation/time in susceptible genotypes
16: DEGs with decreasing down regulation/time in susceptible genotypes
17: DEGs up regulated in resistant genotypes and down regulated in susceptible genotypes
18: DEGs downregulated in resistant genotypes and up regulated in susceptible genotypes

Figure 2.11. Curated list of GO term enrichment on *M. phaseolina* responsive DEGs. Full DEG lists obtained from *ShinyGO* (v0.741; http://bioinformatics.sdstate.edu/go/) were simplified and reduced with *REVIGO* (http://revigo.irb.hr/), then further categorized by larger groups of similar biological functions. GO terms related to stress responses, with an emphasis on biotic stress, as well as terms representative of trends in enriched biological processes, are depicted here. Similar GO terms may be bundled into one row, in which case it is indicated by GO term IDs separated with a slash (/). For merged rows, the lowest *p*-value is depicted if overlapping of enrichment occurs for a DEG group. A full list of GO terms found for each group of DEGs can be found in the supplementary data. The numbers on the x axis denote each DEG group for which analysis was conducted.

Despite having contradictory patterns of expression over time, down-regulated genes with decreasing amplitude and increasing amplitude were both enriched in GO terms related to cell wall organization and biogenesis. Given this, it seems less likely that an actively induced biological process is contributing to the dampening of defense responses in resistant genotypes. Out of 120 genes in *resistant increasing DEGs*, 12 were associated with cell wall-related GO terms, and of those 12, three were up-regulated in response to *M. phaseolina.* Two of the three up-regulated genes encode pectin metabolism genes, *PECTIN METHYL ESTERASE INHIBITOR-PECTIN METHYLESTERASE 18* (*PMEI-PME18*; AT1G11580) and *PECTIN METHYLESTERASE 41* (*PME41*; AT4G02330). The third gene AT2G16385 encodes *CASPARIAN STRIP INTEGRITY FACTOR 1,* which was only expressed in 48-hpi samples of Col-0 and resistant genotypes Copac-1 and Tuewa1-2. Suppressed genes include *XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 2* (*XTH2*; AT4G13090), a pectin lyase-like superfamily protein (AT2G19150), and *CALLOSE SYNTHASE 1* (*CALS1*; AT1G05570).

Although GO term enrichment was not identified, other biological processes may also be important, as indicated by strong differential expression, either basal or in response to *M. phaseolina*, seen in several transcription factors. For example, AT4G34400, or *TARGET OF FLC AND SVP1* (*TFS1*), a transcription factor that promotes floral transition. In susceptible genotypes, this gene was consistently up-regulated irrespective of the time point at which samples were collected. This was

interesting considering that the basal expression of *FLOWERING LOCUS C* (*FLC*; AT5G10140) was extremely strong only in resistant genotypes, and late flowering phenotypes have been linked to resistance against *F. oxysporum* (Lyons *et al*., 2015). What's more puzzling is that Copac-1 and Tuewa1-2 are not considered late flowering accessions, according to previously documented phenotypes (https://arapheno.1001genomes.org/phenotype/262/). Pearson correlation coefficients between growth inhibition phenotype and days-to-flowering (in 16°C and 10°C) were negative, -0.23 and -0.20 respectively, and not significant according to a p-value threshold of 0.01 (Figure 2.12).

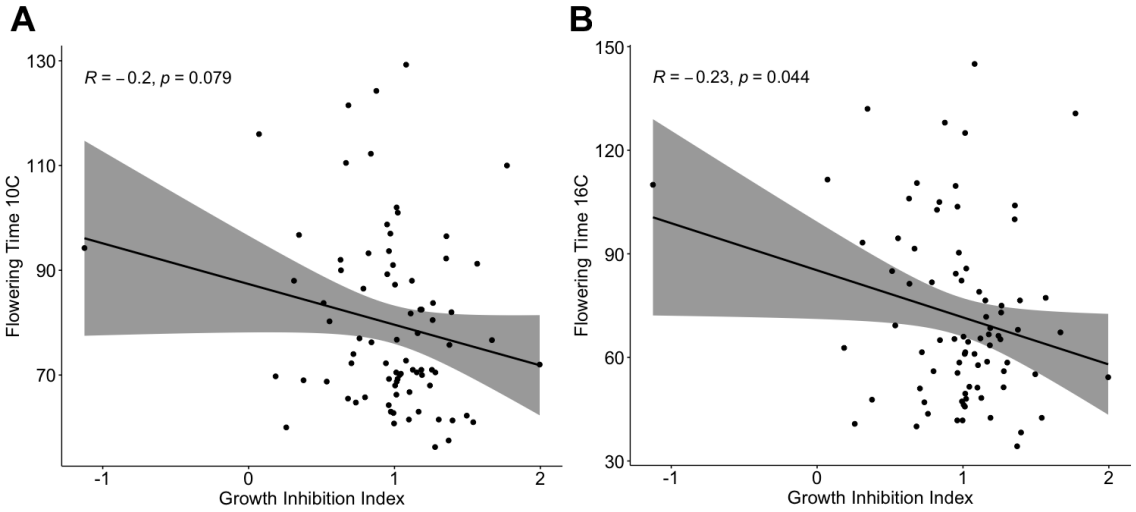

Figure 2.12. Growth inhibition index (GII) shows slightly negative correlation with flowering time. GII values for 80 accessions (for which both GII and flowering time data was available) were plotted against days to flowering for *A. thaliana* grown in (A) 10°C conditions and (B) 16°C conditions. Out of 87 accessions tested in the accession screen, 7 were not included as flowering time data were not available. Pearson correlation coefficients are calculated to check statistical significance. The solid line represents the regression line, and confidence intervals are represented by gray areas on the plot.

Major differences in *M. phaseolina* induced transcript level regulation are seen between susceptible and resistant genotypes

Conversely, *susceptible increasing DEGs* were identified by the following criteria: (1) increasing in amplitude of up or down regulation with time in susceptible genotypes and (2) differential expression stayed constant or decreased in amplitude of up or down regulation with time in resistant genotypes. Genes with no samples with $\log_2$ fold change <1 or >-1 were also filtered out. Of the 3,992 genes that remained, 2,159 were down-regulated in susceptible genotypes and 1,833 were up-regulated. In up-regulated genes, enrichment of GO terms suggesting activation of biotic stress, response to oxygen-containing and organonitrogen compounds, and protein metabolism and modification, were seen. Interestingly, the GO terms “Autophagy” (p=1.40E-14), and “Cellular response to hypoxia” (p=1.10E-13) were also enriched. In down-regulated genes, enrichment of GO terms related to gene expression including “Translation” (*p*=9.20E-96), “Ribonucleoprotein complex biogenesis” (*p*=1.80E-53), “Ribosome assembly” (*p*=4.30E-24), and “DNA replication” (*p*=2.50E-20) were observed.

In order to capture the full scope of trends in genes uniquely regulated in susceptible genotypes, genes with positive and negative DE decreasing with respect to infection time were identified. In total 261 genes were found in this set-42 up-regulated and 219 down-regulated. GO term enrichment on the up-regulated genes included “Sulfate assimilation” (*p*=1.70E-02), “Chitin metabolic proc.” (*p*=1.70E-02), “Amino sugar catabolic proc.” (*p*=1.70E-02), and “Carbohydrate

derivative catabolic proc." (*p*=1.70E-02). Genes down-regulated in this set were enriched in GO terms including "Cell cycle" (*p*=1.60E-16), "Microtubule-based proc." (*p*=2.30E-07), "Mitotic cell cycle" (*p*=2.70E-07), "S-glycoside biosynthetic proc." (*p*=5.30E-06), "Glycosinolate biosynthetic proc." (*p*=5.30E-06), and "Leucine biosynthetic proc." (*p*=8.30E-06).

To further characterize the DEG groups identified, genes in each differential expression category were checked to see if they were also identified as JA or SA responsive in the meta analysis conducted by Zhang *et al.* (2020) (Figure 2.13). To our surprise, the greatest percentage of increasingly induced genes in resistant genotypes were seen in SA↑JA↓, SA↓JA↓, and JA↓ groups, potentially indicating active antagonism against phytohormone, specifically JA, regulated gene induction. For genes that are increasingly suppressed in resistant genotypes, it appears that the greatest percentage of JA/SA-controlled genes represented in this group are either suppressed by JA or induced by JA. Because these genes are suppressed in response to *M. phaseolina*, perhaps gene expression is favoring JA responses and antagonizing expression of SA-inducible responses. Furthermore, out of the DEGs that were increasingly induced in susceptible genotypes, 18.2% overlapped with SA-inducible genes according to Zhang *et al.* (2020). The second and third largest groups represented are JA↓ and SA↑JA↓, suggesting that SA-inducible gene expression is favored, while JA-mediated suppression of gene expression is antagonized in response to *M. phaseolina*. Additionally, many DEGs with increasing suppression in susceptible genotypes are

described as JA↓ or SA↓JA↓. Out of the DEGs that show decreasing up regulation, the greatest percentage of JA/SA responsive genes were in the JA↓ and SA↑ category. 24.7% of genes that showed decreasing down regulation in response to *M. phaseolina* are also down-regulated by both SA and JA.

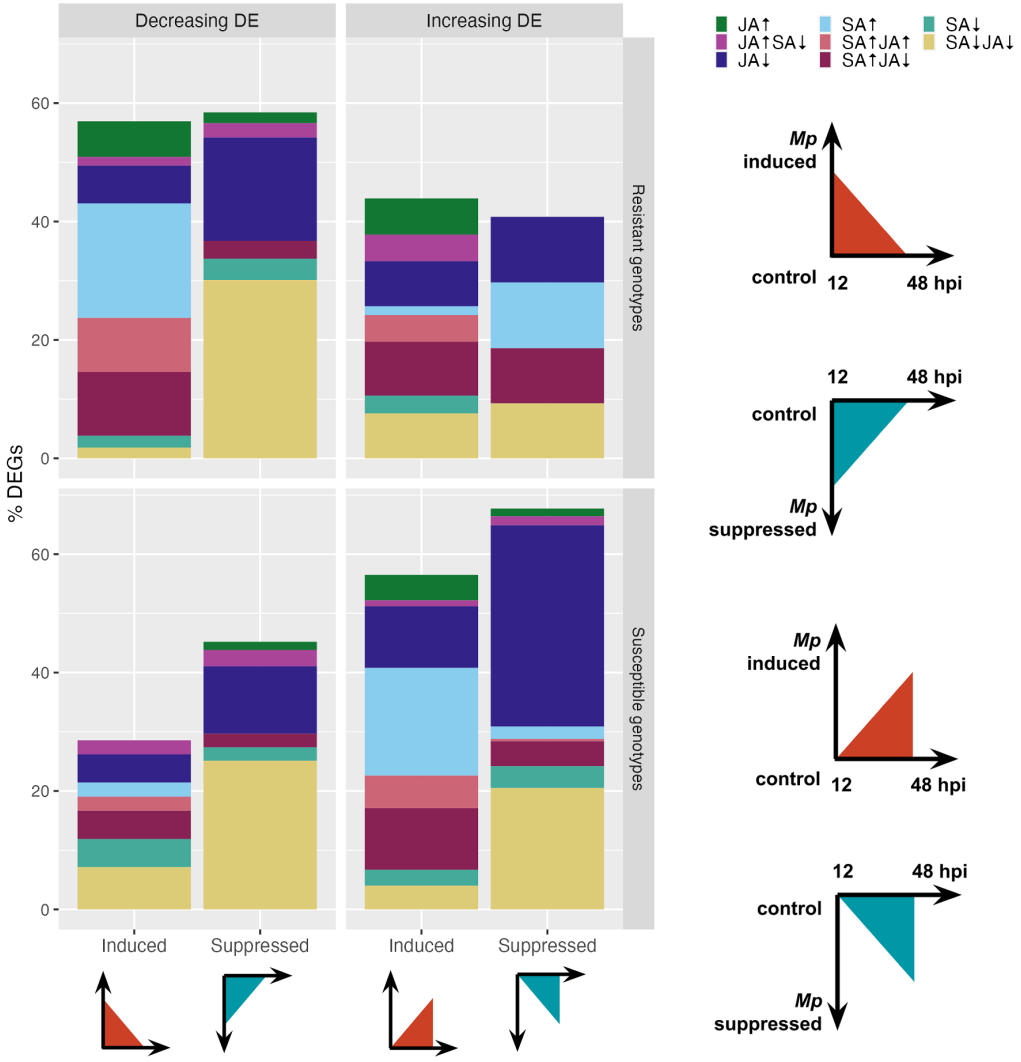

Figure 2.13. Percentage of genes in each DEG category that are found in phytohormone-responsive genes categorized by Zhang *et al.* (2020). Percent of *M. phaseolina*-responsive DEGs in the four categories identified from RNA-seq analysis, which are responsive to SA and/or JA. Genes responsive to JA and/or SA as defined by Zhang *et al.* (2020) are categorized as follows: induced by JA (JA↑), induced by JA and suppressed by SA (JA↑SA↓), suppressed by JA (JA↓), induced by SA (SA↑), induced by SA and JA (SA↑JA↑), induced by SA and suppressed by JA (SA↑JA↓), suppressed by SA (SA↓), and suppressed by SA and JA (SA↓JA↓). Figures on the right summarize trends in DE seen in these DEG groups. From top to bottom: genes with positive DE decreasing in amplitude, negative DE decreasing in amplitude, genes with positive DE increasing in amplitude and genes with negative DE increasing in amplitude, either induced or suppressed in response to *M. phaseolina*.

Contradictory DEGs

Within the pool of total DEGs found across all samples, 227 genes had contradictory expression patterns based on the phenotype. Genes were first filtered based on whether they had significant fold change in at least one resistant sample and one susceptible sample. The average log_2-fold change was calculated for resistant samples and susceptible samples. Genes passed filtering if the mean log_2-fold change in resistant samples was >0, and if the average log_2-fold change in susceptible samples was <0. Of these genes, 120 were up-regulated in resistant genotypes and down-regulated in susceptible genotypes. This group of genes was enriched in several GO terms related to “proteasomal protein catabolic process” (*p*=2.45E-02). In contrast, 107 genes were up-regulated in susceptible genotypes and down-regulated in resistant genotypes. These genes were enriched in the GO terms “response to other organism” (*p*=1.45E-02), “biological process involved in interspecies interaction between organisms” (*p*=1.62E-02), “response to external biotic stimulus” (*p*=1.45E-02), and “response to biotic stimulus” (*p*=1.47E-02).

Discussion

Analysis of the *M. phaseolina* inducible *A. thaliana* transcriptome revealed that the susceptible accessions Ak-1 and UKSE06-252 respond more strongly to infection, compared to the relatively resistant accessions Copac-1 and Tuewa1-2. This is seen in both the number of DEGs and the amplitude of regulation, either

induction or suppression, which increases in correspondence to time post-inoculation. At 12 hpi, transcript profiles in all genotypes show robust induction of defense-related genes at comparable levels, while at 48 hpi there is a clear divergence of genes that have restored their basal expression levels and genes with further increased amplitudes of DE. Moreover, basal level root gene expression was shown to be variable across resistant and susceptible genotypes. Since a significant portion of these basally DEGs are also responsive to *M. phaseolina*, it can be predicted that basal gene expression is an important factor in determining the fate of the host plant once infected with this pathogen. Supporting this hypothesis, GO-term enrichment (Biological Processes) indicated genes involved in defense and biotic stress to be more abundantly expressed at higher basal levels in resistant genotypes in comparison to susceptible genotypes. This multi-variable mRNA-seq assay involving five genotypes, three time points and two conditions allows for a novel three-dimensional assessment of host plant response against *M. phaseolina.*

Who has the upper hand?

Herein lies a question of causation that must be addressed in order to interpret results obtained from further experimentation on this pathosystem. Is the suppression of defense gene induction in resistant genotypes at later infection time points a result of active suppression, either by the host itself or the pathogen? Alternatively, is resistance the result of successful immunity, which consequently leads to dampening of defense gene induction? It seems unlikely that the pathogen

is deliberately suppressing defense-gene induction, given that genotypes exhibiting such transcript-profile trends are more resistant to *M. phaseolina*. However, there are many examples of pathogenic fungi that suppress host-defense genes through effector proteins, and therefore this possibility cannot be ruled out. In this investigation, we sought to filter genes by categories of expression patterns in order to gain insight to this “chicken-or-egg” problem.

Genes with low basal read counts in resistant genotypes and high basal read counts in susceptible genotypes, and vice versa, were assessed whether they were also responsive to *M. phaseolina* (up-regulated or down-regulated). Out of the *M. phaseolina*-responsive DEGs, genes which showed a reduction in differential expression in correspondence to time post-inoculation were also identified. Finally, genes with increasing differential expression in response to *M. phaseolina* across time, both uniquely in resistant and susceptible genotypes, were assessed. Several known defense-related biological processes including phytohormone response and cell wall component metabolism, but also other processes, such as hypoxia and response to oxidative stress, which have not been previously emphasized in studies on host responses to M. *phaseolina*, arose through this analysis. Understanding the regulatory mechanisms underlying the expression patterns associated with these genes will provide new insights to the factors that determine host resistance against this pathogen.

Defense phytohormones SA, JA

The Eulgem lab has previously shown through transcriptomics analysis that multiple hormone-response pathways, namely JA, SA, and ET contribute cooperatively to *A. thaliana* resistance against *M. phaseolina (Schroeder et al., 2019)*. Gaige *et al.* (2010) observed in *Medicago truncatula* that JA and ET responses to *M. phaseolina*, although present in both shoot and root, were weaker in the root tissue, to which the authors hypothesized may be caused by active interference by the pathogen (Gaige *et al*., 2010). This suggests that transcript accumulation of genes seen by both Schroeder *et al*. (2019) and in this experiment may be substantially more intense in the shoot tissue. Involvement of JA and ET, as well as auxin. In root, responses to *M. phaseolina* have also been supported in *Medicago truncatula*, where Mah *et al.* (2012) proposed that the pathogen suppresses auxin responses in the host. Although multiple transcriptomics studies have suggested that *M. phaseolina* interferes with host gene expression, no direct evidence of this occurring exists to date. The lack of a temporal aspect in gene expression profiling makes it difficult to untangle the causational relationship in the context of defense-gene regulation, especially because crosstalk between phytohormone-response pathways is highly complex. Chowdhury *et al.* (2017) observed a distinct morphological change in *M. phaseolina* hyphae, as well as activation of SA responses followed by induction of JA-regulated genes, and accumulation of ROS in sesame (*Sesamum indicum L.*). The timing of these responses, indicating the fungal biotrophy to necrotrophy switch (BNS), was

delayed in relation to susceptibility of the variety tested, which was 36-38 hpi for the resistant variety and 24 hpi for the susceptible variety. In this study, 19% of genes found to be up-regulated exclusively in resistant genotypes but decreasing in magnitude of DE in correspondence to time, were SA-inducible genes (Figure 2.11). Only 2% of genes showing similar patterns of expression in susceptible genotypes were SA inducible, however SA-inducible genes had a strong presence (18%) in up-regulated genes that increase in magnitude of DE with time. These results suggest that between 12 and 48 hpi, SA pathway induction is winding down in resistant genotypes, whereas induction is being amplified in susceptible genotypes. JA-mediated gene suppression seems to be showing a similar trend. Although both SA- and JA-regulated gene expression was already detectable in the 12 hpi timepoint in this experiment, subtle shifts in phytohormone-regulated gene expression, presumably caused by the BNS in *M. phaseolina*, were captured in this investigation.

Several key players of SA and JA signaling had distinct expression patterns in *A. thaliana* roots among genotypes which are relatively resistant, such as Copac-1 and Tuewa1-2, relatively susceptible, such as Ak-1 and UKSE06-252, and Col-0. For example, the *Arabidopsis thaliana* regulator of systemic acquired resistance (SAR) *NONEXPRESSER OF PR GENES 1* (*NPR1*) responds to SA and activates transcription of *PR* genes including *PR1* and *PR2 (J. M. Zhou et al., 2000)*. *M. phaseolina*-responsive *NPR1* up regulation is seen in most samples, but is absent in 12/48 hpi Tuewa1-2, 24/48 hpi Copac-1, and 48 hpi Col-0. The loss of

up regulation corresponds to that of *WRKY70*, an *NPR-1* dependent transcription factor that also acts as a repressor in the JA-mediated pathway (Li *et al*., 2004). While differential expression of *PR1* was not found in any of the comparisons, *PR2* exhibited strong up-regulation exclusively in Tuewa1-2 samples at all timepoints and at 48 hpi Copac-1 (Figure 2.8). *PR5* also showed strong up-regulation at 48 hpi in Copac-1 and Ak-1 (data not shown). Pathogen-induced regulation of *PR2, PR5* and *PR1* in the root has been observed previously in other pathosystems such as nematodes, showing that *PR2* and *PR5* can be regulated not only by SA-independent processes (Wubben *et al*., 2008), but also potentially due to pathogen interference (Molinari *et al*., 2014). Whether *M. phaseolina* is capable of attenuating SA-mediated responses in the susceptible genotypes remains to be seen.

PR2 encodes a β-1,3-glucanase (BGL), which can hydrolyze β-1,3-glycosidic bonds in the cell wall during normal growth and development, and is also known to protect plants against fungal pathogens (Datta & Muthukrishnan, 1999; Oide *et al*., 2013). Together with chitinases, BGLs can hydrolyze fungal cell walls and release β-1,3-glucan and chitin oligosaccharides, which act as plant signals for immune response induction (Balasubramanian *et al*., 2012). Chitin metabolism was among the top enriched GO terms associated with genes of decreasing DE (both up- and down-regulated), and a majority of the significantly enriched GO terms in down-regulated gene sets unique to resistant genotypes were related to the cell wall. Furthermore, among genes with high basal expression

in resistant genotypes were cell wall modifiers like (*MODIFYING WALL LIGNIN-2*; AT4G19370) and chitinases (*CHITINASE*; AT2G43570). *PR2* can also inhibit callose accumulation, and this process is regulated by ABA suppression of *PR2* (Oide et al., *2013*). This suggests that *PR2* may have dual (antagonistic and synergystic) roles in fungal pathogen defense. Overexpression of *BGL2* (*PR2*) increased susceptibility to the necrotrophic fungal pathogens *Leptosphaeria maculans, Alternaria brassicicola* and *Botrytis cinerea,* but reduced susceptibility to *Pseudomonas syringae* DC3000 (Oide *et al*., 2013). In the literature (Doxey *et al*., 2007)*, PR2* is suggested to be a leaf-expressed gene, but it is robustly upregulated in our root RNA-seq data. Zero reads were detected in the control samples, indicating that this gene is induced only after *M. phaseolina* exposure. Doxey *et al.* (2007) grouped 50 *PR2*-like genes based on expression patterns and found that many of these genes are strongly co-expressed. *PR2* is grouped with three other β-1,3-glucanases, but significant fold change in response to *M. phaseolina* was not seen for these genes. Notably genes in β-1,3-glucanase cluster M, predicted to primarily have roles in cell wall morphogenesis and cell division, were down-regulated across all timepoints most strongly in susceptible genotypes. Given these results, the suppression of cell wall-related genes in response to *M. phaseolina* may be linked to suppression of host growth and development to prioritize immune responses. Alternatively, the pathogen may be interfering with host defense mechanisms involving cell wall fortification. Specifically in the susceptible genotypes, genes with down regulation of

decreasing amplitude are enriched in GO terms related to mitotic cell cycle, which is plausible if the plant is slowing its overall metabolism to focus resources on pathogen defense. On the other hand, up regulation of genes like *PR2*, *CHITINASE*, and genes involved in callose deposition in resistant genotypes are likely to have more active roles in preventing *M. phaseolina* penetration of the root tissue. It is also important to note that the *M. phaseolina* genome also encodes a large number of glycoside hydrolases, and therefore carbohydrate metabolism may play a key role in *A. thaliana*-*M. phaseolina* interactions (Islam *et al*., 2012). Further experimentation would be required to determine if *M. phaseolina* effectors are involved in the regulation of these cell wall-related genes.

Hypoxia

Another GO term with prominent presence in this dataset is hypoxia. Hypoxia is seen in the GO-term enrichment of genes commonly up-regulated across all genotypes, genes up-regulated in resistant genotypes with a decreasing trend, as well as in genes up-regulated in susceptible genotypes with an increasing trend. Although to our knowledge *M. phaseolina* is not known to induce oxygen deprivation in host plants, it has been recently shown that *B. cinerea* causes increased respiration, which leads to a locally hypoxic site (Valeri *et al*., 2021). GO-term enrichment in the RNA-seq analysis performed on soybean DEGs in response to *M. phaseolina* also noted the presence of “cellular response to hypoxia” (Yan *et al*., 2021). Hypoxia response is coordinated by a conserved set of ethylene response factors (ERF) in the group ERF-VII, which are transcription

factors that are stable only in the absence of oxygen (Giuntoli & Perata, 2018). Expression of ERF-VII *RELATED TO AP2 2* (*RAP2.2*) is known to enhance resistance against *B. cinerea* (Zhao *et al*., 2012). Another ERF-VII transcription factor *RELATED TO AP2.3* (*RAP2.3*) interacts with another ERF, *OCTADECANOID-RESPONSIVE ARABIDOPSIS 59* (*ORA59*), in the ethylene mediated pathway (Kim *et al*., 2018). Overexpression of *ORA59* is known to increase resistance to *B. cinerea,* and furthermore it is responsive to JA- and ethylene-mediated transcription of defense-related genes downstream in both pathways (Pré *et al*., 2008). Therefore, *RAP2.3* and *ORA59* are potentially a point of convergence between hypoxia and pathogen response. Strong up regulation of *ORA59* is seen in response to *M. phaseolina* in most samples except 12 and 48 hpi in UKSE06-252. Another hypoxia responsive transcription factor, *HYPOXIA RESPONSIVE ERF 2* (*HRE2*), is also induced strongly in all samples tested. *HRE2* initiates adventitious root formation, which can also be suppressed with ethylene, allowing for a shift in the root system to gain access to more oxygen (Eysholdt-Derzsó & Sauter, 2019). Seedlings from which RNA was collected in this experiment were largely exposed to open air, as they were laid atop the *M. phaseolina* hyphae, placed vertically so condensation would not accumulate, and wrapped with breathable millipore tape. The only plausible explanation for such robust hypoxia-related gene expression would be that the pathogen is causing a hypoxic state at the site of infection. This may be a key detail previously unknown

for this pathogen, as hypoxia response is also mediated by genes involved in ethylene and JA pathway with multiple functions.

Oxidative stress

Waterlogging, which causes hypoxia and ROS accumulation, induces programmed cell death (PCD) via autophagy (Guan *et al*., 2019). Genes up-regulated with increasing magnitude in susceptible genotypes are enriched in the GO term “autophagy” (*p*=1.40E-14), along with “Cellular response to hypoxia” (*p*=1.10E-13). Although “autophagy” was not enriched, genes up-regulated with decreasing magnitude in resistant genotypes are also highly associated with GO terms related to hypoxia and oxidative stress. Autophagy is a widely conserved stress-induced degradation and recycling process that discards apoptosis-inducing constituents in the cell, but is also known to have dual functions in positively regulating hypersensitive response- induced cell death (Coll *et al*., 2014; Hofius *et al*., 2009). In recent work, mounting evidence suggests that during pathogen attack, autophagy acts as a balancing mechanism between ROS accumulation and cell survival, and that autophagy favors defenses against necrotrophic pathogens (Leary *et al*., 2018; Tang & Bassham, 2018; S. Zhou *et al*., 2018). Autophagy also plays a crucial role in preventing excessive hypersensitive response-triggered cell death by modulating SA-induced immune responses (Yoshimoto *et al*., 2009). Perhaps the induction of hypoxia by *M. phaseolina* causes ROS accumulation, which triggers autophagy in susceptible *A. thaliana* genotypes as disease progresses.

Enrichment of hypoxia- and oxidative stress-response genes in resistant genotypes were found in gene sets whose positive expression reverts to control levels at 48 hpi. It is possible that an early transient ROS accumulation occurs in resistant genotypes, which is either suppressed due to the pathogen or does not accumulate to levels that necessitate autophagy. ROS accumulation in response to biotic stress has several functions, but most notably it, along with NO, induces PCD at the site of pathogen infection to prevent further invasion and to produce immune signals to induce a systemic response (Mur *et al*., 2008; Torres, 2010). *NAC DOMAIN CONTAINING PROTEIN 42,* or *JUNGBRUNNEN1* (*JUB1*), a gene which encodes a NAC transcription factor responsive to ROS, specifically H_2O_2, is strongly up-regulated in response to *M. phaseolina*, but shows a decreasing trend in resistant genotypes in contrast to susceptible genotypes where it shows an increasing trend. Moreover, average basal expression is lower in susceptible genotypes. It is known that overexpression of the gene strongly delays leaf senescence by suppressing GA synthesis and enhances tolerance to various abiotic stresses (Shahnejat-Bushehri *et al*., 2016). Up-regulation of detoxification-related genes are also observed, for example certain classes of glutathione-S-transferases (GST) are responsive to biotic stress and positively contribute to pathogen response (Gullner *et al*., 2018). Three GSTs, *GLUTATHIONE S-TRANSFERASE 7* (*GSTF7*), *GLUTATHIONE S-TRANSFERASE TAU 15* (*GSTU15*), and *GLUTATHIONE S-TRANSFERASE TAU 10* (*GSTU10*), were identified as having higher basal expression in resistant genotypes and responsive

to *M. phaseolina* infection. *GSTF7* and *GSTU10* are known to be robustly upregulated in response to *Alternaria brassicicola*, while *GSTU10* and *GSTU15,* which belong in the same expression group *tau*, were induced by *Pseudomonas syringae* pv. tomato DC3000 expressing the *avrRpt2* effector (Gullner *et al*., 2018). *WRKY70*, also mentioned previously to act downstream of SA/JA and induce *PR2* expression, is also associated with positive regulation of *GSTF7* transcription (Li *et al*., 2004). All three *GST*s' promoter sequences contain potential binding sites for *WRKY70, such as* WT-box motifs (GACTTTT) and W box motifs (YTGACY) (Eulgem & Somssich, 2007; Machens *et al*., 2014).

Taken together, if these *GST* genes contribute to *M. phaseolina* resistance, it may suggest that their enriched basal expression in resistant genotypes prime the plant for defense induction. It is important to note that both the GO terms "cell death" and "plant-type hypersensitive response" are also enriched in resistance genotype DEGs in up-regulated gene sets with decreasing trends, suggesting that a similar response seen in susceptible genotypes also occurs in resistant genotypes. But if so, induction of PCD-related genes likely peaked before 12 hpi in resistant genotypes. It would require transcript profiling at the single cell level at an earlier time point to determine whether sequential onset of SA and ROS accumulation, autophagy, hypersensitive response, and JA gene regulation occurs at the site of hyphae intrusion into the host tissue.

Ethylene-response factors

ETHYLENE RESPONSE FACTOR 96 (*ERF96*) is induced robustly (5-6 log_2-fold) in resistant genotypes, but is not DE in susceptible genotypes. This ET-responsive transcription factor is known to enhance resistance to necrotrophic pathogens and is repressed by SA (Catinot *et al*., 2015). In response to vascular fungal pathogen *Verticillium longisporum*, induction was only seen in roots (Fröschel *et al*., 2019). *ERF96* binds directly to the promoter of *ORA59* and activates expression of this closely related *ERF* gene (Catinot *et al*., 2015), a core regulator of JA/ET-defense pathways. Notably, *ERF96* recognizes GCC box elements (AGCCGCC), which are found within the 1000-bp upstream regions of several genes with unique expression patterns in resistant genotypes (AT3G43250, AT5G53820, AT4G02970, AT3G51450, AT5G12370, AT2G48140, AT1G27420). Like *ERF96*, many *ERF* genes are upregulated in response to *M. phaseolina*. Previously, work by Schroeder *et al.* (2019) suggested that ET has a critical role in defense response against *M. phaseolina*. Data shown here supports this and reveals additional potential roles of ET in pathogen response involving hypoxia.

A. thaliana germplasm as a resource to study *M. phaseolina* pathogenicity

An important finding in this analysis is that *A. thaliana* accessions, which differ in their levels of resistance/susceptibility to *M. phaseolina,* exhibit significant variability in basal gene expression, including genes that may be crucial to plant

defense. The underlying genetic diversity in *A. thaliana* is potentially a source for discovery of resistance haplotypes, which can be exploited through transgenic approaches. It is apparent from our root transcriptome analysis that *A. thaliana* employs robust and acute immune responses. *M. phaseolina*-responsive genes have multiple known defense-related functions, but there is also a significant portion among them with unknown functions. The present study suggested many candidate genes and biological processes not previously associated with *M. phaseolina* response and should serve as a foundational resource for identifying and studying *M. phaseolina*-resistance loci.

Additionally, our results suggest the possibility of a shift in peak defense gene induction for susceptible genotypes relative to resistant genotypes. Earlier transcriptomic induction of defense responses in resistant genotypes was also seen in sesame-*M. phaseolina* interactions, as described by Yan *et al*. (2021) and Radadiya *et al.* (2021). It would require transcriptome profiling at the single cell level to verify this and distinguish local responses from systemic gene induction which could have different roles in determining susceptibility. Since *M. phaseolina* is a hemi-biotrophic pathogen that changes its virulence strategy during infection, a multi- dimensional understanding of disease progression, both in terms of cell type and timeframe, is critical. Knowledge on key biological processes that determine the fate of the host plant will potentially allow for breeding of crops based on particular traits, like hypoxia resistance. Moreover, transgenic approaches may be easier to implement on model systems like *A. thaliana*, which could be

translated to crop species, accelerating research in this field. Untangling the causational relationship between host-pathogen responses will likely elucidate fungal factors that contribute to *M. phaseolina* virulence. If *A. thaliana* responses found in our work are mediated by *M. phaseolina* effectors or other secretants like ROS, they could be targeted for novel fungicide alternatives.

Materials and methods

Plant materials and growth conditions

A. thaliana accessions Columbia (Col-0; CS1092), Achkarren (Ak-1; CS6602), UKSE06-252 (CS78800), Copac (Copac-1; CS76420), and Tubingen - Wanne (TueWa1-2; CS76405) obtained from Arabidopsis Biological Resource Centre (ABRC) were used for transcriptome analysis. Seeds were surface sterilized by soaking five minutes in 70% ethanol, then 10 minutes in 6% sodium hypochlorite, and rinsing five times with sterile water. Seeds (15 to 20) were sown on 100- x 15- mm polystyrene plates with solid 0.87% agar media containing ½ MS (Murashige and Skoog), 0.25% sucrose and 0.05% MES. pH was adjusted to 5.7 with potassium hydroxide. Plates were wrapped in micropore tape and stratified in the dark at 4°C for four days. Next, plates were transferred to a growth room set to long-day conditions (16 h of light/8 h of dark, 23°C, 100 μE m^{-2} s^{-1}) and kept for 10 days. Plates were set up vertically so that roots would grow in a uniform direction towards one side of the plate.

M. phaseolina inoculation

Dried disks of *M. phaseolina* culture on potato dextrose agar (PDA) were used as inoculum. The original propagule was isolated by Phil Roberts at the UC Riverside Agricultural Operations Field 11 (Muchero *et al*., 2011), and propagated by members of the lab of Thomas Eulgem. Disks were cut into small pieces and placed in sterilized mortar and pestle with a small amount of sterile water. *M. phaseolina* is ground until a black slurry forms. This was then placed in ½ MS agar media that has been autoclaved to a liquid state and cooled. The concentration of distinguishable microsclerotium was adjusted to approximately 70 per 100 µL under the microscope. 3 mL of inoculum ½ MS media mix was pipetted onto one side of solid agar ½ MS media so that the inoculum solidifies on one side of the square plate. Plates were wrapped in micropore tape and aluminum foil to limit UV exposure. *M. phaseolina* culture plates were placed in an incubator at 34°C for four days, then transferred to the growth room for two.

At 10 days growth under light for *A. thaliana* and six days growth after transfer to culture medium, 15 seedlings were transferred per plate, to either *M. phaseolina* growth plate or ½ MS plate as a control. At 12, 24, and 48 hpi, roots were removed from plates using a surgical blade from xx plates, placed in 1 mL microtubules, and flash-frozen in liquid nitrogen. Tissue was stored at -80°C.

mRNA-sequencing

Root tissue was ground in liquid nitrogen with a pellet pestle, and total RNA was isolated using TRIzol™ Reagent (Invitrogen™, https://www.thermofisher.com/) per manufacturer's instructions. Total RNA is first processed using the RNeasy MinElute Cleanup Kit (QIAGEN #74204, https://www.qiagen.com/) and the TURBO DNA-free™ Kit (Invitrogen™, https://www.thermofisher.com/), then mRNA was isolated using NEBNext® Poly(A) mRNA Magnetic Isolation Module according to the manufacturer's instructions. mRNA samples were quantified and assessed for quality on the Agilent 2100 Bioanalyzer at the UC Riverside Genomics Core Facility. A total of 90 mRNA libraries, representing five genotypes, two experimental conditions, three time points, and three biological replicates were prepared with NEBNext® Ultra™ II Directional RNA Library Prep Kit for Illumina® with sample purification beads and NEBNext® Multiplex Oligos for Illumina®.

Libraries were pooled and sequenced at the UC Berkeley QB3 facility on the Illumina Novaseq S4 platform. Reads were pre-processed and checked for quality with *fastp* (0.23.2) (Chen *et al*., 2018). The Copac-1, Tuewa1-2, Ak-1, and UKSE06-252 *A. thaliana* reference genomes were constructed based on the TAIR10 Col-0 assembly by applying variant calls, obtained for each genotype from the 1001 Genomes Project (https://1001genomes.org/tools.html), using the *FastaAlernateReferenceMaker* function from GATK (4.3.0.0) (Van der Auwera & O'Connor, 2020). Col-0 gene annotations from TAIR10 were then mapped onto

the genomes using Liftoff (1.6.3) (Shumate & Salzberg, 2020) to generate genotype-specific reference genomes. RNA-seq reads were mapped onto the *A. thaliana* genome of each accession and *M. phaseolina* reference genome MS6 (GCA_000302655) (https://fungi.ensembl.org/) with STAR (2.7.10b) (Dobin *et al*., 2013) to distinguish reads of fungal origin from that of the host. Alignments were sorted using the *sort* function from SAMtools (1.15) (Danecek *et al*., 2021), before calculating read coverage with *featureCounts* from the Subread package (2.0.3) (Liao *et al*., 2013). Genes with significant differential expression between two experimental conditions were identified by DESeq2 (1.36.0.) (Love *et al*., 2014). Sequencing data alignment, counting, and differential expression analysis was performed by Professor Tokuji Tsuchiya at Nihon University, Japan. DEGs with adjusted *p* values less than 0.01 and $\log_2$ fold change greater than 1 or less than -1 were considered for further analysis.

GO-term enrichment analysis

GO-term enrichment was conducted using *ShinyGO* (v0.741) (http://bioinformatics.sdstate.edu/go/) using the default parameters to find enrichment of Biological Process (BP) terms (Ge *et al*., 2020). Genes with a false discovery rate (FDR) less than 0.05 and fold change greater than 2 were considered for analysis. To limit redundancy in terms, *REVIGO* (http://revigo.irb.hr/) was applied to lists larger than 10. GO BP terms for each gene group described are summarized in Supplementary Data 1.

Literature Cited

Anders, S., & Huber, W. (2010). Differential expression analysis for sequence count data. *Genome Biology*, *11*(10), R106.

Baird, R. E., Wadl, P. A., Allen, T., McNeill, D., Wang, X., Moulton, J. K., Rinehart, T. A., Abbas, H. K., Shier, T., & Trigiano, R. N. (2010). Variability of United States isolates of *Macrophomina phaseolina* based on simple sequence repeats and cross genus transferability to related genera within botryosphaeriaceae. *Mycopathologia*, *170*(3), 169–180.

Balasubramanian, V., Vashisht, D., Cletus, J., & Sakthivel, N. (2012). Plant β-1,3-glucanases: their biological functions and transgenic expression against phytopathogenic fungi. *Biotechnology Letters*, *34*(11), 1983–1990.

Burkhardt, A. K., Childs, K. L., Wang, J., Ramon, M. L., & Martin, F. N. (2019). Assembly, annotation, and comparison of *Macrophomina phaseolina* isolates from strawberry and other hosts. *BMC Genomics*, *20*(1), 802.

Catinot, J., Huang, J.-B., Huang, P.-Y., Tseng, M.-Y., Chen, Y.-L., Gu, S.-Y., Lo, W.-S., Wang, L.-C., Chen, Y.-R., & Zimmerli, L. (2015). ETHYLENE RESPONSE FACTOR 96 positively regulates *Arabidopsis* resistance to necrotrophic pathogens by direct binding to GCC elements of jasmonate - and ethylene-responsive defence genes. *Plant, Cell & Environment*, *38*(12), 2721–2734.

Chen, S., Zhou, Y., Chen, Y., & Gu, J. (2018). fastp: an ultra-fast all-in-one FASTQ preprocessor. *Bioinformatics* , *34*(17), i884–i890.

Chezem, W. R., Memon, A., Li, F.-S., Weng, J.-K., & Clay, N. K. (2017). SG2-Type R2R3-MYB Transcription Factor MYB15 Controls Defense-Induced Lignification and Basal Immunity in *Arabidopsis*. *The Plant Cell*, *29*(8), 1907–1926.

Chowdhury, S., Basu, A., & Kundu, S. (2017). Biotrophy-necrotrophy switch in pathogen evoke differential response in resistant and susceptible sesame involving multiple signaling pathways at different phases. *Scientific Reports*, *7*(1), 17251.

Coll, N. S., Smidler, A., Puigvert, M., Popa, C., Valls, M., & Dangl, J. L. (2014). The plant metacaspase AtMC1 in pathogen-triggered programmed cell death and aging: functional linkage with autophagy. *Cell Death and Differentiation*, *21*(9), 1399–1408.

Coser, S. M., Chowda Reddy, R. V., Zhang, J., Mueller, D. S., Mengistu, A., Wise, K. A., Allen, T. W., Singh, A., & Singh, A. K. (2017). Genetic Architecture of Charcoal Rot (*Macrophomina phaseolina*) Resistance in Soybean Revealed Using a Diverse Panel. *Frontiers in Plant Science*, *8*, 1626.

Cunningham, F., Allen, J. E., Allen, J., Alvarez-Jarreta, J., Amode, M. R., Armean, I. M., Austine-Orimoloye, O., Azov, A. G., Barnes, I., Bennett, R., Berry, A., Bhai, J., Bignell, A., Billis, K., Boddu, S., Brooks, L., Charkhchi, M., Cummins, C., Da Rin Fioretto, L., … Flicek, P. (2022). Ensembl 2022. *Nucleic Acids Research*, *50*(D1), D988–D995.

Danecek, P., Bonfield, J. K., Liddle, J., Marshall, J., Ohan, V., Pollard, M. O., Whitwham, A., Keane, T., McCarthy, S. A., Davies, R. M., & Li, H. (2021). Twelve years of SAMtools and BCFtools. *GigaScience*, *10*(2). https://doi.org/10.1093/gigascience/giab008

Datta, S. K., & Muthukrishnan, S. (1999). *Pathogenesis-Related Proteins in Plants*. CRC Press.

Dobin, A., Davis, C. A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M., & Gingeras, T. R. (2013). STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* , *29*(1), 15–21.

Doxey, A. C., Yaish, M. W. F., Moffatt, B. A., Griffith, M., & McConkey, B. J. (2007). Functional divergence in the *Arabidopsis* beta-1,3-glucanase gene family inferred by phylogenetic reconstruction of expression states. *Molecular Biology and Evolution*, *24*(4), 1045–1055.

Eulgem, T., & Somssich, I. E. (2007). Networks of WRKY transcription factors in defense signaling. *Current Opinion in Plant Biology*, *10*(4), 366–371.

Eysholdt-Derzsó, E., & Sauter, M. (2019). Hypoxia and the group VII ethylene response transcription factor HRE2 promote adventitious root elongation in *Arabidopsis*. *Plant Biology* , *21 Suppl 1*(Suppl Suppl 1), 103–108.

Fröschel, C., Iven, T., Walper, E., Bachmann, V., Weiste, C., & Dröge-Laser, W. (2019). A Gain-of-Function Screen Reveals Redundant ERF Transcription Factors Providing Opportunities for Resistance Breeding Toward the Vascular Fungal Pathogen *Verticillium longisporum*. *Molecular Plant-Microbe Interactions: MPMI*, *32*(9), 1095–1109.

Gaige, A. R., Ayella, A., & Shuai, B. (2010). Methyl jasmonate and ethylene induce partial resistance in *Medicago truncatula* against the charcoal rot pathogen *Macrophomina phaseolina*. *Physiological and Molecular Plant Pathology*, *74*(5), 412–418.

Ge, S. X., Jung, D., & Yao, R. (2020). ShinyGO: a graphical gene-set enrichment tool for animals and plants. *Bioinformatics* , *36*(8), 2628–2629.

Giuntoli, B., & Perata, P. (2018). Group VII Ethylene Response Factors in *Arabidopsis:* Regulation and Physiological Roles. *Plant Physiology*, *176*(2), 1143–1155.

Guan, B., Lin, Z., Liu, D., Li, C., Zhou, Z., Mei, F., Li, J., & Deng, X. (2019). Effect of Waterlogging-Induced Autophagy on Programmed Cell Death in *Arabidopsis* Roots. *Frontiers in Plant Science*, *10*, 468.

Gullner, G., Komives, T., Király, L., & Schröder, P. (2018). Glutathione S-Transferase Enzymes in Plant-Pathogen Interactions. *Frontiers in Plant Science*, *9*, 1836.

Hofius, D., Schultz-Larsen, T., Joensen, J., Tsitsigiannis, D. I., Petersen, N. H. T., Mattsson, O., Jørgensen, L. B., Jones, J. D. G., Mundy, J., & Petersen, M. (2009). Autophagic components contribute to hypersensitive cell death in *Arabidopsis*. *Cell*, *137*(4), 773–783.

Islam, M. S., Haque, M. S., Islam, M. M., Emdad, E. M., Halim, A., Hossen, Q. M. M., Hossain, M. Z., Ahmed, B., Rahim, S., Rahman, M. S., Alam, M. M., Hou, S., Wan, X., Saito, J. A., & Alam, M. (2012). Tools to kill: genome of one of the most destructive plant pathogenic fungi *Macrophomina phaseolina*. *BMC Genomics*, *13*, 493.

Kim, N. Y., Jang, Y. J., & Park, O. K. (2018). AP2/ERF Family Transcription Factors ORA59 and RAP2.3 Interact in the Nucleus and Function Together in Ethylene Responses. *Frontiers in Plant Science*, *9*, 1675.

Koike, S. T., Arias, R. S., Hogan, C. S., Martin, F. N., & Gordon, T. R. (2016). Status of *Macrophomina phaseolina* on Strawberry in California and Preliminary Characterization of the Pathogen. *Guoshu Xuebao = Journal of Fruit Science*, *16*(sup1), 148–159.

Leary, A. Y., Sanguankiattichai, N., Duggan, C., Tumtas, Y., Pandey, P., Segretin, M. E., Salguero Linares, J., Savage, Z. D., Yow, R. J., & Bozkurt, T. O. (2018). Modulation of plant autophagy during pathogen attack. *Journal of Experimental Botany*, *69*(6), 1325–1333.

Liao, Y., Smyth, G. K., & Shi, W. (2013). featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics* , *30*(7), 923–930.

Li, J., Brader, G., & Palva, E. T. (2004). The WRKY70 transcription factor: a node of convergence for jasmonate-mediated and salicylate-mediated signals in plant defense. *The Plant Cell*, *16*(2), 319–331.

Llanos, A., François, J. M., & Parrou, J.-L. (2015). Tracking the best reference genes for RT-qPCR data normalization in filamentous fungi. *BMC Genomics*, *16*(1), 71.

Love, M. I., Huber, W., & Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology*, *15*(12), 550.

Lyons, R., Rusu, A., Stiller, J., Powell, J., Manners, J. M., & Kazan, K. (2015). Investigating the Association between Flowering Time and Defense in the *Arabidopsis thaliana-Fusarium oxysporum* Interaction. *PloS One*, *10*(6), e0127699.

Machens, F., Becker, M., Umrath, F., & Hehl, R. (2014). Identification of a novel type of WRKY transcription factor binding site in elicitor-responsive cis-sequences from *Arabidopsis thaliana*. *Plant Molecular Biology*, *84*(4-5), 371–385.

Mah, K. M., Uppalapati, S. R., Tang, Y., Allen, S., & Shuai, B. (2012). Gene expression profiling of *Macrophomina phaseolina* infected *Medicago truncatula* roots reveals a role for auxin in plant tolerance against the charcoal rot pathogen. *Physiological and Molecular Plant Pathology*, *79*, 21–30.

Molinari, S., Fanelli, E., & Leonetti, P. (2014). Expression of tomato salicylic acid (SA)-responsive pathogenesis-related genes in Mi-1-mediated and SA-induced resistance to root-knot nematodes. *Molecular Plant Pathology*, *15*(3), 255–264.

Muchero, W., Ehlers, J. D., Close, T. J., & Roberts, P. A. (2011). Genic SNP markers and legume synteny reveal candidate genes underlying QTL for *Macrophomina phaseolina* resistance and maturity in cowpea [*Vigna unguiculata* (L) Walp.]. *BMC Genomics*, *12*, 8.

Mur, L. A. J., Kenton, P., Lloyd, A. J., Ougham, H., & Prats, E. (2008). The hypersensitive response; the centenary is upon us but how much do we know? *Journal of Experimental Botany*, *59*(3), 501–520.

Oide, S., Bejai, S., Staal, J., Guan, N., Kaliff, M., & Dixelius, C. (2013). A novel role of PR2 in abscisic acid (ABA) mediated, pathogen-induced callose deposition in *Arabidopsis thaliana*. *The New Phytologist*, *200*(4), 1187–1199.

Pré, M., Atallah, M., Champion, A., De Vos, M., Pieterse, C. M. J., & Memelink, J. (2008). The AP2/ERF domain transcription factor ORA59 integrates jasmonic acid and ethylene signals in plant defense. *Plant Physiology*, *147*(3), 1347–1357.

Radadiya, N., Mangukia, N., Antala, V., Desai, H., Chaudhari, H., Dholaria, T. L., Dholaria, D., Tomar, R. S., Golakiya, B. A., & Mahatma, M. K. (2021). Transcriptome analysis of sesame-*Macrophomina phaseolina* interactions revealing the distinct genetic components for early defense responses. *Physiology and Molecular Biology of Plants: An International Journal of Functional Plant Biology*, *27*(8), 1675–1693.

Schroeder, M. M., Lai, Y., Shirai, M., Alsalek, N., Tsuchiya, T., Roberts, P., & Eulgem, T. (2019). A novel *Arabidopsis* pathosystem reveals cooperation of multiple hormonal response-pathways in host resistance against the global crop destroyer *Macrophomina phaseolina*. *Scientific Reports*, *9*(1), 20083.

Shahnejat-Bushehri, S., Nobmann, B., Devi Allu, A., & Balazadeh, S. (2016). JUB1 suppresses *Pseudomonas syringae*-induced defense responses through accumulation of DELLA proteins. *Plant Signaling & Behavior*, *11*(6), e1181245.

Shumate, A., & Salzberg, S. L. (2020). Liftoff: accurate mapping of gene annotations. *Bioinformatics* , *37*(12), 1639–1643.

Su, G., Suh, S. O., Schneider, R. W., & Russin, J. S. (2001). Host Specialization in the Charcoal Rot Fungus, *Macrophomina phaseolina*. *Phytopathology*, *91*(2), 120–126.

Suzuki, R., & Shimodaira, H. (2006). Pvclust: an R package for assessing the uncertainty in hierarchical clustering. *Bioinformatics* , *22*(12), 1540–1542.

Tang, J., & Bassham, D. C. (2018). Autophagy in crop plants: what's new beyond *Arabidopsis*? *Open Biology*, *8*(12). https://doi.org/10.1098/rsob.180162

Torres, M. A. (2010). ROS in biotic interactions. *Physiologia Plantarum*, *138*(4), 414–429.

Valeri, M. C., Novi, G., Weits, D. A., Mensuali, A., Perata, P., & Loreti, E. (2021). *Botrytis cinerea* induces local hypoxia in *Arabidopsis* leaves. *The New Phytologist*, *229*(1), 173–185.

Van der Auwera, G. A., & O'Connor, B. D. (2020). *Genomics in the Cloud: Using Docker, GATK, and WDL in Terra*. "O'Reilly Media, Inc."

Wubben, M. J. E., Jin, J., & Baum, T. J. (2008). Cyst nematode parasitism of *Arabidopsis thaliana is* inhibited by salicylic acid (SA) and elicits uncoupled SA-independent pathogenesis-related gene expression in roots. *Molecular Plant-Microbe Interactions: MPMI*, *21*(4), 424–432.

Yan, W., Ni, Y., Liu, X., Zhao, H., Chen, Y., Jia, M., Liu, M., Liu, H., & Tian, B. (2021). The mechanism of sesame resistance against *Macrophomina phaseolina* was revealed via a comparison of transcriptomes of resistant and susceptible sesame genotypes. *BMC Plant Biology*, *21*(1), 159.

Yi, H., & Richards, E. J. (2007). A cluster of disease resistance genes in *Arabidopsis* is coordinately regulated by transcriptional activation and RNA silencing. *The Plant Cell*, *19*(9), 2929–2939.

Yoshimoto, K., Jikumaru, Y., Kamiya, Y., Kusano, M., Consonni, C., Panstruga, R., Ohsumi, Y., & Shirasu, K. (2009). Autophagy negatively regulates cell death by controlling NPR1-dependent salicylic acid signaling during senescence and the innate immune response in *Arabidopsis*. *The Plant Cell*, *21*(9), 2914–2927.

Zhang, N., Zhou, S., Yang, D., & Fan, Z. (2020). Revealing Shared and Distinct Genes Responding to JA and SA Signaling in *Arabidopsis* by Meta-Analysis. *Frontiers in Plant Science*, *11*, 908.

Zhao, Y., Wei, T., Yin, K.-Q., Chen, Z., Gu, H., Qu, L.-J., & Qin, G. (2012). *Arabidopsis* RAP2.2 plays an important role in plant resistance to *Botrytis cinerea* and ethylene responses. *The New Phytologist*, *195*(2), 450–460.

Zhou, J. M., Trifa, Y., Silva, H., Pontier, D., Lam, E., Shah, J., & Klessig, D. F. (2000). NPR1 differentially interacts with members of the TGA/OBF family of transcription factors that bind an element of the PR-1 gene required for induction by salicylic acid. *Molecular Plant-Microbe Interactions: MPMI*, *13*(2), 191–202.

Zhou, S., Hong, Q., Li, Y., Li, Q., & Wang, M. (2018). Autophagy contributes to regulate the ROS levels and PCD progress in TMV-infected tomatoes. *Plant Science: An International Journal of Experimental Plant Biology*, *269*, 12–19.

General Conclusions

Crop pathogens cause severe economic losses and threaten food security. Soil-borne microbes are notoriously difficult to control as they are easily spread via transmission of organic materials or contaminated waters, and live deep in the soil where surface treatments are often not penetrable (Brown & Hovmøller, 2002). The soil microbiome, which includes nematodes, bacteria, and fungi, form a complex and diverse ecosystem. These ecosystems are continuously disrupted and restructured, either by human involvement or natural forces, dynamically evolving with the environment. The increasing instability of climatic conditions decreases agricultural productivity and can worsen infections by pathogenic microorganisms (Anderson *et al*., 2004; Bailey-Serres *et al*., 2019). Microbial populations, both pathogenic and beneficial, have a great impact on crop yield, and therefore its stochastic nature poses a great challenge for growers. While crop evolution relies on artificial selection, natural forces drive the rapid evolution of microbial pathogens, often outcompeting that of crops (Möller & Stukenbrock, 2017). As high-yielding crop cultivars are typically genetically uniform, outbreaks of disease can devastate entire fields. In addition to making strategic decisions between crop cultivars, growers must continuously adapt by applying various methods of pathogen control, including agronomic practices, chemical and biological control.

Crop yields skyrocketed during the Green Revolution, primarily due to the global adoption of high yield crop varieties, as well as synthetic pesticides and fertilizers (Kush, 2001). However, in recent years additional research has raised concerns regarding increased agrichemical use. Off-target effects, surface runoff, and effects on human health have discouraged the use of potent chemical treatments to control pathogen outbreaks, and consequently consumer interest has shifted towards organic farming (Parrott & Marsden, 2002; Singh, 2000). Specialty crops, such as fruits, nuts, and vegetables, make up a larger share of the organic sector than field crops, despite the inherently high risk involved in their cultivation (Greene *et al*., 2017). Many of these crops lack genetically improved varieties, which increases their vulnerability to various biotic and abiotic threats (Bate *et al*., 2021). A decline in genetic diversity is also seen in calorie-rich field crops, as large economic incentives centered on increasing yield have accelerated their genetic homogeneity (Khush, 2001). Although there is an intense debate surrounding the pros and cons of biotechnology applications in agriculture since the Green Revolution, crop germplasms are perceived to be a crucial reserve for genetic variants to be utilized for the development of new desirable cultivars (Govindaraj *et al*., 2015). In a future where protection of crops from infectious diseases and sustainability coincide as equally important goals, development of additional resistant cultivars through the use of advanced biotechnology is indispensable. The genetic diversity maintained in crop germplasms are likely to be the key to combating emerging pathogens.

Disease caused by the generalist soil-borne fungal pathogen *Macrophomina phaseolina* (Tassi) Goid. has been reported globally on various host species including both field and specialty crops, at least since the early 1900's. It is most commonly described as charcoal rot, in reference to the dark colored mass of hyphal cells called microsclerotia, which is typically observed in infected plant tissue. In soybean, charcoal rot is considered to be one of the leading causes of yield loss due to disease, next to soybean cyst nematodes (Bandara *et al*., 2020; Wrather & Koenning, 2009). Despite decades of publications describing infection on various hosts and efforts to control this pathogen, no fungicide treatment has been registered as an effective control agent to date. In the past, soil fumigation with chloropicrin and methyl bromide was commonly used to control *M. phaseolina* populations within strawberry fields in California. The use of methyl bromide has been phased out due to concerns over its effects on the atmospheric ozone (Chamorro *et al*., 2016; Guthman & Others, 2017). Despite this, evidence suggests that infestation and overall chemical use has increased since the phase-out (Guthman & Others, 2017). Significant yield loss due to *M. phaseolina* has also been documented in orphan crops, a term referring to crops that are often neglected in research and international markets, such as cowpea and sorghum (Mahmoud *et al*., 2018; Muchero *et al*., 2011). *M. phaseolina* pathogenicity is therefore a world-wide food security concern that must be addressed urgently.

In all crops mentioned above (soybean, strawberry, sorghum, and cowpea), genotypes with variable susceptibility to *M. phaseolina* have been identified (da

Silva, Zaccaron, *et al*., 2019; Mahmoud *et al*., 2018; Muchero *et al*., 2011; Nelson *et al*., 2021). Although crop varieties with elevated resistance to *M. phaseolina* have been seen in several species, complete resistance has not been observed, and phenotypes measured across populations with genetic heterogeneity exhibit a continuous distribution. This suggests that variability in *M. phaseolina* response phenotypes are the result of quantitative contributions from multiple genetic loci. Next-generation sequencing and advanced genomic markers have been utilized in efforts to identify quantitative trait loci (QTL) responsible for elevated tolerance to this pathogen. Coser *et al*. (2017) identified five soybean loci from field screening and eight loci from greenhouse screening associated with *M. phaseolina* resistance. Interestingly, there was no overlap seen between association analyses based on field and greenhouse screens, suggesting that observations of resistance phenotypes are dependent on the environment (Coser *et al*., 2017). Moreover, Da Silva *et al*. (2019) conducted QTL mapping on a soybean population grown in greenhouse conditions and identified three distinct loci. Using the same population, Da Silva and colleagues identified three additional loci using bulked segregant analysis, none of which overlapped with the loci identified in the previous QTL mapping approach. From the information that we could glean from these reports, results from Coser *et al.* (2017) and Da Silva *et al.* (2019) do not overlap, suggesting that quantitative genetics approaches may be sensitive to differences in experimental conditions.

Phenotypes are the product of genetic factors, environmental factors, and interactions between genetic and environmental factors (Schmidt *et al*., 2019). To our knowledge, the extent of contributions from environmental factors on variability of *M. phaseolina* resistance has not been discussed in any *M. phaseolina* pathosystem. This is an important step to uncovering the genetic basis of *M. phaseolina* resistance, especially since the majority of the literature on this pathogen report assays conducted in field conditions, where environmental variance may have played a major role in determining virulence. In this work, we attempt to minimize environmental factors that may contribute to host response against *M. phaseolina* infection by using a sterile plate-based assay combined with an objective phenotype quantification method. Additionally, by using natural inbred lines of the model plant *Arabidopsis thaliana,* which are available through the Arabidopsis Biological Resource Center (ABRC), we demonstrate a highly reproducible pathogenicity assay for *M. phaseolina*. Using phenotypic data obtained from this assay, a number of genomic regions with suggestive associations to resistance were identified, as well as several biological processes that may be critical for plant defense. Additional experimentation will be necessary to test whether these associations are indeed linked to causal genes or biological processes. Our plate-based assays system assay has proven to be useful in testing knockout mutants for further functional studies in the work by Schroeder *et al.* (2019).

One major advantage of utilizing a model species like *A. thaliana* is the abundance of published literature on specific genes and their functions. In our root transcriptomic analysis capturing *M. phaseolina*-induced gene expression responses, enrichment of genes related to biotic stress response were pronounced, which could be further categorized into specific biological processes such as cell wall modification, oxidative stress response, and defense-responsive phytohormone pathways. Processes that have not been thoroughly described in the context of *M. phaseolina* defense, such as hypoxia response, were also shown to be up-regulated. Furthermore, by examining transcriptomic profiles at different timepoints of *M. phaseolina* infection, a temporal dimension was added to our analysis, which indicated that the magnitude of defense responses seem to decline earlier in relatively resistant accessions. This is an interesting finding considering that, previously, Chowdhury *et al.* (2017) observed that a resistant variety of soybean exhibited transcript-level evidence for a shift from biotrophic to necrotrophic pathogen response which was delayed in comparison to the susceptible variety. What mechanism causes this genotype-specific shift in timing of responses is still unknown, but may be a key factor in *M. phaseolina* resistance. Nonetheless, it is apparent from our findings in this study and that of Chowdhury *et al.* (2017) that host plant response to *M. phaseolina* dynamically changes throughout the progression of infection, or lack thereof.

Moreover, it is known that many plant immune responses are specialized in the root (Chuberre *et al*., 2018). As *M. phaseolina* is a soil-borne fungal pathogen,

our work focused on responses in the root, but it would be interesting to see whether there are systemic signals that could also be detected in the shoot tissue. This would be easily achievable using the same plate-based assay (Schroeder *et al*., 2019). It is also important to understand that the transcriptomic profile described in our analysis represents gene expression in the whole root, and therefore transcripts isolated in specific root cell types or expressed in low concentrations may have been overlooked. Currently, efforts to further dissect *A. thaliana* root response to *M. phaseolina* at the single-cell level utilizing fluorescence-activated cell sorting (FACS) is underway in our lab. In addition to a temporal dimension, such experiments add another spatial level examination of *M. phaseolina* response. Based on mounting evidence, which suggests resistance traits against *M. phaseolina* in *A. thaliana* are the consequence of a complex interaction of many genetic loci, it is important to gain a detailed understanding of genotype-dependent temporal and spatial gene expression patterns. Our heritability estimates also suggested the presence of epistatic interactions among causal loci. Identification of such interaction may require additional mathematically and computationally rigorous analyses such as those reviewed by Niel *et al.* (2015).

Using the *A. thaliana* genes identified in our analyses, candidate crop loci may be identified based on synteny or sequence similarity, and tested for their contributions to *M. phaseolina* tolerance. Translational genomics has been shown to be useful in applying knowledge obtained from model species like *A. thaliana* to

improve crop plants for which less genomic data is available (Salentijn *et al*., 2007). Although individual genes may have a relatively small contribution to the phenotype, biological processes in which these *A. thaliana* genes play a role should also be informative as a resource for alternative gene targets for functional analysis. For crop species that can be propagated through grafting, genes of interest identified in these experiments can potentially be used to develop rootstock varieties with high tolerance to *M. phaseolina.*

Disease resistance breeding in crop plants is complex. A decline in genetic diversity in agriculture subjects many crops to higher risks of catastrophic yield loss, and therefore the preservation of crop germplasms is a critical aspect of future crop genetics. Traits that are linked to increased resistance can be a product of multiple small effect loci that interact with each other and the environment. Quantitative disease resistance against a certain pathogen can, at times, be race non-specific, in that they confer protection against other pathogens (R. P. Singh *et al*., 2011). Pleiotropic effects and epistasis could also contribute to the complexity of genetic resistance, which may be harder to detect in genomic analysis. The polyploid nature of many crop species makes functional analysis of genes more complex in comparison to model species like *A. thaliana,* which has a simple diploid genome. Each allelic component must be pieced together without sacrificing other valued traits such as fruit quality, biomass, and responsiveness to light (Nelson *et al*., 2018). In order to address these confounding factors and effectively harness the power of genetics to combat successful pathogens such as

M. phaseolina, a comprehensive understanding of host plant responses and their extent of contribution to resistance is essential. In this investigation, we demonstrate that *A. thaliana* could be a useful model for identifying components of the genetic architecture contributing to *M. phaseolina* resistance, which can potentially be applied to crop improvement.

Literature Cited

Anderson, P. K., Cunningham, A. A., Patel, N. G., Morales, F. J., Epstein, P. R., & Daszak, P. (2004). Emerging infectious diseases of plants: pathogen pollution, climate change and agrotechnology drivers. *Trends in Ecology & Evolution*, *19*(10), 535–544.

Bailey-Serres, J., Parker, J. E., Ainsworth, E. A., Oldroyd, G. E. D., & Schroeder, J. I. (2019). Genetic strategies for improving crop yields. *Nature*, *575*(7781), 109–118.

Bandara, A. Y., Weerasooriya, D. K., Bradley, C. A., Allen, T. W., & Esker, P. D. (2020). Dissecting the economic impact of soybean diseases in the United States over two decades. *PloS One*, *15*(4), e0231141.

Bate, N. J., Dardick, C. D., de Maagd, R. A., & Williams, R. W. (2021). Opportunities and challenges applying gene editing to specialty crops. *In Vitro Cellular & Developmental Biology - Plant*, *57*(4), 709–719.

Brown, J. K. M., & Hovmøller, M. S. (2002). Aerial dispersal of pathogens on the global and continental scales and its impact on plant disease. *Science*, *297*(5581), 537–541.

Chamorro, M., Seijo, T. E., Noling, J. C., De los Santos, B., & Peres, N. A. (2016). Efficacy of fumigant treatments and inoculum placement on control of *Macrophomina phaseolina* in strawberry beds. *Crop Protection* , *90*, 163–169.

Chowdhury, S., Basu, A., & Kundu, S. (2017). Biotrophy-necrotrophy switch in pathogen evoke differential response in resistant and susceptible sesame involving multiple signaling pathways at different phases. *Scientific Reports*, *7*(1), 17251.

Chuberre, C., Plancot, B., Driouich, A., Moore, J. P., Bardor, M., Gügi, B., & Vicré, M. (2018). Plant Immunity Is Compartmentalized and Specialized in Roots. *Frontiers in Plant Science*, *9*, 1692.

Coser, S. M., Chowda Reddy, R. V., Zhang, J., Mueller, D. S., Mengistu, A., Wise, K. A., Allen, T. W., Singh, A., & Singh, A. K. (2017). Genetic Architecture of Charcoal Rot (*Macrophomina phaseolina*) Resistance in Soybean Revealed Using a Diverse Panel. *Frontiers in Plant Science*, *8*, 1626.

da Silva, M. P., Klepadlo, M., Gbur, E. E., Pereira, A., Mason, R. E., Rupe, J. C., Bluhm, B. H., Wood, L., Mozzoni, L. A., & Chen, P. (2019). QTL Mapping of Charcoal Rot Resistance in PI 567562A Soybean Accession. *Crop Science*. https://doi.org/10.2135/cropsci2018.02.0145

da Silva, M. P., Zaccaron, A. Z., Bluhm, B. H., Rupe, J. C., Wood, L., Mozzoni, L. A., Mason, R. E., Yingling, S., & Pereira, A. (2019). Bulked segregant analysis using next-generation sequencing for identification of genetic loci for charcoal rot resistance in soybean. *Physiological and Molecular Plant Pathology*, 101440.

Govindaraj, M., Vetriventhan, M., & Srinivasan, M. (2015). Importance of genetic diversity assessment in crop plants and its recent advances: an overview of its analytical perspectives. *Genetics Research International*, *2015*, 431487.

Greene, C., Ferreira, G., Carlson, A., Cooke, B., & Hitaj, C. (2017). Growing organic demand provides high-value opportunities for many types of producers. *Amber Waves: The Economics of Food, Farming, Natural Resources and Rural America*, *1*. https://www.ers.usda.gov/amber-waves/2017/januaryfebruary/growing-organic-demand-provides-high-value-opportunities-for-many-types-of-producers

Guthman, J., & Others. (2017). Land access and costs may drive strawberry growers' increased use of fumigation. *California Agriculture*, *71*(3), 184–191.

Khush, G. S. (2001). Green revolution: the way forward. *Nature Reviews. Genetics*, *2*(10), 815–822.

Mahmoud, A. F., Abou-Elwafa, S. F., & Shehzad, T. (2018). Identification of charcoal rot resistance QTLs in sorghum using association and in silico analyses. *Journal of Applied Genetics*. https://doi.org/10.1007/s13353-018-0446-5

Möller, M., & Stukenbrock, E. H. (2017). Evolution and genome architecture in fungal plant pathogens. *Nature Reviews. Microbiology*, *15*(12), 756–771.

Muchero, W., Ehlers, J. D., Close, T. J., & Roberts, P. A. (2011). Genic SNP markers and legume synteny reveal candidate genes underlying QTL for *Macrophomina phaseolina* resistance and maturity in cowpea [Vigna unguiculata (L) Walp.]. *BMC Genomics*, *12*, 8.

Nelson, J. R., Verma, S., Bassil, N. V., Finn, C. E., Hancock, J. F., Cole, G. S., Knapp, S. J., & Whitaker, V. M. (2021). Discovery of three loci increasing resistance to charcoal rot caused by *Macrophomina phaseolina* in octoploid strawberry. *G3* . https://doi.org/10.1093/g3journal/jkab037

Nelson, R., Wiesner-Hanks, T., Wisser, R., & Balint-Kurti, P. (2018). Navigating complexity to breed disease-resistant crops. *Nature Reviews. Genetics*, *19*(1), 21–33.

Niel, C., Sinoquet, C., Dina, C., & Rocheleau, G. (2015). A survey about methods dedicated to epistasis detection. *Frontiers in Genetics*, *6*, 285.

Parrott, N., & Marsden, T. (2002). The real green revolution. *London: Greenpeace Environmental Trust*, 11–16.

Salentijn, E. M. J., Pereira, A., Angenent, G. C., van der Linden, C. G., Krens, F., Smulders, M. J. M., & Vosman, B. (2007). Plant translational genomics: from model species to crops. *Molecular Breeding: New Strategies in Plant Improvement*, *20*(1), 1–13.

Schmidt, P., Hartung, J., Bennewitz, J., & Piepho, H.-P. (2019). Heritability in Plant Breeding on a Genotype-Difference Basis. *Genetics*, *212*(4), 991–1008.

Schroeder, M. M., Lai, Y., Shirai, M., Alsalek, N., Tsuchiya, T., Roberts, P., & Eulgem, T. (2019). A novel *Arabidopsis* pathosystem reveals cooperation of multiple hormonal response-pathways in host resistance against the global crop destroyer *Macrophomina phaseolina*. *Scientific Reports*, *9*(1), 20083.

Singh, R. B. (2000). Environmental consequences of agricultural development: a case study from the Green Revolution state of Haryana, India. *Agriculture, Ecosystems & Environment*, *82*(1), 97–103.

Singh, R. P., Huerta-Espino, J., Bhavani, S., Herrera-Foessel, S. A., Singh, D., Singh, P. K., Velu, G., Mason, R. E., Jin, Y., Njau, P., & Crossa, J. (2011). Race non-specific resistance to rust diseases in CIMMYT spring wheats. *Euphytica/ Netherlands Journal of Plant Breeding*, *179*(1), 175–186.

Wrather, A., & Koenning, S. (2009). Effects of Diseases on Soybean Yields in the United States 1996 to 2007. *Plant Health Progress*, *10*(1), 24.