# THE LINK BETWEEN SUSCEPTIBILITY TO *MACROPHOMINA PHASEOLINA* AND THREE PHYTOHORMONES IN *MEDICAGO TRUNCATULA*

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Adam James Hefel

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The following faculty members have examined the final copy of this thesis for form and content, and recommend that it be accepted in partial fulfillment of the requirement for the degree of Master of Science, with a major in Biological Sciences.

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Bin Shuai, Committee Chair

_____

William Hendry, Committee Member

_____

Moriah Beck, Committee Member

# DEDICATION

To my family, friends, and the members of the biology
department who have influenced me in
many profound ways

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# ABSTRACT

*Macrophomina phaseolina* is a major agricultural pest causing the disease charcoal rot in many species of crops. The disease manifests as yellowing (chlorosis) and death (necrosis) of plant tissue by secreting plant toxins and clogging its vasculature with small black cellular aggregates called microsclerotia. This research aimed at identifying molecular mechanisms that can promote resistance to the pathogen. Using the exogenous application of phytohormones abscisic acid (ABA), ethylene (ET), and jasmonic acid (JA), we assessed the role of each of these hormones in promoting defense or susceptibility. In a previous study using *M. truncatula* as a model organism, application of ET and/or JA promoted resistance in the A17 ecotype but not in the R108 ecotype. To investigate the mechanism for this phenotypic difference, plants from each ecotype were treated with these hormones and their expression of ET and JA responsive genes were analyzed using real-time PCR. We investigated ABA for its role in defense because charcoal rot incidence is higher during hot, dry months and ABA is involved in defending plants against the symptoms of drought. *Medicago truncatula* plants were transferred to media containing ABA before being inoculated with the pathogen and then observed for the development of disease symptoms.

The results of this study indicate that the expression of ET markers was similar between A17 and R108 plants treated with ethephon, while JA markers demonstrated a difference between these ecotypes following MeJA application. Thus, we speculate that the difference between R108 and A17's inducible resistance phenotypes results from misregulation of JA responsive gene in R108. Our results also indicated that exogenous ABA application can lead to increased susceptibility to *M. phaseolina* in A17 plants, as demonstrated by more rapid development of disease symptoms.

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LIST OF ABBREVIATIONS

| | |
|---|---|
| µl | Microliter |
| µM | Micromolar |
| ABA | Abscisic Acid |
| C | Celsius |
| cm | Centimeter |
| $C_T$ | Cycle threshold |
| $DDH_2O$ | Distilled deionized water |
| DNA | Deoxyribonucleic acid |
| dNTP | Deoxynucleotide triphosphate |
| DTT | Dithioreitol |
| ET | Ethylene |
| EtOH | Ethanol |
| g | Grams |
| hpi | Hours post infection |
| JA | Jasmonic Acid |
| kb | Kilobase |
| mRNA | Messenger RNA |
| mins | Minutes |
| ml | Milliliter |
| MS | Murashige-Skoog |

| | |
|---|---|
| NaCl | Sodium chloride |
| NaCitrate | Sodium citrate |
| NaOAc | Sodium acetate |
| PCR | Polymerase chain reaction |
| RAPD | Random Amplified Polymorphic DNA |
| RNA | Ribonucleic Acid |
| RT-PCR | Reverse transcriptase PCR |
| RT-qPCR | Quantitative RT-PCR |

# CHAPTER 1

# INTRODUCTION

## 1.1. *Macrophomina phaseolina* and Charcoal rot

*Macrophomina phaseolina* is a major agricultural pest that infects over 500 species of plants (1). Identified as the causal agent for dry wilt, *M. phaseolina* destroys plant tissue leading to symptoms of chlorosis, wilt, and eventually death during periods of drought. As the fungus multiplies, it forms small black aggregates of hyphae called microsclerotia and the resulting appearance of many small black bits in infected plant tissue led to its name of charcoal rot. Those black bits are melanized bodies capable of resisting desiccation and damage from UV light and, as dead infected plants are incorporated into the soil, the microsclerotia provide the source of inoculum that will infect future plants (2, 3). Microsclerotia can survive in soil for 2-15 years where dry, arid soils promote longer survival (2, 4). While microsclerotia are the primary inoculum of this fungus, there are reports of this species' ability to form conidia, depending on the host species (2) and media (5). The development of conidia means this fungus can be spread by air however seed contamination is often blamed for its spread (3). The impact of charcoal rot is global (1, 3), while having devastating effects on many crop species including *Glycine Max* (soybean) (6), *Medicago sativa* (alfalfa) (7), *Zea mays* (corn) (8), *Gossypium sp.* (cotton) (8) and *Helianthus sp.* (sunflower) (1). Disease severity and incidence are often greater where traditional tillage practices are applied which may account for the fungus' impact on agriculture. Along with a large spatial distribution (9). *M. phaseolina* is also considered a generalist pathogen with many hosts including immune-compromised human patients (10). While it infects many different hosts, there does appear to be specialization among them indicated by random amplified polymorphic DNA (RAPD) analysis of *M. phaseolina* isolated from different plots containing

different species of host plants (8). Essentially RAPD patterns in *M. phaseolina* were similar among isolates found from a particular host plant species, and different patterns were observed among isolates of different hosts (8). Additionally, it was noted that colonization of corn roots was greater with fungus that was previously isolated from corn than any other isolate, which may prove problematic if the same crop is planted successively (8).

*M. phaseolina* belongs to the *Botryosphaeriaceae* family of fungi that is composed of saprophytic, endophytic and necrotrophic members. In general, this family is found on many woody species of plants and one member, *Neoscytalidium dimidiatum,* is frequently found under human nails (11, 12). Saprophytes are fungi that take nutrients from dead plant material such as fallen trees. They accomplish this with a large number of proteolytic and polysaccharide hydrolytic enzymes. Since many members of this family of fungi are saprophytes, *M.phaseolina* may be armed with a common set of tools for degrading cell walls (13). While Dhingra and Sullivan described *M. phaseolina* as a saprophyte, it is most often described as a necrotroph which destroys living plant tissue in order to acquire nutrients (2). In addition to variations in lifestyle, in nature and in culture, *M. phaseolina* exhibits variations in appearance that led to different taxonomic identifications such as *Macrophomina phaseoli or Rhizoctonia bataticola* (3). When grown on agar media, it often appears as brown/black spots with white hyphae. It is found in soil and seeds (2, 14) and is associated with a variety of symptoms ranging from cankers to lesions and generalized rot (2, 3).

During hot dry months, *M. phaseolina* will begin growing from microsclerotia as germ tubes. Germ tubes then seek out roots and form appresoria which secrete cell wall degrading enzymes and use mechanical pressure to penetrate the cell walls of host plant tissue (2, 3). Formation of appresoria is driven by signals through interaction with plant cells and sequencing

of *M. phaseolina genome* revealed that this process may depend on the cell surface receptor Pth11 (15).This receptor apparently has a role in pathogenesis of the fungus *Magnaporthe grisea* by driving the formation of appresoria via recognition of plant cell surfaces (16). As the infection progresses, mycelia of *M. phaseolina* eventually enter the vasculature through the cortex and into the xylem where it interferes with water transport (2, 3). The obstruction of water transport and enzymatic digestion eventually leads to generalized wilt and death of the plant. Some of the enzymes secreted by *M. phaseolina* that play a role in disease development include endoglucanases, hemicellulases, amylases and proteases (3). Endoglucanases of *M. phaseolina* are similar to plant endoglucanases which are responsible for cell wall degradation and they are important for disease progression (17). In addition to secreting digestive enzymes, *M. phaseolina* secretes a potent phytotoxin, phaseolinone (18) that is implicated in disease initiation (19). Sett and colleagues found that non-pathogenic strains of *M. phaseolina* can cause disease in the presence of phaseolinone (19). In addition to phaseolinone, other phytoxic metabolites including asperlin, phomalactone, phomenon and phaseolinic acid, are important for disease severity (3, 18, 20). In 2007, researchers found isolates of *M. phaseolina* infecting soybean that did not produce detectable levels of phaseoloinone but did produce another plant toxin known as (-)-Botryodiplodin (21). This combination of toxins and digestive enzymes are what makes *M. phaseolina* particularly dangerous to agricultural crops.

Charcoal rot, root rot, dry wilt, and seedling blight are all terms to describe the disease caused by *M. phaseolina*. A study by Wrather and colleagues investigated the leading causes of soybean yield reduction and their findings put *M. phaseolina* at the top of the list of infectious agents, second only to soybean nematode cyst in 2003 (22). In 2003 alone *M. phaseolina* was estimated to be responsible for nearly 2 million tons of soybean crop reduction (22). In soybean,

*M. phaseolina* infects mature plants as well as seeds. When *M. phaseolina* infects seedlings, the disease can be seen as black or brown spotting near cotyledon margins (14). In mature plants, leaves turn yellow at the tips before eventually wilting (3). The most severe effects of charcoal rot can be seen under drought conditions. Originally listed as a minor pest in India, in 2004 *M. phaseolina* was described as a major pathogen as the result of drought during growing seasons (3). Some growers in Missouri described as much as 50% yield loss in certain years, while growers in India reported as much as 80% yield loss (3). Since the disease caused by *M. phaseolina* has major global and economic impacts, current research is aimed at controlling this pathogen by a variety of means.

The current control efforts for charcoal rot include irrigation (23), soil heating (24), competitive microbes (25), and crop rotation (2). Irrigation can lead to lower densities of fungus in soil, but the act of flooding fields may be costly and waste important resources (23). Lodha and colleagues used polystyrene mulching to raise soil temperatures to 57 °C which effectively reduced the number of sclerotia found in the soil up to 30cm deep. However, this strategy also requires annual amendment of cruciferous residues to be successful (24). The compounds Iridodial β-monoenol acetate and actinidine isolated from *Nepeta clarkei* have significant negative effects on growth of *M. phaseolina*. However, it may require frequent application which could prove costly or ineffective (26). Since these management approaches often require regular maintenance or are not effective, another candidate strategy may be identifying or engineering disease-resistant cultivars (6). Currently, the number of *M. phaseolina*-tolerant varieties of soybean are limited and none are completely disease resistant (6). Therefore, genetic engineering may be the most effective path to develop more disease-resistant cultivars. To achieve this goal, a better understanding of host-pathogen interactions at the molecular level is necessary.

### 1.2 *Medicago truncatula* as a model system

Many agricultural legumes including alfalfa and soybean are hosts to *M. phaseolina.* Legumes are plants belonging to the *Fabaceae* family which constitute approximately 27 % of all annual crop production (27). Legumes also provide a major source of protein and 35% of processed vegetable oils (27). Alfalfa (*Medicago sativa)* is one of the most important legumes and it is ranked 3$^{rd}$ or 4$^{th}$ for most important crop in the US with an annual production of more than 72 megagrams (27). In 2014 an estimated 38 million acres of alfalfa were harvested according to the USDA (28). Legumes such as soybean and alfalfa are commonly used in crop rotation practices for nitrogen fixation to produce nitrogen rich soil for future crops (27).This is possible because legumes readily form nodules containing rhizobia which are modified bacterial cells that can take atmospheric nitrogen and convert it to more usable forms. Legumes are unique in their ability to form nodules and they may also have unique strategies for responding to pathogenic microbes. Therefore, studying legume models may offer unique advantages when making predictions about other legumes.

Phylogenetic research estimates that all legumes share a common ancestor from 60 million years ago (29). This same analysis also reveals that a close relative of alfalfa (*Medicago sativa*) called the burr-medic (*Medicago truncatula)* has micro synteny with *Glycine max* with the highest chromosomal similarity of 10 out of 20 genes for chromosomes Mt1 and Gm17 (30). *M. truncatula* is a Mediterranean plant that is adapted to dry soil and, in Australia, *M. truncatula* is often used as an important foraging crop. Not only is *M. truncatula* an important foraging crop, in 1999 it was proposed as a model legume due to several advantages in studying plant-pathogen interactions including a fully sequenced genome and the ability to form rhizobia (30, 31). *M. truncatula* possesses a relatively small genome that is beneficial for studying resistance-

associated genes. By identifying resistance in different accessions of *Medicago*, it is possible to identify what genes are involved in resistance and what mechanisms promote a resistant phenotype (32). *M. truncatula* is also useful since it is relatively small in size with a relatively short germination time, is self-fertile, and a large number of genotypes of it are available. As a result of donations and collections there are approximately 5,500 accessions of *M. truncatula* available from the Australian *Medicago* Genetic Resource Center (33). These accessions consist of various ecotypes and mutants that aid researchers in studying plant pathogen interactions. There are advantages to using a model like this instead of studying soybean crops for experimentation. For instance, soybean can be difficult to manage in a laboratory environment with its large size and its lengthy maturation time as well as significant genome variation in soybean cultivars. By using *M. truncatula* as a model for plant pathogen interactions, we hope to better understand the role of phytohormones in plant resistance since it appears that phytohormones regulate defense strategies towards various types of stress. With identification of induced resistance or susceptibility to *M. phaseolina,* we can make predictions about their applications in resistant cultivars.

Our research is aimed at identifying important genes that can promote resistance to the pathogen *M. phaseolina.* This should aid future research by providing targets for genome modifications that may result in resistant phenotypes. This may, in turn, provide agriculture with *M. phaseolina*-resistant cultivars that can be grown for production of food or other important resources. In addition, resistant cultivars may help reduce the density of *M. phaseolina* in different agricultural systems and protect plants that may not be resistant to the pathogen.

## Chapter 2

## EXPLORING THE MECHANISM FOR RESISTANCE PHENOTYPE DIFFERENCES BETWEEN A17 AND R108 ECOTYPES OF *MEDICAGO TRUNCATULA*.

### 2.1 Introduction to the necrotrophic response

As previously mentioned, *M. phaseolina* is a necrotrophic pathogen that destroys plant tissue in order to acquire nutrients. This strategy is different from biotrophic pathogens which will infect plants and acquire nutrients from living tissue without causing significant damage (16). The significance of these different lifestyles is supported by evidence suggesting there are two unique defense responses that coordinate biotrophic and necrotrophic defenses (34). To direct energy to specific defense strategies, plants use a complicated network of hormones also known as hormone crosstalks (34-36). One way that plants may recognize fungal pathogens is through the chitin oligosaccharide elicitor that is a membrane-bound protein that recognizes the presence of chitin (a principal component of the fungal cell wall) to direct signaling towards defense or the formation of symbiotic relationships (37). Current findings suggest that, in response to necrotrophic pathogens, plants upregulate the production and release of ethylene (ET) and jasmonic acid (JA) (34, 38). These hormones likely work synergistically, as indicated in a study by Lorenzo and colleagues showed that *ETHYLENE RESPONSE FACTOR-1* (*ERF1*) acts as a synchronization point of synergism in *Arabidopsis* as its expression is upregulated in plants treated with ET or JA (39). This gene encodes a transcription factor that coordinates JA/ET signaling where insensitivity to either pathway blocks *ERF1* induction (39). Additionally, ERF1 overexpression appears to confer resistance to the necrotrophic fungal pathogens *Plectosphaerella cucumerina* and *Botrytis cinerea* with a significant decrease in disease severity for *Arabidopsis thaliana* overexpression mutants (40). In fact, some pathogen defense proteins

such as the plant defensin gene (PDF1.2) and the pathogen resistance (PR) proteins PR-3 and PR-4 require functional sensing of ET and JA in order to be expressed (41). A study by Penninckx and colleagues using mutants that are insensitive to either JA or ET demonstrated that blocking ET or JA signaling leads to susceptibility to the necrotroph *B. cinerea;* indicating that resistance requires expression of both JA and ET pathways(42).

While JA and ET appear to work synergistically, other hormones can act antagonistically. Salicylic acid (SA), which is implicated in the biotrophic response, negatively regulates the JA/ET pathway (35, 43, 44). As demonstrated in Figure 1, the hormone signaling crosstalks involve complex relationships of gene regulation that leads to specific defense strategies. By better understanding the crosstalk interactions and their role in pathogen defense, we may be able to more readily identify or isolate pathogen resistant cultivars.

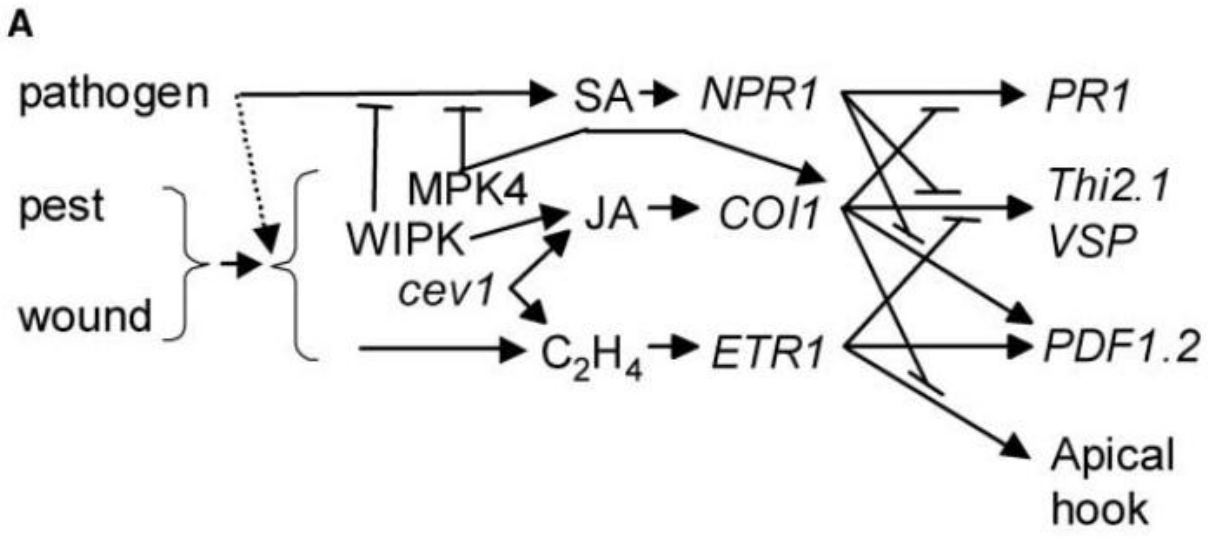


Figure 1: A model that demonstrates the cross-talk nature of the phytohormones ethylene ($C_2H_4$), jasmonate (JA) and salicylic acid (SA). Arrows represent positive and bars represent negative effects. From Turner 2002(45)

To better understand how JA and ET promote resistance to *M. phaseolina,* a previous study by former graduate student Andres Gaige investigated resistance to *M. phaseolina*

following application of ethephon (a molecule designed to be metabolized by plants into functional ethylene) and methyl-jasmonate (MeJA) in *M. truncatula* ecotype A17. This study concluded that A17 demonstrates partial resistance to *M. phaseolina* with JA and ET treatment because treated plants had delayed disease progression (46). Interestingly, while A17 demonstrated induced partial resistance with MeJA and/or ethephone treatments, the R108 genotype showed no induced resistance after hormone treatment (47). Subsequent gene expression analysis using root tissue infected by *M. phaseolina* was performed to analyze expression of genes specific to the JA and ET pathways by Tyler Doerkson (48). Targets included ethylene response genes such as *Ethylene response factor (ERF)*, *Chitinase IV (Chit IV)*, *Hevein like protein (HEV)* and the JA associated genes such as *Chalcone Synthase (CHS), Pathogenesis related 10 (PR10),* and *Defensin 1 (Def1)*. Selection of these genes was based on previous studies that demonstrated they were upregulated by the respective hormone (49). Tyler's study concluded that expression of these defense-related genes was generally reduced and delayed in R108 compared to A17 and, for *DEF1,* there was a marked decrease in expression after infection in R108. We hypothesize that this difference is due to an inability of R108 to sense these hormones and therefore we expect to see a difference in the transcription patterns of JA or ET-associated genes in the A17 and R108 ecotypes following MeJA or ethephon application. By understanding the mechanism that drives the partial resistance in A17 and not R108, we hope to better understand what genes are important for resistance to the pathogen *M. phaseolina.*

### 2.1.1 ET

Ethylene is the simplest alkene and a very potent plant hormone. Originally described as a plant effector in the 19$^{th}$ century, some of its roles include premature senescence, fruit ripening,

and seed development (50). Ethylene is produced in plants from the amino acid methionine that is first converted into the metabolic intermediate molecule S-Adenosyl methionine (AdoMET). ACC synthase (ACS) converts AdoMET into 1-aminocyclopropane-1-carboxylic acid (ACC) that is then converted into ET by ACC oxidase (ACO). ACS appears to act as a key regulator for this metabolic pathway (50, 51). ACS is upregulated by stimuli that also support ET production; indicating that it plays a role in regulating ET biosynthesis (51). Production of ethylene requires only one molecule of ATP for recycling methionine so large amounts of ethylene can be generated from a small amount of methionine (50).

There are four families of ethylene receptors (ETR1-4), and they are found in the plasma membrane of all plant organs and tissues (52). One pathway for ethylene signaling involves auto-phosphorylation of a histidine kinase domain in ETR1 that inactivates it and the kinase CONSTITUTIVE TRIPLE RESPONSE1 (CTR1) (53, 54). This then allows for downstream expression of defense-related genes including pathogen resistance (*PR)* and plant defensin 1 (*PDF1)* families (55). The resulting gene expression activities offer resistance to necrotrophic pathogens. Exogenous ethylene application appears to promote resistance to *B. cinerea* in *Arabidopsis* plants as fewer necrotic leaves were observed in plants that were exposed to the gaseous hormone (56). Thomma and colleagues also noted that functional ET receptor *ETHYLENE-INSENSITIVE 2* (*EIN2*) is necessary for resistance against the same pathogen (56). Ethylene also appears to have a role in development of nodules in legumes. A study by Penmetsa and colleagues identified an ethylene-insensitive mutant of *M. truncatula* that appeared to have an increase of nodules by an order of magnitude compared with the wild-type. This mutation was identified as *sickle* for its unusually shaped nodules and it supports findings that ET may regulate various aspects of plant development (57).

ET signaling works in tandem with JA signaling as demonstrated by studies which show mutants that are insensitive to JA are susceptible to necrotrophic pathogens. Thomma and colleagues reported that JA insensitive mutants of *Arabidopsis* are susceptible to the necrotrophic pathogen *A. brassicola.* As previously mentioned, JA and ET signaling are both necessary for the expression of ERF1 in *Arabidopsis* (39, 58). Since JA and ET appear to play an important role in defense against necrotrophic pathogens such as *M. phaseolina*, we must study the contributions of both hormones to pathogen defense.

### 2.1.2 JA

JA is a phytohormone derived from linolenic acid (LA) that was first isolated in 1962 by Demole and colleagues from the essential oil of *Jasminium grandiflorum*, also known as Spanish jasmine (59, 60) . It was later characterized as having growth inhibition and senescence-promoting physiological effects in several studies (60). JA appears to have a role in promoting plant defense against necrotrophic pathogens. Synthesis of JA can be triggered by wounding via the lipoxegenase (LOX) pathway. LA, the precursor of JA, is most often found esterified in membrane phospholipids where it can be released by phospholipases before entering the LOX pathway. It is believed that LA abundance determines the rate of JA biosynthesis since application of LA results in accumulation of JA (59). This could mean that phospholipases that can free LA from plasma membranes may regulate JA accumulation. One such phospholipase is the DAD1 phospholipase A1, which appears to regulate JA biosynthesis in flower development (61). Wounding may also stimulate production of JA by co-localization of the enzymes and substrates in LOX pathway that would normally compartmentalize separately (59). Synthesis of JA from LA involves several enzymatic reactions in which the first step is conversion of LA to 13(S)-hydroperoxy linelinolic acid (HPOT) by LOX.

The receptor mechanism for JA signaling is not fully understood but it is known that JA signaling leads to degradation of proteins that are members of the *JASMONATE ZIM-DOMAIN* (*JAZ*) family. JAZ proteins inhibit JA signaling by blocking transcription of JA response genes. When JA is present, JAZ proteins are ubiquitinated and degraded, thereby allowing expression of JA responsive genes (62). Degradation of JAZ proteins appears to be controlled by the Skp1-Cdc53-F-box protein complex ($SCF^{COI1}$) that requires that JA be present to target degradation of JAZ proteins. In addition to requiring JA and all members of the $SCF^{COI1}$ complex, a notable study of a JA insensitive mutant *CORONATINE INSENSITIVE PROTIEN-1 (COI-1)* revealed that COI-1 is a F-box protein that is necessary to recruit JAZ proteins to the degradation complex in the presence of JA-ILE, which is one of several JA conjugates (63).

JA signaling has a role in the response to wounding by insects. When plants are wounded, they accumulate JA. Additionally, *Arabidopsis* plants that are deficient in LA appear to be susceptible to fungal gnats because exogenous JA applications restore resistance to the pest (59). As previously mentioned, JA and ET appear to synergistically promote resistance to necrotrophic pathogens. Since JA and ET both appear to be necessary for necrotroph resistance, it may prove beneficial to understand how exogenous applications of these hormones can affect plant defense. To study how ET and JA signaling promotes resistance to the pathogen *M. phaseolina*, two *M. truncatula* ecotypes that differ in their response to these hormones were used.

### 2.1.3 *Medicago truncatula* Ecotypes A17 and R108

The first *M. truncatula* ecotype to have a completely sequenced genome was A17 jemalong that comes from Australia and was first described in 1955 (33). One of the identifiable characteristics of this ecotype is the presence of an anthocyanin wedge on the leaflets. The R108

ecotype was developed by Hoffmann and colleagues from the 108-1 model line and it offers advantages of better regenerative power and the ability to more easily create rhizobia symbiosis than A17 (64). Another difference between A17 and R108 is their response to iron deficiency (65) . Under iron deficient conditions, A17 upregulates ethylene biosynthesis and the expression of a gene named *MtFRD3* that is responsible for loading an iron chelator into the xylem but R108 does not demonstrate this same expression pattern (65). In a resequencing study by Wang and colleagues, it was noted that R108 has a deletion in the *YELLOW STRIPE1-LIKE* (*YSL*) iron transporter gene which may result in its sensitivity to iron deficiency (66). A17 also appears to have an aberrant chromosomal arrangement as indicated by linkage maps from A17 crosses which may also lead to the phenotypic differences between these ecotypes (67). These two genotypes vary phenotypically yet are the same species and therefore they can provide a useful tool for understanding roles of various genes involved in plant stress responses.

### 2.2 Research hypothesis

In the study by Tyler Doerkson, three ET associated genes were used to study the ET response included *Ethylene response factor (ERF), Chitinase IV (Chit IV), and Hevein like protein (HEV)* genes. In *Arabidopsis thaliana*, ERF1 is transcription factor that appears to regulate ET and JA dependent signaling and research suggests that this protein may be necessary the synergism shared by these hormone pathways (39). Over expression of *ERF* appears to confer resistance to the necrotrophic fungus *B. cinerea (40).* Chitinase IV is an enzyme that can break down chitin, one of the principle components of the fungal cell wall. Expression of chitinases can potentially reduce the effectiveness of different fungal pathogens by degrading the fungal cell wall (68). Finally *HEL* was selected as a marker for the ET response since expression of this protein coordinates with exogenous ET application. HEL is predicted to be a fungal

metalloprotease inhibitor but its function has not been confirmed (69). In addition, three JA genes studied for differences in expression were *Chalcone Synthase(CHS), Pathogenesis related 10 (PR10),* and *Defensin 1(Def1*) genes. CHS is an enzyme that appears to catalyze the synthesis of several phytoalexins that have antimicrobial properties. Its expression appears to be in response to biotic stress in defense against microbial pathogens (70). *M. truncatula* plants that have decreased *CHS* expression from RNAi inhibition are susceptible to the pathogen *Phytophthora medicaginis* (49). *PR10* is upregulated in response to necrotrophic pathogens, and has a complex role in diseases resistance due to its antifungal and ribonuclease activities (49). Finally, *DEF1* is also upregulated in *M. truncatula* seedlings following exogenous JA application and this protein has antifungal properties (71, 72).

In Tyler Doerkson's thesis, *M. truncatula* A17 and R108 plants were inoculated with the pathogen *M. phaseolina* before the roots were analyzed for differences in expression of the aforementioned genes. Those results indicated reduced expression of JA and ET response genes in R108 compared to A17 where there was a marked reduction in the expression of *Def1*. Therefore, we hypothesized that R108 may have difficulty sensing the hormones ET and JA during infection as compared to A17 and therefore does not exhibit a strong necrotrophic response following hormone application.

## 2.3 Results and discussion

### 2.3.1 Expression of ET-associated Genes in A17 and R108 plants treated with ethylene

To test the hypothesis in the current study, plants were grown in sterile magenta boxes containing Murashige-skoog (MS) basal salt media as in the previous study. However, instead of studying expression patterns in response to the pathogen, we examined expression patterns for plants treated with either MeJA or ethephon. Plants were sampled 12hrs after spraying with the

respective hormones. RNA was extracted from root tissue and used to create cDNAs for quantitative PCR studies to assess expression levels of the six target genes (*ERF, CHIT IV, Defl, PR10, HEL,* and *CHS)*. Those expression levels were compared between treatment types and ecotypes to determine whether A17 and R108 have different responses to the hormones. The expression of all three ET-associated genes revealed a pattern of up-regulation 12 hours after treatment in both the A17 and R108 ecotypes. This pattern was most significant for the expression of *ERF* that demonstrated an 11- and 15-fold increase in expression for A17 and R108 respectively (Figure 2). This result indicates that both ecotypes have similar patterns for sensing ethylene. This is not expected as treatment with ethylene did not confer resistance in the R108 ecotype (48). This result may indicate an imbalance in the crosstalk pathways that coordinate ET/JA associated resistance, and perhaps essential PR proteins that confer resistance are not expressed in R108.

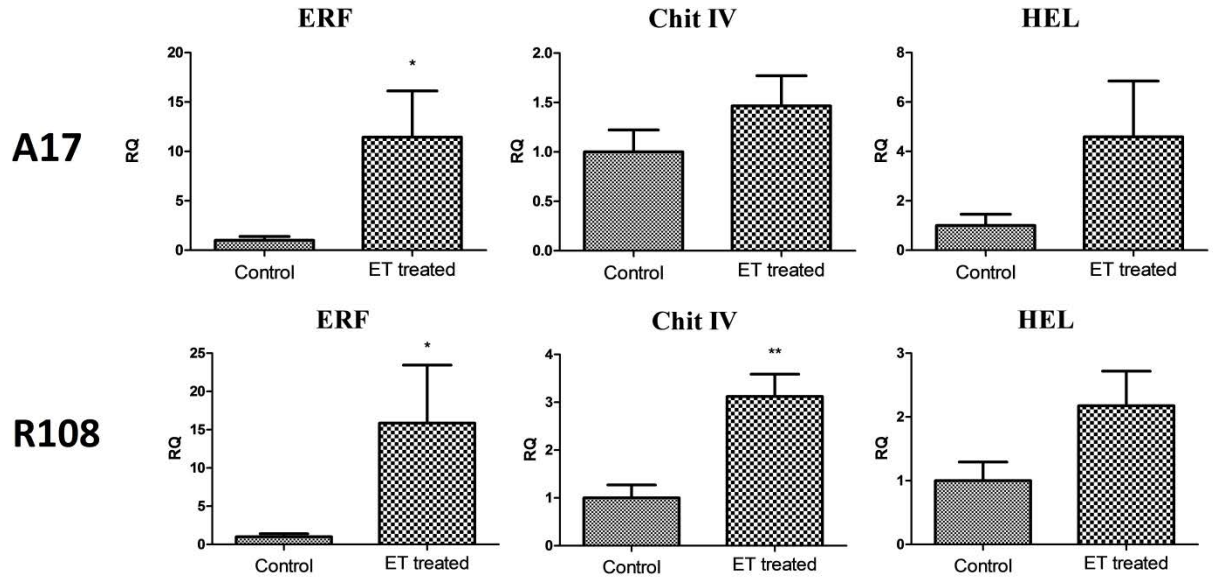


Figure 2: Mean relative quantity of gene expression of ET associated genes in ET treated and control plants. Bars indicate standard error. * and ** represent significance with p-values < 0.05 and 0.01 respectively.

### 2.3.2 JA-associated gene expression in A17 and R108 plants treated with methyl-jasmonate

JA-associated gene expression in A17 plants showed that application of MeJA did not successfully lead to upregulation of JA-associated genes *CHS* or *PR10* after the 12 hour incubation (Figure 3). This indicates that the 12 hour treatment may not have been sufficient time for expression of these JA-associated genes in A17 plants. However, A17 showed a marked increase in *DEF1* transcripts after treatment with JA with a significant (p-value <0.05) and nearly 3-fold difference between the control and the MeJA treated plants. This pattern was not seen in R108 treated with MeJA. While *DEF1* was too difficult to amplify in R108 plants treated with MeJA, *CHS* and *PR10* transcripts exhibited a notable decrease in expression after MeJA treatment. *CHS* expression in particular was significantly (p<0.01) reduced by nearly 7-fold. This indicates that R108 may decrease expression of these JA-associated genes in response to MeJA treatment while A17 increases the expression of these markers.

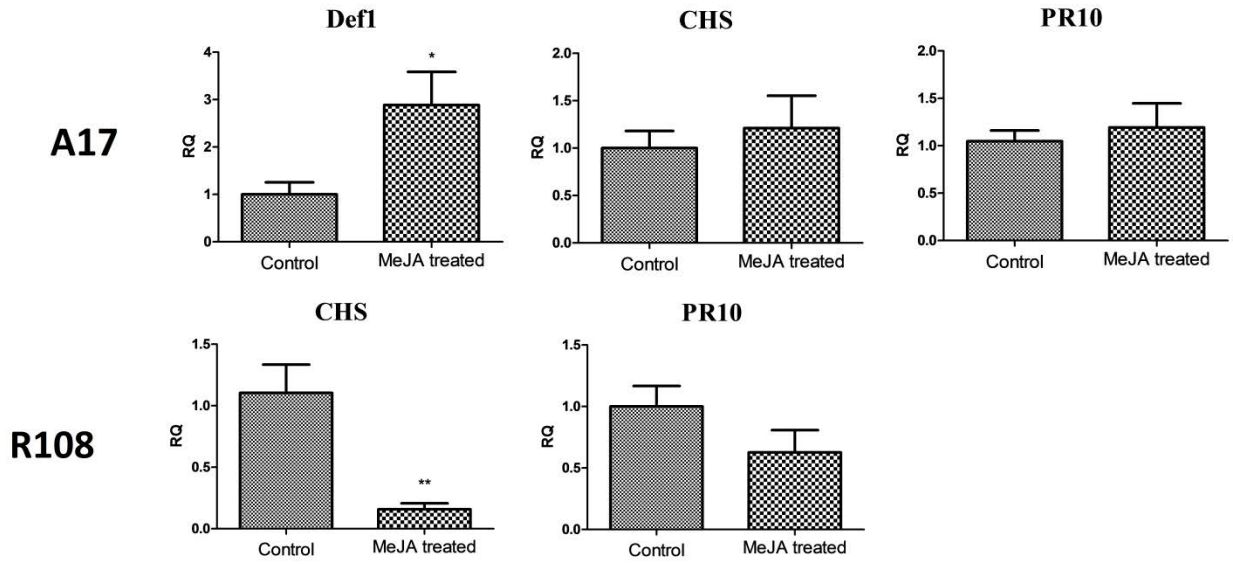


Figure 3: Mean relative quantity of gene expression of JA associated genes in MeJA treated and control plants. Bars indicate standard error. * and ** represent significance with p-values less than 0.05 and 0.01 respectively.

### 2.3.3 Possible mechanisms for phenotypic differences between R108 and A17 ecotypes

Samac and colleagues demonstrated that an *Arabidopsis* knock out mutant of *CHS* was susceptible to the biotrophic pathogen *Colletotrichum trifolii* (49). It may be the case that *CHS* expression is also critical in the defense against *M. phaseolina* such that loss of *CHS* expression causes susceptibility. In the study by Gaige *et al.*, it should be noted that R108 plants treated with MeJA alone exhibited slightly greater disease development than untreated plants and plants treated with ET or MeJA + ET . It should also be noted that, in Tyler's thesis, the expression of *CHS* was actually reduced after 24 hours of infection in *M. truncatula* plants (48). One explanation for this phenomenon may be that *Medicago* plants respond to JA treatment with the up-regulation of repressor JAZ proteins as demonstrated in *Arabidopsis.* This pattern represents a negative feedback loop in which JA biosynthesis promotes JA gene expression repression (73). It could be the case that both A17 and R108 express JAZ in response to JA treatment but, while A17 may counteract this repression with appropriate degradation of JAZ proteins, R108 may be defective in its ability to degrade all or specific JAZ proteins as quickly as A17. Alternatively, R108 may be more sensitive to MeJA and express repressor proteins more than A17. With inability to relieve repression of JA associated genes, JA treatment may lead to JA signaling repression in R108. To test this, future research could investigate JAZ protein expression in the A17 and R108 ecotypes following JA treatment. Another explanation for this unusual phenomenon may be that the MeJA conjugate is not bioactive and, instead, must be converted to a bioactive form such as JA-ILE in order to coordinate JA-associated defense expression. Katsir and colleagues explain that while MeJA and JA-ILE both elicit a physiological response, only JA-ILE can directly promote binding of the COI1 protein to the ubiquitin protease complex $SCF^{COI1}$ (73). It is possible that MeJA treatment does not mimic the physiological response of

plants to necrotrophic pathogens. Instead, some other conjugate of JA may be involved in necrotroph defense. Seo and colleagues note that MeJA treatment promotes *Jasomonate methyltransferase* (*JMT*) expression (74). JMT is an enzyme that converts JA into MeJA, and its overexpression leads to what Mitra and colleagues describe as a MeJA sink in which JA is rapidly converted into MeJA which, in turn, may shift JA signaling towards different strategies of defense or growth (75). Perhaps there is a delay in expression of some of the JA genes observed in A17 that could be a result of the conversion of MeJA into another bioactive form that may explain the insignificant increase in *CHS* and *PR10* expression. In R108, it may be that MeJA promotes *JMT* expression to create a situation where JA-ILE or other JA conjugates are depleted and thereby reduce defense gene expression. Little is known about the JA pathway and it is possible that JA-ILE and MeJA promote the same signaling pathway; therefore future research should investigate *JMT* expression in these two ecotypes. Perhaps *JMT* expression differences between the ecotypes result in different levels of the various JA conjugates that drive the difference in phenotypes.

These results indicate that R108 and A17 ecotypes both sense and respond to ET in a similar manner. Both ecotypes appeared to upregulate *ERF* expression significantly while only R108 demonstrated a significant upregulation of *CHIT IV*. This may be due to the large amount of variability in expression patterns for A17; regardless, A17 appears to upregulate *CHIT IV* as well. Ethylene signaling appears to involve the expression of ERFs that act as transcription factors for downstream signals. Perhaps the lack of significance in upregulation of *HEL* is due to a delayed response requiring early expression of ERFs for *HEL* transcription to occur. If this were the case, then I would expect *HEL* expression to increase 48 hours after hormone application. Doerksen's thesis reported that *HEL* upregulation occurred 48 hours after

inoculation in A17 and R108. This further supports the idea that *HEL* expression is a delayed event. While ET expression can lead to partial resistance in A17, in R108, exogenous ET did not confer resistance, indicating an imbalance in the phytohormone crosstalks resulting from disruption of JA signaling.

Treatment with either ethephon or MeJA does not confer resistance in R108. These results demonstrate no notable difference between A17 and R108's ability to sense ethylene, which may indicate that coordinated expression of both JA-responsive genes and ET-responsive genes are necessary for resistance to the nectrotrophic pathogen *M. phaseolina.* Perhaps the reason why R108 did not respond to ET treatment with the partially resistant phenotype was due to a lack of synergism in the two pathways. As described in Lorenzo et al, disruption of JA or ET signaling can block *ERF1* expression in *A. thaliana* (39). According to the results from Tyler Doerksen's study, both ecotypes significantly upregulate *ERF* expression 24 hours after inoculation. However, in R108, the expression appeared to decrease after 48 hours (48) which may be due to a shift in the crosstalks controlling the necrotrophic response. A shift in the crosstalk signaling network would also explain why treatment with exogenous ET promoted resistance in A17 and not R108. It may be that coexpression of these hormones in *M. truncatula* promotes synergism in downstream signaling as described in *A. thaliana* with the gene *ERF1* (39). When looking for genes that promote resistance to charcoal rot, perhaps the focus should be on those requiring JA and ET signaling and on resistance genes that are not expressed in plants defective in JA or ET signaling. These resistance genes may require the degradation of JAZ proteins as well as ERF transcription factors for their expression. There may also be a homologous gene such as *ERF1* in *M. truncatula* that acts as a transcription factor for a plethora of resistance genes that promote resistance to charcoal rot.

# CHAPTER 3

# EXPLORING THE EFFECTS OF ABA ON PLANT RESISTANCE TO *M. PHASEOLINA*

## 3.1 Introduction

*M. phaseolina* preferentially infects plants during periods of drought (2). Seedling disease caused by *M. phaseolina* in soybean is greatest at temperatures between 30-35 °C (4). The effects of drought on pathogen susceptibility are not fully understood, but they may involve *M. phaseolina's* effects on water transport (76). Current research suggests that plants respond to drought by up-regulating the biosynthesis and release of the phytohormone abscisic acid (ABA) (77). ABA is produced in plant leaves and roots and is released into the xylem (78). ABA binds to receptors that are members of the *PYRABACTIN RESISTANCE* (PYR)*, PYR1-Like* (PYL)*,* and *REGULATORY ELEMENTS OF ABA RECEPTORS* (RCAR) families (79) and causes these receptors to bind and inhibit phosphatases of the type 2C protein phosphotase (PP2C) family (80). Inhibition of PP2C leads to an increase in phosphorylated transcription factors that induce expression of ABA-responsive genes (81). During the drought response, ABA biosynthesis leads to a signal cascade that promotes the release of ions from guard cells that results in a loss in turgor pressure and thus relaxation of the guard cells to prevent water loss through transpiration (82). ABA also has a role in regulating seed dormancy and ABA insensitive mutants were identified in *Arabidopsis* by the ability of seeds to germinate in inhibitory concentrations of exogenous ABA (83). In 1990, researchers proposed a model for the effects of ABA-insensitivity at 3 different loci (*abi-1, abi-2, and abi-3*) wherein *abi-1* and *abi-2* affect vegetative growth but *abi*-3 has greater effects on seed dormancy (83). Other relevant mutations include one in barley called *cool* that causes ABA insensitivity in guard cells that results in excessive transpiration (84).

ABA also has a role in plant pathogen defense. However, this role is unclear as ABA can promote either resistance or susceptibility to different necrotrophic pathogens. In 2007, Adie and colleagues investigated *A. thaliana* for genes that promote resistance or susceptibility to the necrotrophic pathogen *Pythium irregulare* and they discovered that ABA is a key signaling element necessary for defense against this pathogen. In multiple ABA signaling mutants, they observed the spread of disease in inoculated leaf tissue and determined that mutants deficient in ABA signaling had a greater mean disease area than wild-type plants (85). ABA accumulation was also upregulated in most of these mutants in response to the pathogen. However, this pattern was not repeated in the other necrotrophs as these researchers also demonstrated that ABA biosynthesis deficient mutants and insensitive mutants showed increased susceptibility to another necrotrophic pathogen *Alterneria brassicola* while demonstrating increased resistance to the necrotroph *B. cinerea* (85). Their research indicates that ABA expression may be necessary for the expression of JA/ABA-related genes that can promote resistance to some but not all necrotrophs. Another example of how ABA can affect pathogen resistance was when Sanchez-Vallet and colleagues found that disruption of ABA signaling in *A. thaliana* ABA-insensitive mutants could induce resistance to the necrotrophic pathogen *Plectosphaerella cucumeria*. If ABA receptors are knocked-out or biosynthesis is disrupted, *A. thaliana* plants demonstrated more resistance to the pathogen than did the wild-type. Moreover, constitutive ABA signaling resulting from disruption of phosphatases that negatively regulate ABA signaling leads to greater susceptibility (86). ABA's role in providing resistance or susceptibility against necrotrophic pathogens is unclear. It has been proposed that ABA's role in stomata closure may provide innate immunity by preventing pathogen entry (85, 87). So, ABA may provide drought resistance and pathogen resistance by similar mechanisms. Since ABA appears to offer resistance to some

necrotrophic pathogens, we chose to evaluate its potential to provide resistance against *M. phaseolina.*

Since pathogen-resistant phenotypes may prove to be the most cost effective form of control for *M. phaseolina*, Mengistu and colleagues evaluated soybean ecotypes for resistance to *M. phaseolina*. Their research indicated that drought years promoted susceptibility in most of the ecotypes and that no ecotypes were completely resistant during their 3 years of testing (6). Drought resistant soybean cultivars have been investigated for their resistance to the pathogen because disease severity is higher during drought. Wrather and colleagues screened five genotypes of drought-resistant soybean for resistance to *M. phaseolina* as indicated by differences in annual yield. Their results indicate that, during a drought year, only one drought resistant genotype may be resistant to *M. phaseolina*. However, a more interesting result was noted during a period of greater moisture availability where some drought resistant cultivars appeared to have partial resistance to the pathogen (88). Among different *M. phaseolina* tolerant cultivars, there was a significant difference between drought-resistant and drought-sensitive cultivars in their annual yields due to increased resistance to the pathogen in the drought-resistant cultivars (88). Since drought resistance may be a predictor for resistance to *M. phaseolina* and drought resistance occurs through ABA signaling, we hypothesized that exogenous ABA application may provide resistance to the pathogen in *M. phaseolina*-infected plants.

**3.2 Results and Discussion**

*M. truncatula* A17 Jemalong plants were transferred to media containing varying concentrations of ABA, inoculated with *M. phaseolina* infected wheat seeds, and then monitored for the development of chlorotic and necrotic symptoms. We found that plants treated with 100 μM ABA frequently developed chlorotic symptoms prior to inoculation. Therefore, this

concentration was not used for experimentation. Plants treated with 0.1, 1.0, 5, 10, or 20 μM of ABA did not develop symptoms during the time of seven days of observation, therefore these concentrations were used for ABA treatment for plants inoculated with *M. phaseolina*. After several rounds of testing, a discernible pattern developed in which plants treated with 10 or 20 μM ABA developed disease symptoms more rapidly than those treated with 0, 0.1, or 1 μM ABA. One test in particular showed a significant difference at 96 hours post-inoculation (hpi), in which plants treated with 10μM ABA developed chlorosis whereas none of the inoculated control plants did (Figure 4).

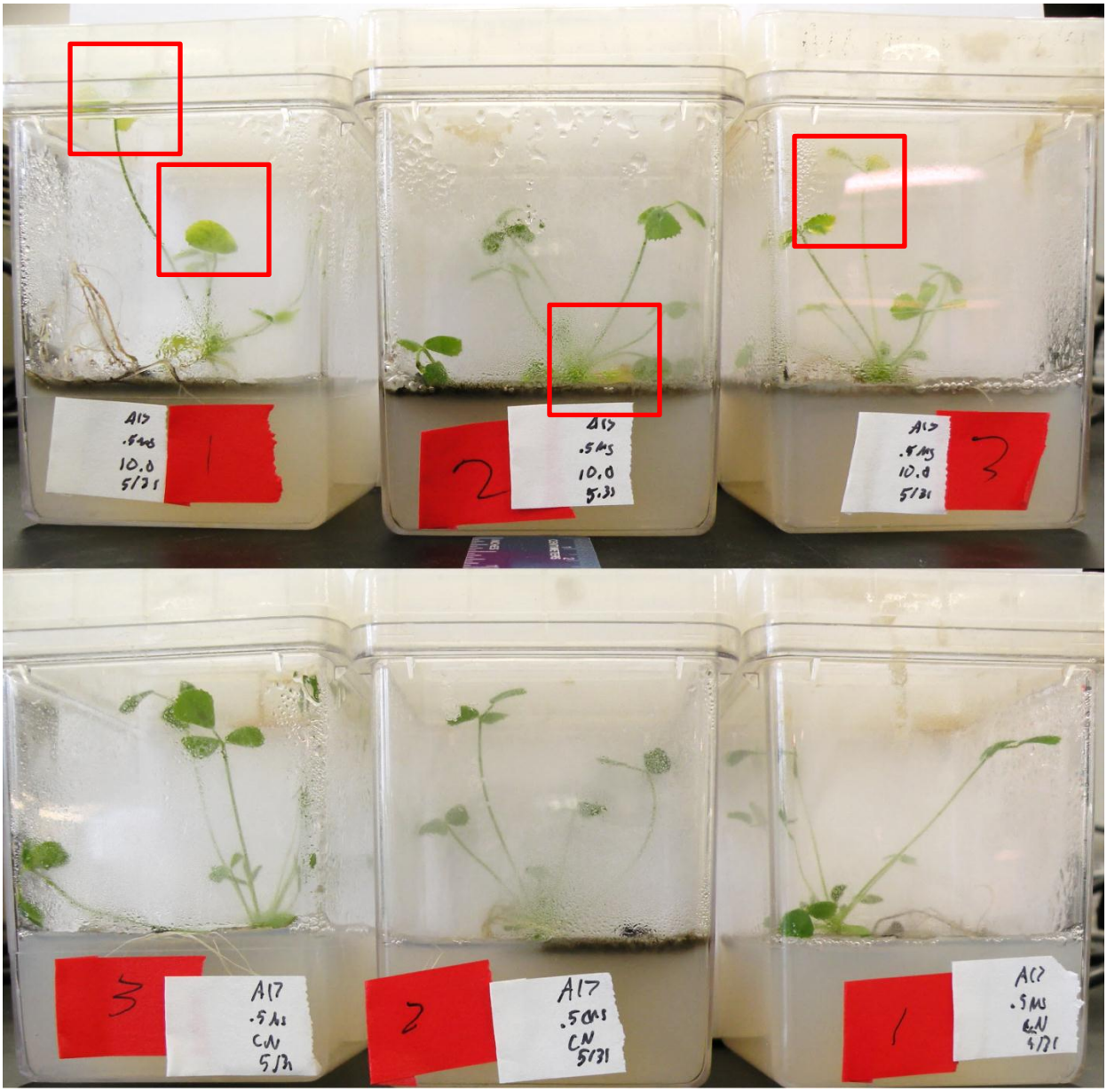

Figure 4: Plants treated with 10 µM ABA showing more rapid disease progression 96 hours after inoculation. Top panel, plants inoculated with *M. phaseolina* and grown on media containing 10 µM ABA. Bottom panel, plants inoculated with *M. phaseolina* and grown on media containing 0.1% methanol . Red rectangles indicate areas of chlorosis. Note: no areas of chlorosis are detected in mock inoculated plants.

The progression of disease in one particular trial using 0, 5, 10, and 20 µM ABA is shown in Figure 5.

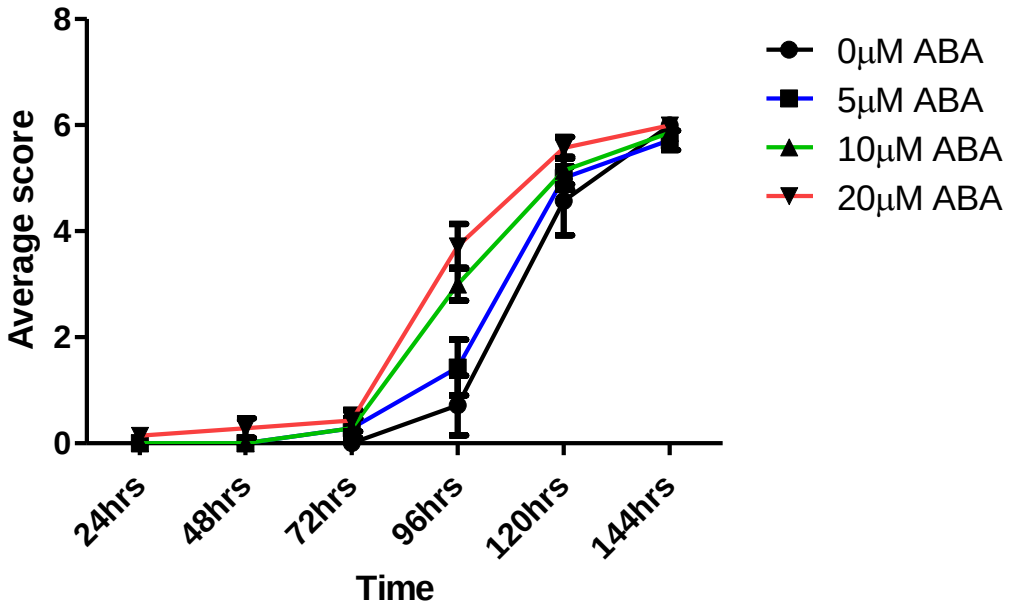


Figure 5: Graph depicting average disease score in ABA treated *Medicago truncatula* plants over the course of infection (n=7). Note the difference in treatment levels at the 96 hour mark.

The graph shows that plants treated with 10 and 20μM ABA developed significantly greater average disease symptoms at 96 hpi than in 5 and 0 μM ABA treated plants (n=7, p-value $< 0.05$). Combined data of 5 trials indicated that plants treated with 10 or 20 μM ABA had a significant difference in disease severity at 96hpi (Figure 6).

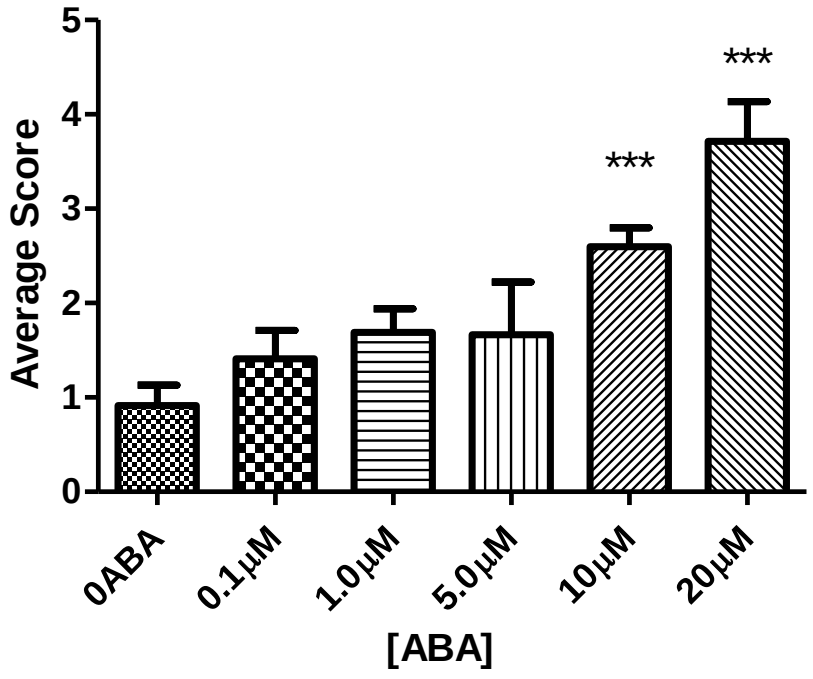


Figure 6: Average disease severity at 96 hpi for *M. truncatula* plants infected with *M. phaseolina* and treated with various concentrations of ABA. *** indicates a significant difference between control treatments and the respective ABA treatment with a p-value < 0.001 calculated with Dunn's multiple comparison post-tests.

Using the Kruskall-Wallis test for non-parametric data, the p-value of less than 0.001, indicates that, at 96 hpi, there was a statistically significant difference among the treatment levels. Using Dunn's multiple comparison test, we observed a statistically significant difference for mean disease severity between the 10 and 20 μM ABA versus the control, 0.1 or 1.0μM ABA treatment groups.

This information indicates that ABA treatment greater than or equal to 10 μM causes earlier development of disease symptoms in *M. truncatula* plants that are infected with *M. phaseolina*. It should be noted, however, that more than 9 days after transferring control plants to media containing either 10 or 20 μM ABA, the plants began developing a yellow appearance between margins of the leaves (Figure 7). This may indicate continued ABA signaling lead to water deficiency. Furthermore, it may be that this treatment created continued ABA signaling

that is detrimental. These observations support previous findings that disruption in ABA signaling results in enhanced resistance against the pathogen *P. cucumerina* or *B. cinerea* in *Arabidopsis*. *A. thaliana* plants deficient in ABA biosynthesis or ABA signaling also demonstrate resistance to the pathogen *P. cucumerina* with longer life post infection (86). In addition, Radwan and colleagues suggest that ABA may promote radical oxidative stress (ROS) and a hypersensitivity response (HR) which, in turn, may lead to cell death (by promoting *M. phaseolina growth*) and induction of SA related defense signaling (89). ABA's role in promoting plant immunity is likely wide spread, effecting many genes in which stress signals ABA release and thus, initiating a cascade of downstream events that will coordinate defense strategies (38). While Adie and colleagues showed ABA is necessary for defense signaling against some necrotrophic pathogens, this may be from an early and possibly temporary signal associated with the initial infection (85).

Figure 7: Picture showing yellowing between margins of un-inoculated control plant 9 days after transfer to 10 µM ABA treated media.

Since *M. phaseolina* preferentially infects plants during drought, drought may also stimulate ABA signaling in a way that is detrimental. Therefore, *M. phaseolina* may rely on ABA signaling for more efficient pathogenesis in plants. Research showing that some fungi including *B. cinerea* and *Cercospora cruenta* can synthesize ABA using a different biochemical pathway suggests a convergent development for this biosynthetic pathway (90, 91). These biosynthetic pathways may prove to have different origins yet the same end product and thus fungi that produce ABA may use it to promote pathogenesis. This offers an advantage to pathogenic fungi that rely on cell lysis for nutrients. In the case of *M. phaseolina,* dry weather and drought may promote plant susceptibility to this pathogen by promoting ABA signaling.

Therefore, if ABA is responsible for the susceptibility; then cultivars that are deficient in ABA signaling may be partially resistant to the pathogen. Additionally, future research should look for soybean cultivars that demonstrate ABA insensitivity as well as drought resistance because they may be less susceptible to the pathogen in hot or dry weather.

# Chapter 4

## SUMMARY AND CONCLUSIONS

The findings in this study suggest that susceptibility to *M. phaseolina* occurs when *M. truncatula* plants do not demonstrate a significant increase in the transcript abundance of JA marker genes. In this case, the lack of induced resistance in R108 plants from ET, MeJA, and ET + MeJA application appears to result from down regulated JA signaling at the transcriptional level. However, transcription levels of these genes may not necessarily correlate with their expression at the protein level. There may in fact be differences in gene regulation at the post-transcriptional level that explain in these different phenotypes between A17 and R108. Analysis of the abundance of these proteins produced by the JA marker genes may provide insight into the importance they have in defense against the pathogen. Because R108 appears to respond to ethephon treatment with similar levels of ET marker transcription compared to A17, functional JA signaling may induce partial resistance to *M. phaseolina* with ethephon treatment. Future investigation may use *M. truncatula* accessions that are deficient in JA signaling or JA sensitivity to study the effects that MeJA and ethephon application have on induction of partial resistance to *M. phaseolina*.

Abscisic acid appears to promote susceptibility to *M. phaseolina.* In this study, we noted earlier onset of the disease caused by *M. phaseolina* in *M. truncatula* plants that were grown on ABA-treated media. We speculate that *M. phaseolina* relies on functional ABA signaling for pathogenesis and that plants deficient in ABA signaling may be better protected from the disease caused by *M. phaseolina.* ABA's role in the hypersensitivy response may cause disease to develop more rapidly since *M. phaseolina* relies on plant cell death for pathogenesis. If this is the

case, mutants deficient in a hypersensitivity response may also be better protected from the disease caused by *M. phaseolina.*

# CHAPTER 5

# MATERIALS AND METHODS

## 5.1 Plant materials

A17 Jemalong and R108 seeds were grown and harvested from original lines at the Samuel Roberts Nobel Foundation (provided by Dr. Srinivasa Rao Uppalapati). The seeds for *M. truncatula* ecotypes A17 and R108 were soaked in concentrated $H_2SO_4$ for 8 minutes to scarify the tough outer coating of the seed. After soaking in acid, the seeds were rinsed 5 times with distilled deionized water ($DDH_2O$) and then transferred to a 20% bleach solution containing 1 drop of Tween-20 for every 20ml of solution in order to sterilize the seeds. After 10 min, the seeds were rinsed 5 times with sterilized $DDH_2O$. Seeds were then transferred to plates containing half-strength Murashige-skoog basal salt (MS, Sigma) media containing 1% sucrose and 1% agar where they were spread out with about 10 seeds per plate. After germinating for 3 days in the dark, the seedlings were transferred to half strength MS agar (pH 5.7, 1% agar, 1% sucrose) contained in Magenta boxes. Growth conditions included a 12 hour photoperiod, 44% relative humidity, with a constant temperature of 27 °C.

## 5.2 ET and JA treatments

The three types of treatments for studying the effects of JA and ET on gene expression included one for each hormone and one for a mock-treated control. After 2 weeks of growth in the Magenta boxes and at the beginning of the dark cycle, control plants from each ecotype were sprayed with a solution of 0.1% methanol containing 0.01% Tween-20. JA treatment included 0.1% methyl jasmonate (Sigma) with 0.01% Tween-20, and ET treatment was with 0.003M Ethephon (Sigma) and 0.01% Tween-20 solution. After 12 hours, right when the light cycle

started, the plants were removed from Magenta boxes, the roots were cut and lightly blotted to remove agar pieces, transferred to labeled tubes, and flash frozen in liquid nitrogen. Samples were kept at -80 °C until RNA isolation.

**5.3 RNA extraction**

To extract total RNA, root samples were placed in mortars that were pre-chilled with liquid nitrogen and then grinded to fine powder using a pre-chilled pestle. After grinding, 1 ml of TriZol (Invitrogen) reagent was added to the sample that was homogenized by further grinding. The homogenized sample was transferred to a 1.5 mL microcentrifuge tube that was centrifuged for 10 minutes at 4 ºC and 12,000 x g to pellet cellular debris. The supernatant was transferred to a clean tube and, after incubating at room temperature for 5 minutes, 200 µl of chloroform was added. The solution was vortexed vigorously for 10 seconds and incubated at room temperature for 3 minutes. To separate the aqueous RNA phase from the organic phase, samples were centrifuged at 12,000 x g for 10 minutes at 4 °C. Five hundred microliters of supernatant was then carefully removed and transferred to a new tube containing 250 µl of isopropanol and 250 µl of a high salt solution (0.8M NaCitrate and 1.2 M NaCl). The solution was mixed well by inverting the tube several times and allowed to incubate at room temp for 10 minutes before centrifuging to pellet the RNA. After centrifugation, the supernatant was discarded and 1ml of 70% EtOH was added to wash the RNA pellet. The RNA was pelleted by centrifuging at 7,400 x g for 5 minutes at 4 °C. After removing any trace amount of EtOH, the pellet was air-dried and resuspended in 20µl of RNase free $H_2O$ (Ambion). Sample quality and quantity was analyzed using a NanoDrop spectrophotometer (ThermoScientific).

**5.4 DNase treatment**

To ensure that the RNA samples were not contaminated with genomic DNA, they were treated with DNase and then resuspended in RNase free $H_2O$. First, 10 µg of RNA were mixed with 1 µl of Turbo DNase (Ambion), 5 µl of 10X DNase buffer, and enough water to bring the reaction volume up to 50 µl. The samples were mixed and then allowed to incubate at 37 °C for 30 mins. After digestion of genomic DNA, the sample was transferred to a new centrifuge tube and the reaction volume was brought to 250 µl. Next, 250 µl of 25:24:1 (phenol:chloroform:isoamyl alcohol) (Fisher) was added to the reaction tube which was then vortexed for 10 seconds before incubating at room temperature for 3 minutes. After incubation, the sample was centrifuged at 12,000 x g for 10 minutes at 4 °C. Then, 200 µl of the top layer (aqueous phase) was transferred to a new tube and 400 µl of 100% EtOH and 20 µl of 3 M NaOAc (pH 5.2) were added. The tube was then mixed vigorously for 10 seconds and allowed to incubate for 30 minutes in a -20 °C freezer. After incubation, the tube was centrifuged as before and the supernatant was discarded. The pellet was washed with 1mL 70% EtOH. The ethanol was removed and the pellet was dried before resuspension in 10µl of RNase free $H_2O$.

**5.5 Reverse Transcription (RT)**

The first step in preparing samples for quantitative PCR was to convert the RNA samples into cDNAs using reverse transcriptase. RNA quantity and quality were analyzed using a NanoDrop spectrophotometer (ThermoScientific). Using a 0.5 ml PCR tube, 2 µg of the DNase-treated RNA sample, 1 µl of 20 µM OligodT primer, and enough RNase-free $H_2O$ to bring the reaction volume to 12µl were mixed. The reaction tube was incubated at 65 °C for 5 mins and then chilled on ice. A master mix containing 4 µl of 5X first strand buffer, 2µl of 100 mM DTT, 1 µl of 10 mM dNTPs, and 1µl of SuperscriptIII Reverse transcriptase (Invitrogen) was added to each sample and the RT reaction was carried out at 50 °C for 1 hour followed by 10 min

incubation at 70 °C to denature the enzyme. To test for genomic DNA contamination and to confirm the synthesis of cDNA, PCR was prepared with 2µl of cDNA or DNase treated RNA sample and 1mM of forward and reverse UBQ primers. For this PCR, UBQ primers were used with a PCR protocol that includes an initial 95 °C denaturation step followed by 35 cycles of a 1.5 minute 95°C denaturation step, a 45 second 60°C annealing step, and a 45 seconds 72°C extension step. This was followed by a 5 minute final extension step before running products on a 2% agarose gel with a 1kb ladder standard.

**5.6 RT-qPCR**

Three technical replicates were included for each sample. A cocktail mix containing all reagents for the replicates was prepared to minimize pipetting errors. For each replicate, the cocktail contained 5 µl of 2X Power SYBR Green PCR master mix (Applied Biosystems), 1 µl of 1:10 diluted cDNA (MilliQ $H_2O$ for negative controls), 1µL of the respective primers (1 µM of forward and reverse primers, see appendix for sequences), and 3 µl of $H_2O$. After mixing all reagents, the samples were aliquoted to the reaction plate with 10 µl per well. RT-qPCR reaction was carried out in a StepOne Plus Real-Time PCR system (Applied Biosystems) and the data were analyzed using StepOne Plus software. The comparative $C_T$ method was used to determine the relative fold change in transcript abundance of a particular gene in each sample. $C_T$ values were determined computationally by the StepOne software that determines that fractional cycle at which the signal threshold is reached. To normalize $C_T$ values the endogenous control gene *UBQ* (*Ubiquitin*) was used. Normalized $C_T$ values ($\Delta C_T$) of biological replicates were averaged to produce mean $\Delta C_T$ values. $\Delta C_T$ values were then compared between the methanol control (calibrator) and hormone (sample) treatment types to produce $\Delta\Delta C_T$ values that were used to calculate the relative quantity (RQ) of transcript abundance in fold difference. RT-qPCR $C_T$

values were calculated by StepOne Plus software and the data were exported to GraphPad Prism (GraphPad Software Inc.) to perform the student t-test using a 2-way significance p-value.

$$\Delta C_T \text{ sample} = C_T \text{ (Gene of interest)} - C_T \text{ (UBQ)}$$

$$\Delta C_T \text{ calibrator} = C_T \text{ (Gene of interest)} - C_T \text{ (UBQ)}$$

$$\Delta\Delta C_T = \Delta C_T \text{ sample} - \Delta C_T \text{ calibrator}$$

$$\text{Relative quantity} = 2 \text{ ^ } (-\Delta\Delta C_T)$$

**5.7 ABA treatment**

ABA-treated plants were grown using similar methods to the ET and JA-treated plants. However, after the plants grew in the Magenta boxes for 12 days, they were transferred to Magenta boxes containing 1% agar, 1% sucrose, half strength MS, and varying concentrations of ABA or a control treatment of just 0.1% methanol at a pH of 5.7. These plants were grown in this media for 2 days before being inoculated with wheat seeds that were infected with *M. phaseolina*. Sterile wheat seeds were used for mock inoculation. There were 5 replicates with 3, 5, or 7 plants per treatment per replicate for a total of 23 control plants, seven 20μM ABA-treated plants, twenty-five 10 μM ABA-treated plants, six 5 μM ABA-treated plants, sixteen 1.0 μM ABA-treated plants, and seventeen 0.1 μM ABA-treated plants. Each replicate also included 3 uninoculated controls in order to verify that the treatment itself was not detrimental. Any plants that were contaminated with microbial growth other than of *M. phaseolina* were excluded from observation.

**5.8 Inoculum preparation**

*M. phaseolina* isolate #210 was maintained on potato dextrose agar (PDA) and used to prepare the wheat seed inoculum. Wheat seeds were soaked in $DDH_2O$ overnight with several water changes. These seeds were then sterilized in an autoclave with 20 minutes sterilization

time using Erlenmeyer flasks containing approximately 20 ml of water and sealed with cotton plugs. After removal from the autoclave, the seeds were allowed to cool before adding four or five 1 $cm^3$ PDA plugs with *M. phaseolina* culture. Control seeds were placed in an incubator without fungus being added. Seeds were incubated at 27 °C for 5 days and the flasks were shaken every day to allow even distribution of the fungus.

**5.9 Wheat seed inoculation, scoring matrix, and data analysis**

Two days after the plants have been transferred to ABA containing media, one wheat seed was dropped into the magenta box using sterile technique. For the control plants that are to be left uninfected, a sterile wheat seed was dropped into their box. The disease progression was then studied and photographed for up to seven days after the inoculation procedure. Infected plants were graded using a scoring matrix which scores plants that displayed no signs of disease as a score of 0, plants with 1-10% chlorosis or 1-5% necrosis received a 1, plants with 10-20% chlorosis or 5-10% necrosis scored a 2, plants with 20-40% chlorosis or 10-20% necrosis scored a 3, plants with 40-60% chlorosis or 20-40% necrosis scored a 4, plants with 60-80% chlorosis or 40-60%.necrosis scored a 5, and dead plants or those with greater than 80% chlorosis or greater than 60% necrosis were scored a 6. Results for the five ABA replicates were combined and analyzed using GraphPad Prism and the Kruskal-Wallis one-way analysis of variance and Dunn's multiple comparison post-test.

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# APPENDIX

# APPENDIX

Primers used in RT-qPCR to study gene expression. F = forward, R = reverse.

| Primer name | Primer sequence (5' to 3') |
|---|---|
| MtChit4-F | GGTGATGCATATTGTGGCACAGGG |
| MtChit4-R | GCAGCAGCAACCTCACGTTTGGAG |
| MtCHS-F | CCACGACACCATCCTAAATTGTATC |
| MtCHS-R | TGGTGTGACTAATGCCTTTTTGAC |
| MtDef1-F | GGACCATGCTTTAGTGGTTGTG |
| MtDef1-R | CCTGCCGCTAACTGCATTCT |
| MtERF-F | GATCAACGACGCTAACAACCTC |
| MtERF-R | CCATCTTCAGAACCATCAT |
| MtHEL-F | CCTTGTGGGAACGATGTTAGTG |
| MtHEL-R | AGGTGGTATGTTGCCCTAACGT |
| MtPR10-F | TGTTGGCCTTCCAGACACAA |
| MtPR10-R | CCATTTGGACCTGCAGACAA |
| MtUBQ-F | GAACTTGTTGCATGGGTCTTGA |
| MtUBQ-R | CATTAAGTTTGACAAAGAGAAAGAGACAGA |