INVESTIGATING THE GENETIC VARIATIONS AMONG *MEDICAGO TRUNCATULA* ECOTYPES IN RESPONSE TO PATHOGENIC FUNGUS *MACROPHOMINA PHASEOLINA*

A Thesis by

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Bachelor of Arts, Bethel College Ks, 2008

Submitted to the Department of Biology
and the faculty of the Graduate School of
Wichita State University
in partial fulfillment of
the requirements for the degree of
Master of Science

July 2012

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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

_____
Jim Bann, Committee Member

DEDICATION

To all my friends and family;

The bold, stubborn, insightful and pragmatic
The loving, affectionate, caring and dramatic

And most of all;

To the everlasting Arm and ever present Comfort
Faithful when I was faithless
Faithful ever still
Savior, Creator, Lover and Friend

Without you none of this was possible

ACKNOWLEDGEMENTS

Thanks to my advisor Dr. Bin Shuai, for her support, advice and countless revisions of my work. Thanks to my committee members for hearing the same talk and reading the same manuscript more than once. Thanks, to my lab mates, Andrés, Carrie, Priya and Kar Men for teaching, advising, supporting and laughing. And thanks to all my graduate student friends along the way for living inside and out of the lab.

ABSTRACT

Macrophomina phaseolina, an internationally distributed fungal pathogen, causes a disease known as “charcoal rot” (also known as dry-weather wilt and summer wilt) that clogs vascular tissue and produces yellow, wilted plants with visible fungal sclerotia. Primarily acting in dry, hot conditions, it inflicts extensive economic losses in Midwestern crops such as soybean, alfalfa, sorghum, and cotton. To better understand the molecular mechanisms of the disease a model pathosystem was established using *Medicago truncatula* to study host-pathogen interactions. 319 ectoypes of *M. truncatula* were screened using a root-dip method to identify potential variations in host susceptibility (either unusual susceptibility or resistance) to *M. phaseolina* in hopes of revealing naturally existing genetic variations that can lead to host resistance. In this screen, all ecotypes showed normal disease progression compared to a reference genotype, Jemalong A17. Previous study showed that treatment with jasmonic acid (JA) and ethylene (ET) confer increased resistance to A17 but not to R108, another widely used *M. truncatula* genotype. To test the hypothesis that the difference in disease progression in A17 and R108 after exogenous treatment with JA and ET was due to differential regulation of the JA/ET pathways in these genotypes, expression of genes representing the JA and ET pathways were compared in A17 and R108 after inoculation with *M. phaseolina*. The results showed that genes indicative of the JA pathway were induced both faster and stronger in A17 than in R108, and marker genes representing ET pathway were more strongly upregulated in A17 than in R108. Since many of the marker genes used in this study are related to fungal defense and considering that the JA and ET pathways regulate plant defense genes, including those with antifungal properties, it is likely that the varying induction of JA and ET pathways is involved in the different hormonal responses between A17 and R108 to *M. phaseolina* infection. Testing that hypothesis requires further study of how these marker genes respond to exogenous JA and/or ET treatment in A17 and R108.

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LIST OF ABBREVIATIONS

µl	Microliter
µmol	Micromolar
C	Celcius
cDNA	Complementary DNA
C_T	Cycle Threshold
dpi	days post inoculation
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide Triphosphate
dpi	Days Post Inoculation
g	Gram
hr	Hour
KCl	Potassium Chloride
M	Molar
mg	Milligram
$MgCl_2$	Magnesium Chloride
$MgSO_4$	Magnesium Sulfate
ml	Milliliter
mm	Millimeter
mM	Millimolar

mRNA	Messenger Ribonucleic Acid
NaCl	Sodium Chloride
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
MS	Murashige and Skoog

CHAPTER 1

INTRODUCTION

1.1 *Macrophomina phaseolina* and charcoal rot disease

In 1890, upon examining black-rot of sweet potato, B.D. Halstead described the hardened mycelium called sclerotia of necrotrophic fungus, *Macrphomina phaseolina,* as minute, irregular masses appearing as bits of charcoal (Dhingra & Sinclair, 1978). Causing a disease called charcoal rot (AKA dry-weather wilt, summer wilt), *M. phaseolina* has a wide host range of more than 500 cultivated and wild plants including *Arachis hypogaea* (peanut), *Glycine Max* (soybean), *Helianthus annuus* (sunflower), *Medicago sativa* (alfalfa) and *Zea mays* (corn) (H. Khan & Shuaib, 2007). *M. phaseolina* is regarded as a non-host specific pathogenic fungus. Even though it has an extreme level of variation both between isolates from different plant species and isolates from different parts of the same plant, the genus *Macrophomina* is considered to have only one species, *M. phaseolina* (Dhingra & Sinclair, 1978; H. Khan & Shuaib, 2007; Su, Suh, Schneider, & Russin, 2001). While *M. phaseolina* is believed to be asexual, it has been suggested that different sub-populations can interact to form genetic hybrids with novel genetic profiles and pathogenic capabilities (Saleh et al., 2010). This genetic exchange between isolates is thought to occur through the fusion of vegetative cells that may form heterokaryons where parasexual recombination of nuclear genes can take place (Pearson, Leslie, & Schwenk, 1986; Su et al., 2001). Usually deemed a soil borne fungus due to sclerotia in the soil, it may undergo aerial and seed-borne transmission by conidia and pycnidia that reportedly form naturally on certain hosts (Dhingra & Sinclair, 1978; H. Khan & Shuaib, 2007; Pratt, Mclaughlin, Pederson, Rowe, & Management, 1998). Sclerotia formed by the fungus in parasitized plant tissue function as the long-term unit of survival in the soil and primary inoculum for root infection (Dhingra & Sinclair, 1978). The sclerotia vary in size and shape

depending on isolate, substrate and temperature (Dhingra & Sinclair, 1978; H. Khan & Shuaib, 2007; Pratt et al., 1998). Germinating in minimal nutrient supply, sclerotia of the fungus are multicellular and can remain viable for at least a year in dry culture media and 4 years in infected tea roots in dry storage (Dhingra 1978). In soil, some report that sclerotia survive for more than ten months under dry conditions while another source reports survival for several seasons in soil and plant parts (Bhatia et al., 2008; H. Khan & Shuaib, 2007). Another group reported that sclerotia survive much longer under dry than wet conditions (Dhingra & Sinclair, 1978).

M. phaseolina has been identified in many countries including Argentina, India, the US, and Pakistan, and is found in most crop-producing areas of the tropics, subtropics and subtropical-temperate regions (Dhingra & Sinclair, 1978; H. Khan & Shuaib, 2007). It was estimated in 1978 to be the cause of 10-15% crop loss each year (Dhingra & Sinclair, 1978). Charcoal rot causes yield loss of up to 25% in sunflower and is ranked fourth in soybean diseases in the north central United States (Doupnik, 1993). A 2007 disease estimate for soybean crops in the United States listed charcoal rot responsible for 30,133,000 bushels lost; the third highest among other listed pathogens (Wrather & Koenning, 2009).

Taxonomic classification of *Macrophomina Phaseolina*

Division	*Eumycota*
Sub Division	*Deuteromycotina*
Class	*Coelomycetes*
Order	*Sphaeropsidales*
Family	*Sphaeropsidaceae*
Genus	*Macrophomina*
Species	*phaseolina*

Figure 1. Taxonomic classification of *M. phaseolina* (S. N. Khan, 2007)

A study by Dhingra and Sinclair showed optimum growth of *M. phaseolina* in soil at 35 °C with a soil moisture of 5%, conditions usually associated with drought (Dhingra & Sinclair, 1978). While maximum infection occurs at the seedling stage or when the plant is maturing,

many crops develop the disease after they lose vigor due to environmental stresses such as drought or high temperature (Dhingra & Sinclair, 1978). Increased incidence and severity as well as a broader host range also occur during flowering and seed development (Dhingra & Sinclair, 1978; Pratt et al., 1998; Saleh et al., 2010). A single sclerotium can cause death in a host and disease severity was directly linked to the population of viable sclerotia in the soil (Dhingra & Sinclair, 1978; H. Khan & Shuaib, 2007). The main symptom in various adult plant hosts is dehydration of leaves followed by progressive necrosis of vascular tissue with subsequent collapse of surrounding pith and epidermis (Dhingra & Sinclair, 1978; Pratt et al., 1998). In some hosts, sclerotia were found in all tissues of the plant including just beneath the epidermis where microsclerotia may be so numerous as to give the tissue a grey-black look (Dhingra & Sinclair, 1978; H. Khan & Shuaib, 2007; Pratt et al., 1998).

Generally, germ tubes from microsclerotia that germinate on the root surface form appresoria that penetrate host epidermal cell walls by mechanical pressure and enzymatic digestion or through natural openings (Dhingra & Sinclair, 1978). Invasion of the host requires both pectolytic and cellulolytic enzymes (Dhingra & Sinclair, 1978). After penetration, the fungus primarily grows intercellularly but hyphae can fracture the middle lamella either mechanically or enzymatically causing disintegration of pectin of middle lamellae and outer cell walls, allowing intracellular colonization (Dhingra & Sinclair, 1978). Upon colonization of the epidermal and cortical cells, hyphae colonize the vascular system where large numbers of sclerotia develop in the xylem vessels so as to plug them and thus drastically reduce of water transport (Dhingra & Sinclair, 1978). Toxins produced by the fungus can also cause dehydration of seedling leaves and may play a role in dehydration of adult plants (Dhingra & Sinclair, 1978).

With such wide-spread and economically devastating effects, effective ways to manage charcoal rot will be very beneficial. Suggested agronomical control techniques include irrigation, adjusting planting dates, and changing planting densities (Mengistu, Ray, Smith, &

Paris, 2007). Crop rotation may also prove effective since the population of *M. phaseolina* tends to increase if a host crop is grown year after year (Dhingra & Sinclair, 1978). Saxena and Mathela reported that, in lab conditions, *M. phaseolina* is very susceptible to iridodial β-monoenol ecetate, an essential oil isolated from plant *Nepeta leucophylla* (Saxena & Mathela, 1996). More recently it was found that fluorescent pseudomonads isolated from the rhizosphere of groundnut (*Arachis hypogeal*) suppressed *M. phaseolina* growth in vitro and enhanced germination and grain yield (Bhatia et al., 2008). Other methods that had limited effects include fungicide applications to seed and soil and biological control using hyperparasitism (Boulevard, Reddy, Zablotowicz, & Weed, 2009). However, it is currently thought that host resistance, may be the only feasible method to manage charcoal rot (Mengistu et al., 2007). Traditional plant breading and genetic engineering that generates transgenic lines either knock-outing or overexpressing a gene of interest are common ways of creating resistance strain.

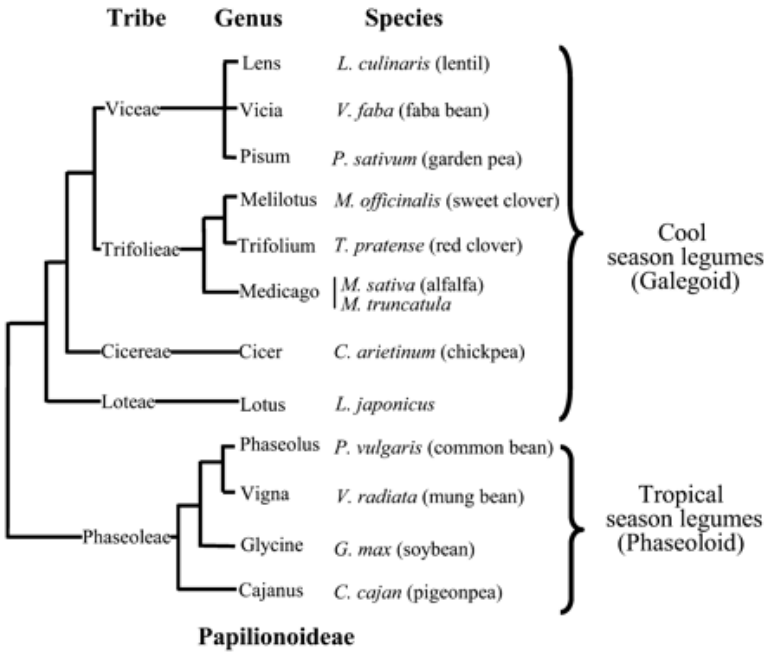

Figure 2 - Phylogenetic relationships of Papilionoideae legumes (Zhu et al., 2005)

1.2 *Medicago truncatula* as a model system for studying charcoal rot disease

The hosts of *M. phaseolina* include many legumes. Legumes (Fabaceae) are second only to grasses in importance as food, feed, and raw materials plus they cover 12-15% of Earth's arable surface and thus result in 27% of the world's primary crop production (Benedito et al., 2008; Graham & Vance, 2003; Tadege et al., 2008). Responsible for 35% of processed

vegetable oil, they are also rich sources of dietary protein in the chicken and pork industries and play an important role in agroforestry and natural ecosystems due to their N-fixing capability (Graham & Vance, 2003). *Medicago sativa*, a prevalent forage legume in temperate climates important for dairy and meat production, is regarded as the most important forage legume in the world and ranks 3rd or 4th most valuable crop in the U.S. (Graham & Vance, 2003; Uppalapati et al., 2009). Legumes can be used in food products such as bread, doughnuts, tortillas and spreads; in liquid form to produce milks, yogurt and infant formula; or in novel products such as soybean candy, licorice and as a thickener in pill formation (Graham & Vance, 2003). Industrially, legume products are utilized to form biodegradable plastics, oils, gum, dye, ink and biodiesel (Graham & Vance, 2003). Medicinally, isoflavones from soybean and other legumes may reduce cancer risk plus serum cholesterol levels while phytoestrogens are proposed as possible alternatives to hormone replacement therapy in postmenopausal women (Graham & Vance, 2003).

Therefore, improvement in crop legumes would be beneficial. However, such improvement is difficult to achieve given that species variety introduces obstacles such as genomic variability, fertility, generation time, sheer plant size and manageability. To overcome these obstacles, model legumes, that are more manageable in the areas mentioned above, are proposed for molecular and genetic studies with the goal of identifying agronomically important genes in other legumes (Kemen, Hahn, Mendgen, & Struck, 2005). Thus far, commonly used model legumes include *Lotus japonicus* and *Medicago truncatula. M. truncatula* is widely used as a model legume due to its small, diploid genome (500 MB), self-fertility, short generation time, amenability to transformation, availability of Affymatrix gene chips, accessible gene atlas, and numerous accessible mutants (Tadege et al., 2008; Tadege, Wang, Wen, Ratet, & Mysore, 2009; Uppalapati et al., 2009). It is also a member of the "galegoid" group in the legume family, which comprises agronomically significant crop species such as pea (*Pisum sativum*), broadbean

(*Vicia faba*), chickpea (*Cicer arietinum*), and alfalfa (*Medicago sativa*) (Kemen et al., 2005). Zhu et al. showed that *M. truncatula* and *M. sativa* share highly conserved sequences and exhibit nearly perfect synteny of genomes (Zhu, Choi, Cook, & Shoemaker, 2005). They also demonstrated that, of 50 soybean contigs, 27 (54%) possessed some level of microsynteny with *M. truncatula* and that the SYM2 region of pea is highly syntenic with *M. truncatula* (Zhu et al., 2005).

It is generally accepted that host resistance is the most cost-effective and long-term strategy for controlling necrotrophic pathogens such as *M. phaseolina* (Mengistu et al., 2007; Tivoli, Baranger, Sivasithamparam, & Barbetti, 2006). However, infection and disease development of *M. phaseolina* including mechanistic interaction and genetic involvement is still very poorly understood. Ellwood et al. stated that resistance to nectrotrophs is quantitative and poorly understood because relatively few resistance genes are characterized, but they believe that *M. truncatula* provides a good platform to identify and isolate R genes and genes involved in resistance signaling pathways due to its genetic tractability (Ellwood, Lichtenzveig, Kamphuis, & Oliver, 2006). Tivoli et al. affirm that *M. truncatula* is an appropriate and agronomically relevant model plant for legumes over the popular Arabidopsis and is proving itself a useful research tool in understanding mechanisms of resistance to individual pathogens as well as general necrotrophic pathogens to forage and grain legumes (Tivoli et al., 2006). To better understand the molecular interactions between *M. phaseolina* and its plant host and help elucidate the potential mechanisms involved in disease response, we established a model pathosystem using *Medicago truncatula* (Gaige, Ayella, & Shuai, 2010).

CHAPTER 2

ECOTYPE SCREEN IDENTIFIED NO VARIATION IN HOST RESPONSE TO *M. PHASEOLINA*

2.1 Introduction

Varying grades of resistance to *Macrophomina phaseolina* were reported in numerous species including *Cyamopsis tetragonoloba* (cluster bean), *Sorghum bicolor* (sorghum), *Sesamum indicum* (sesame), *Corchorus sativus* (jute), *Glycine max* (soybean), *Vigna radiate* (mung bean), *Vigna unguiculata* (cowpea) and *Phaseolus vulgaris* (common bean) (De & Kaiser, 1991; Hernández-Delgado, Reyes-Valdés, Rosales-Serna, & Mayek-Pérez, 2009; H. Khan & Shuaib, 2007; Lodha & Solanki, 1993; Olaya, Abawi, & Weeden, 1996; Singh & Lodha, 1986; Sinhamahapatra & Das, 1992; Smith & Carvil, 1997; Tenkouano, Miller, Frederiksen, & Rosenow, 1993). Resistance to charcoal rot in beans was associated with drought tolerance but there are no reports of this genetic overlap in other legumes except in the sorghum stay-green trait (Mayek, E, Cumpián, & Acosta, 2004; Muchero, Ehlers, Close, & Roberts, 2011; Pastor-Corrales, 1988). However, in common bean, soybean, sorghum, and potato early maturation was associated with increased susceptibility to *M. phaseolina* and has been noticed in other systems as well, most notably potato (Muchero et al., 2011). A screening of common bean genotypes showed that most of the resistant ones belonged to the mesoamerican race that had a black seed coat while susceptible ones were from Jalisco or Durango races with “pinto” or “bayo” seed coats (Mayek-Pérez, López, and Acosta 2002; N Mayek-Pérez et al. 2001).

A 1996 study by Olaya et al. found that resistance to *M. phaseolina* in beans was controlled by two dominant complementary genes designated *Mp-1* and *Mp-2* (Olaya et al.,

1996). Thought to be polygenic in common bean, resistance was attributed to two dominant genes with double-recessive epistatic effects along with possible influences from some minor genes (Hernández-Delgado et al., 2009; N Mayek-Pérez, López-Salinas, Cumpián-Gutiérrez, & Acosta-Gallegos, 2008; Miklas et al., 2000). Quantitative trait loci (QTL) involved in resistance in common bean were found on linkage groups US7 (B7) and US8 (B8) (Miklas et al., 2000). In cowpea, resistance had a quantitative basis (Muchero et al., 2011). In soybean and *Medicago,* QTL contained genic single nucleotide polymorphism (SNP) markers with disease resistance gene annotation and disease resistance-associated genes were located within syntenic regions (Muchero et al., 2011). Pectin metabolism genes were identified in 3 of the QTL intervals, suggesting an important role for pectin-related genes in defense against *M. phaseolina* (Muchero et al., 2011). Pectin, a plant cell wall polysaccharide, is involved in pathogen defense in Arabidopsis, *Malus domestica* (apple) and *Vitis vinifera* (grapevine) (Muchero et al., 2011).

An ecotype screen of 113 *M. truncatula* accessions showed varied resistance strategies in response to biotrophic oomycete *A. euteiches* (Djébali et al., 2009). *M. truncatula* cultivar Jemalong A17 responded to *A. euteiches* infection by expanding its root system that is thought to be facilitated by pericycle cell division and might provide additional structural barriers to allow increased secondary root production and/or prevent stele colonization (Djébali et al., 2009). They also observed that both endoderm and pericycle cell-wall thickenings and lignin deposition on the outer pericycle cell wall contributed to protection of the stele (Djébali et al., 2009). Another study observed that in response to infection by the rust fungus *Uromyces striatus* , 4 resistant *M. truncatula* ecotypes had deposits of lignin and lignin-like phenolics indicated by fluorescence of cells and yellow-blue auto fluorescence of cell walls surrounding the infection (Kemen et al., 2005). Observations were also made of aborted cells that didn't form haustoria and restricted development of haustorium resulting in poor sporulation of the fungus (Kemen et al., 2005). Another group of ecotypes developed brownish cells and strong auto fluorescence

typical of hypersensitive reactions in response to *U. striatus* (Kemen et al., 2005). One accession showed prehaustorial resistance where fungal development stopped immediately after haustorial mother cells formed without necrosis of cells in contact with the fungus (Kemen et al., 2005). This appears to be non-host resistance, a desirable form of resistance due to its effectiveness and durability (Kemen et al., 2005).

Resistance of *M. truncatula* to other fungal pathogens is documented. Genotype A17 is resistant to the biotrophic fungal pathogen *Colletotrichum trifolii* (anthracnose) which is associated with a hypersensitive response (Yang et al., 2007). This is likely mediated by a single dominant gene labeled *RCT1* (Yang et al., 2007). RCT1 was physically mapped to the top of *M. truncatula* linkage group 4 and is a part of a complex locus containing numerous genes homologous to nucleotide binding site and C-terminal leucine-rich repeat (TIR-NBSLRR) type resistance genes (Yang et al., 2007). Partial resistance in A17 to oomycete *Aphanomyces euteiches* is attributed to a 135-kb region rich in proteasome-related genes near the top of chromosome 3, a region well associated with resistance (Djébali et al., 2009). Plants inoculated with *A. euteiches* responded to pathogen attack by expanding their root system, a process thought to be facilitated by pericycle cell division that might provide additional structural barriers to allow for increased secondary root production and/or prevent stele colonization (Djébali et al., 2009). Both endoderm and pericycle cell-wall thickenings and lignin deposition on the outer pericycle cell wall contributed to protection of the stele (Djébali et al., 2009). Previous QTL for resistance to biotic stresses was detected in the same region of chromosome 3 as a minor QTL for partial resistance in *M. truncatula* to the root bacterium *Ralstonia solanacearum* (Djébali et al., 2009).

2.2 Results and Discussion

As stated before, host resistance will most likely offer the most effective and long term way to manage *M. phaseolina*. In order to develop host resistance, more about host-pathogen interactions, such as molecular mechanisms of the disease and host responses, must be known. Uncovering the molecular interactions between the host and the pathogen can lead to identification, location, and functional analyses of whatever gene or genes that may confer resistance. This process is also vital for understanding the basis of resistance that would guide genetic engineering of disease resistant strain. A common way to identify genes responsible for a particular phenotype is to screen populations for that phenotype. From these pools, a desired phenotype is identified and, through subsequent methods such as genetic mapping or functional genomics, the gene or genes responsible for the phenotype are discovered. In this study, the screened population was a collection of naturally occurring *M. truncatula* ecotypes and the particular desired phenotype is resistance to *M. phaseolina*.

319 ecotypes were attained from the U.S National Plant Germplasm System (NPGS http://www.ars-grin.gov/npgs/) and the specific goal was to identify accessions that either showed increased susceptibility or increased resistance to *M. phaseolina*. Susceptible and/or resistant accessions can be molecularly and genetically dissected to further our knowledge on the infection process. Accessions were screened using a root dip method and were monitored and scored according to development of necrotic and chlorotic symptoms by comparing to the reference genotype Jemalong A17. 174 of the 319 accessions were previously screened (Gaige, 2010) and the 145 remaining lines were screened using the same protocol in this study. Depending on availability of plants and fungal inoculum, 3-6 plants were inoculated with *M. phaseolina* and 2-3 plants were used as controls for each accession. For the reference A17 plants, 3 were inoculated and 3 were used as control in each batch of screening. In this study, most accessions exhibited the same degree of disease symptoms compared to A17. The plants

developed disease symptoms by day 1, yellowing and necrotic tissues were more prominent by

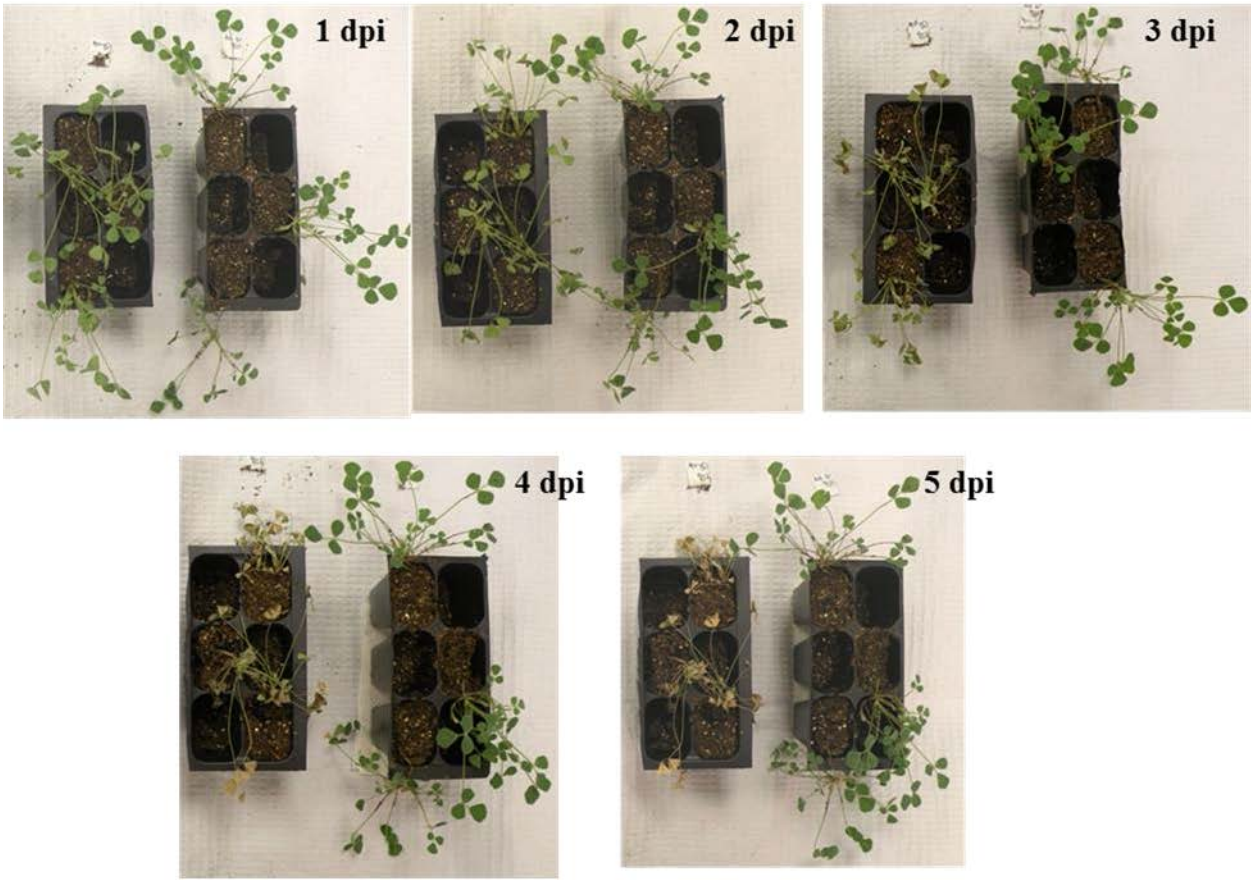

Figure 3. Disease progression in A17 inoculated with *M. phaseolina*. Treatment plants are on the left, control plants on the right. Dpi, day-post-inoculation.

day 3, and plants died at day 4 or day 5 (Figure 3). In a few batches of screens, plants inoculated with *M. phaseolina* did not die completely by day 5, and the young shoots remained green. However, the same was observed for the reference A17 plants that were inoculated with the same batch of inoculum (Figure 4). The reason that shoots remained is probably due to overwatering after inoculation. This seems plausible considering *M. phaseolina* prefers dry conditions. Plants that exhibited these symptoms are recorded as 5s ("s" for shoots) in Appendix B for days when shoots remained. Some of these lines were rescreened to confirm that there was no variation in disease progression. Based on these results, we concluded that no accession exhibited increased susceptibility or resistance to *M. phaseolina* since all ecotypes showed normal disease progression compared to A17.

In this particular screen, disease symptoms for each ecotype were evaluated based on visual examination of the degree of chlorosis and necrosis in infected plant shoots (see methods). While this method can easily identify lines containing resistance, it lacks accuracy and specificity and can also prove misleading when symptoms are non-specific and likely confused with stress-related symptoms (Narayanasamy, 2008). Since there is a negative relationship between fungal biomass and resistance to the pathogen, both factors can be quantified and compared using immunoassays and nucleic-acid-based techniques, both of which do not rely on development of visible symptoms (Narayanasamy, 2008). Enzyme-linked immunosorbent assay (ELISA) is used in various plant-pathogen systems to quantify fungal antigen in a timely manner (Narayanasamy, 2008). In one example, the pathogen *Anisogramma anomala* causing eastern filbert blight disease in *Corylus avellana* (hazelnut) requires an incubation period of 13-22 months for symptom expression while an indirect ELISA protocol was able to detect the pathogen at 3-5 months after inoculation when there was no visible sign of infection (Narayanasamy, 2008). A more specific and quantitative derivative of ELISA called biotin/avidin (BA)-ELISA provides the possibility of categorizing the levels of resistance of cultivars and genotypes (Narayanasamy, 2008).

Figure 4. Example of green shoot that was observed in some of the ecotypes at 5 day-post-inoculation.

While immunoassays quantify resistance, nucleic acid-based techniques are more sensitive, specific, rapid, and reliable for detection, differentiation and quantification (Narayanasamy, 2008). Real-time PCR measures fungal development by directly estimating fungal DNA using total DNA extracted from infected plant tissues even before visual symptoms appear (Narayanasamy, 2008). In inoculated alfalfa plants, pathogen DNA was significantly lower in highly resistant plants than in susceptible ones (Narayanasamy, 2008). In pepper (chili), the pathogen *Phytophthora capsici* causing root rot was detected as early as 8 hours post-

inoculation (Narayanasamy, 2008). Moreover, increased pathogen DNA was more rapid in susceptible pepper (chilli) cultivars and slower in resistant genotypes, thereby providing the ability to determine pathogen development and assess levels of resistance (Narayanasamy, 2008). This technique may also help discriminate between lines displaying slightly different levels of resistance (Narayanasamy, 2008). None of the *M. truncatula* ecotypes screened visibly showed signs of resistance to *M. phaseolina* when compared to genotype A17. However, the collection did have some ecotypes that developed the disease slightly slower or slightly quicker, normally between 1-2 days, than A17. This small degree of difference did not seem significant enough to deem a line resistant or susceptible using visual analysis but different results might be obtained if the collection is subjected to immunoassays or nucleic-acid based techniques as mentioned above resulting in possible grades of resistance that may lead to discovery of QTL where resistance genes may be located. Developing an immunoassay and/or nucleic-acid-based assay that are specific to *M. phaseolina* may help identify subtle differences among different genotypes in the future.

While no ecotype in this screen exhibited altered susceptibility, at least 3 other stock centers that make germplasm available. As of 2006, the *Medicago truncatula* handbook reported that the Australian *Medicago* Genetic Resource Centre (AMGRC) under The South Australian Research & Development Institute (SARDI) has 5,509 accessions from 40 countries that include 4,517 wild accessions, a core collection of 231 accessions, 788 breeding lines, 18 cultivars, 5 mutants, 2 landraces, 175 recombinant inbred lines (*Medicago truncatula* stock centres 2006 – http://www.noble.org/medicagohandbook/). The French National Institute for Agricultural Research (INRA) reports more than 800 populations of *M. truncatula* (of which some were introduced from the core collections of SARDI (Australia) and ICARDA (Syria)) including 16 cultivated varieties, 638 natural populations, 720 inbred lines, 5 populations of about 200 recombinant inbred lines (RILS) and 1,750 mutant lines (*Medicago truncatula* stock centres

2006). As of 2012, the Noble Foundation in Ardmore, Oklahoma USA has reported a population of approximately 80,000 M_1 lines mutagenized by fast-neutron bombardment (FNB) and over 19,000 regenerated lines in R108 background using the Tnt1 retrotransposon (http://medicago-mutant.noble.org/mutant/). Plans are in place to integrate other Tnt1 mutant lines that are being developed by European groups to produce a total of 21,000 lines (http://medicago-mutant.noble.org/mutant/). Seeds for both mutant populations are available at http://medicago-mutant.noble.org/mutant/index.php. With such a large selection of genetic lines, it is hopeful that resistance will be found.

CHAPTER 3

MOLECULAR MECHANISMS UNDERLINING DIFFERENTIAL HORMONAL RESPONSES BETWEEN A17 AND R108

3.1 Introduction

Our previous results showed that treatment with jasmonic acid (JA) and ethylene (ET) confer increased resistance to *M. phaseolina* in A17 but not in R108 plants infected with *M. phaseolina* (Gaige et al., 2010). However, the molecular mechanisms underlying the ecotype differences were unknown. A17 and R108 are two widely used *Medicago* genotypes and are genetically and phenotypically different. We hypothesized that the different hormonal responses between A17 and R108 were due in part to variations in their JA and ET signaling pathways. Studies showed that these two ecotypes have different responses to biotic and abiotic stress. In one study, A17 was resistance to biotrophic fungal pathogens *Colletotrichum trifolii* (anthracnose) and *Erysiphe pisi* (powdery mildew) and moderately susceptible to hemibiotrophic *Phytophthora medicaginis* (damping off or root rot) (Samac et al., 2011). R108 however, was also resistant to *C. trifolii*, but was moderately susceptible to *E. pisi* and *P. medicaginis* (Samac et al., 2011). In A17, all three fungal pathogens produced strong up-regulation of a hevein-like protein, thaumatin-like protein (TLP) and members of the pathogenesis response 10 (PR10) family (Samac et al., 2011). Functional analysis using interfering RNA (RNAi) in R108 revealed that reduced expression of *PR10* and *TLP* had no effect on disease development, whereas reduced expression of *chalcone synthase* (*CHS*) resulted in increased susceptibility to necrotrophic but not biotrophic pathogens (Samac et al., 2011).

3.1.1. *M. truncatula* genotypes A17 and R108

Two genotypes commonly used in *M. truncatula* analysis are Jemalong A17 and R108. A17 is a derivative of the Australian cultivar Jemalong. Chosen as cultivar for its apparent growth vigor, satisfactory pod type and generally good nodulating behavior with native bacterial populations, Jemalong is easily identifiable by its prominent purple-brown anthocyanin wedge-shaped marks extending from near the base to about the top third of the leaflet ("Register of Australian Herbage Plant Cultivars," 1972). In terms of genetic and molecular analyses, it was selected because of its transformability and in vitro regeneration capacity ((Hoffmann, Trinh, Leung, Kondorosi, & Kondorosi, 1997). R108 was named for the symbiotic bacteria with which it associates (*Rhizobium meliloti)* and the ecotype from which it was derived (108-1) (Hoffmann et al., 1997). Like its parent it can establish effective symbiosis with several widely studied *R. meliloti* strains and has a smaller genome in comparison with other genotypes (Hoffmann et al., 1997). However, R108 gained the capacity to readily regenerate in vitro and therefore became a preferred line for investigating symbiosis at genetic and molecular levels (Hoffmann et al., 1997).

Compared with Jemalong, R108 has a 17% smaller genome and 3 week shorter generation time from seed-to-seed (Hoffmann et al., 1997). In the 1990's, it was suggested that lines derived from Jemalong with high symbiotic performance could be used in conjunction with R108 as a flexible genetic approach to dissect and understand symbiotic processes (Hoffmann et al., 1997). Could a similar approach be used today regarding to necrotrophic fungal infection?

3.1.2 JA

Derived from oxylipins, jasmonates are compounds similar in structure and origin to prostaglandins that are important signaling molecules in vertebrates and invertebrates (Kazan & Manners, 2008). In the plant kingdom, they are involved in many vital processes including

defense responses, secondary metabolism of compounds including terpenoids, alkaloids, pheylpropanoids and antioxidants, reproduction, senescence, fruit development and tritrophic interactions (Arimura, Kost, & Boland, 2005; Avanci, Luche, Goldman, & Goldman, 2010; Liechti & Farmer, 2003; Seo et al., 2001; Wasternack, 2007). They are also involved in defense responses including both abiotic (drought, UV irradiation and ozone) and biotic (insects and microbial pathogens) conditions (Kazan & Manners, 2008). It is generally accepted that JA participates in defense response against necrotrophic pathogens and insects (Beckers & Spoel, 2006). Similar to our previous findings for *M. truncatula* genotypes A17, exogenous JA application in rice activated defense-related genes and induced local resistance against the necrotrophic/hemibiotrophic fungus *Magnaporthe grisea* (Mei, Qi, Sheng, & Yang, 2006).

A global gene expression profiling study of *M. truncatula* in response to infection by necrotrophic fungal pathogen *Phymatotrichopsis omnivora* revealed a role for jasmonic acid, ethylene, and the flavonoid pathway in disease development (Uppalapati et al., 2009). More specifically, during interactions of *P.omnivora* and *M. truncatula*, several genes including chitinase Ib chitinase II, endochitinase, exo-1,3-β-glucanase and β1-4,glucanase were induced in *M. truncatula* roots (Uppalapati et al., 2009). Chitinases are generally induced in response to fungal pathogens, abiotic stress or during plant development and over-expression of chitinases and β1-4,glucanase results in fungal resistance in tobacco, potato and wheat (Bieri, Potrykus, & Fütterer, 2003; Jach et al., 1995; Lorito et al., 1998).

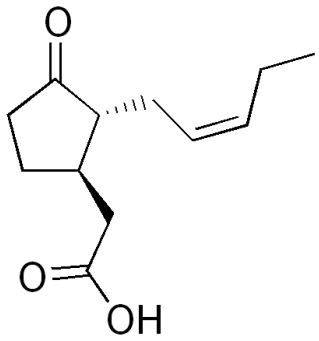

Figure 5. Chemical structure of jasmonic acid.

Most studies that elucidate the JA pathway in plants were conducted in Arabidopsis, tobacco, and tomato (Kazan & Manners, 2008). While little evidence exists of the actual roles of Arabidopsis JA signaling genes in monocots, studies in corn and rice showed involvement of jasmonates in reproduction (Avanci et al., 2010; Kazan & Manners, 2008). Currently, much

more is known about the biosynthesis than the signaling of JA. Given the redundancy of receptors for other plant hormones, it is probable that multiple JA receptors exist in plants (Avanci et al., 2010; Kazan & Manners, 2008). It was demonstrated that JA-related signaling responses are directly associated with a major reset of downstream genes in the pathway (Avanci et al., 2010). After binding to receptors, jasmonates induce a signal-transduction pathway that is capable of either activating or repressing JA-regulated genes (Avanci et al., 2010). Some of these genes create a positive feedback loop by activating JA biosynthesis while, at the same time, a negative feedback loop was identified by activation of JASMONATE ZIM-DOMAIN (JAZ) protein repressors (Kazan & Manners, 2008). The gene *JIN1*/*MYC2* is also involved in regulating JA responses (Kazan & Manners, 2008). In Arabidopsis, JIN1/MYC2 positively regulates JA-medated oxidative stress and flavonoid metabolism in response to insect defense (Kazan & Manners, 2008). However, *JIN1/MYC2* negatively regulates JA-dependent pathogen defense and biosynthesis of secondary metabolites such as indole glucosiniolates (Kazan & Manners, 2008). It was proposed that *JIN1/MYC2* regulates these controls by coordinating a transcriptional cascade that involves the transcription factors *AP2/ERFs*, *MYBs*, and *WRKYs* that all have demonstrated roles in regulating downstream gene expression (Kazan & Manners, 2008). While the components are mostly unknown, it appears likely that phosphorylation/dephosphorylation of proteins have roles in JA signaling cascades (Kazan & Manners, 2008).

3.1.3 ET

Ethylene (ET, Figure 6) is a gaseous plant hormone that is involved in many aspects of plantdevelopment including seed germination, seedling growth, leaf/root/stem/flower development, fruit ripening, organ senescence, and abscission (Wang, Li, & Ecker, 2002). It is also involved in responses to biotic and abiotic stresses such as pathogens, insects, wounding, salt, drought, cold, ozone, hypoxia, and flooding (Guo & Ecker, 2004; Wang, Li, & Ecker,

2002). Biosynthesis of ET depends on the precursors *S*-adenosylmethionine (*S*-AdoMet) and on the 1-aminocyclopropane-1-carboxylic acid (ACC) anenzymes *S*-AdoMet (SAM) synthetase, ACC synthase (ACS) and ACC oxidase (ACO) (Wang et al., 2002). These enzymes are encoded by multigene families whose expression is regulated by a complex network of developmental and environmental cues (Wang et al., 2002). While involved in so many plant processes with a high degree of stimulation, ET response and sensitivity varies greatly according to plant species, tissue, developmental stages, and type of pathogen (Guo & Ecker, 2004; Wang et al., 2002). In disease resistance pathways, ethylene's role remains controversial. Mutants that were deficient in ethylene signaling show both increased susceptibility and increased resistance to different types of pathogens (Wang et al., 2002). An example of this was seen in soybean where ethylene sensitivity produced less severe chlorotic symptoms in response to virulent strains of bacteria *Pseudomonas syringae* pv *glycinea* and oomycete *Phytophthroa sojae* but more severe symptoms in response to necrotrophic fungi *Septoria glycines* and *Rhizoctonia solani* (Hoffman, Schmidt, Zheng, & Bent, 1999). Much of the information available on ethylene signaling derives from studies in Arabidopsis. In Arabidopsis, five ethylene membrane receptors exist and may function as an inverse-agonist model where, in the absence of ethylene, the receptor is constitutively signaling, but when ethylene is bound, the receptor is turned off (Yoo, Cho, & Sheen, 2011). In response to ethylene, transcription factors activate genes including *ETHYLENE RESPONSE FACTOR1* (*ERF1)* and *EBF2* (Yoo et al., 2011). *ERF1* and other transcription factors, in turn, induce the expression of secondary response genes that are eventually involved in modulating plant survival, defense, and growth (Yoo et al., 2011). The signaling components in this linear pathway have been shown to be highly conserved in tomato

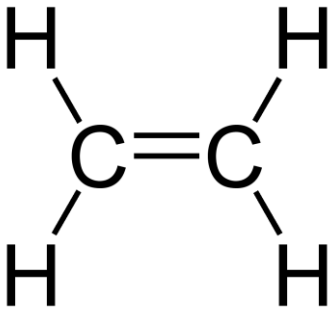

Figure 6. Chemical structure of ethylene

(*Lycopersicon esculentum*), tobacco (*Nicotiana tobacum*), rice (*Oryza sativa*) and maize (*Zea mays*) (Yoo et al., 2011).

Hormonal cross talk in plants is a complex and fine-tuned network of interactions and interplay between JA and ET is no exception. There are reports of both synergistic and antagonistic interactions between JA and ET, depending on stress conditions (Kazan & Manners, 2008). Plant defenses are induced by activation of both pathways and there is evidence that the two pathways co-regulate the expression of genes involved in plant defense (Wang et al., 2002). Downstream interactions are the most plausible scenario since only a small subset of genes are affected by both signals but independent regulation has not been ruled out (Wang et al., 2002).

3.1.4. Genes involved in JA and ET pathways

The different response and disease development of genotypes A17 and R108 to treatment with JA and ET could be due to variations in these hormone pathways. The increased resistance observed in A17 might be due to upregulation of genes in the JA/ET pathways induced by exogenous hormone treatment and therefore enhanced defense responses. Conversely, why R108 plants do not show the same level of resistance could be due to suppression or a diminished response in these pathways. R108 was chosen for its ability to easily form symbiotic relationships with nitrogen fixing bacteria *Rhizobium meliloti* which might indicate it has lower defenses or higher tolerance for microbe invasion.

To investigate this and the possible mechanisms involved, expression of marker genes representing downstream responses to JA and/or ET were compared between the two genotypes. We chose different downstream genes that can be used to represent the activation of either JA or ET signaling pathway. Genes chosen for study that represent the JA pathway were *CHALCONE SYNTHASE* (*CHS*), *PATHOGENESIS RELATED 10 (PR10)*, and *DEFENSIN1 (Def1)*. For the

ET pathway we chose, *ETHYLENE RESPONSE FACTOR (ERF)*, *CHITINASE IV (CHIT IVt)* and *HEVEIN-LIKE (HEL)*.

CHS represents the first committed step to flavonoid biosynthesis and is modulated by MeJA in soybean (Creelman, Tierney, & Mullet, 1992; Samac et al., 2011). In R108 plants, *CHS* expression increased in response to *C. trifolii*, *E. pisi,* and *P. medicaginis* and decreased expression using RNAi was associated with increased susceptibility to necrotrophic fungi (Samac et al., 2011). Expression of *PR10* is regulated by the JA pathway but also by the salicylic (SA) and abscisic (ABA) pathways (Samac et al., 2011). PR10 proteins are constitutively expressed in roots but accumulate in roots and other organs in response to biotic and abiotic stresses and wounding (Samac et al., 2011). While the *PR10* family is generally associated with ribonucleases, it is a large family with numerous subgroups that influence involvement in developmental processes and enzymatic activities in secondary metabolism as well as pathogen defense (Liu & Ekramoddoullah, 2006). Known for its antifungal properties, *Def1* in *M. truncatula* seedlings is strongly induced by MeJA (Hanks et al., 2005). Although a homologous gene in Arabidopsis is both JA and ET inducible, ET treatment did not elicit the same response in *M. truncatula* (Hanks et al., 2005).

In Arabidopsis, transcription factor *ERF* regulates ethylene response after pathogen attack (Berrocal-Lobo, Molina, & Solano, 2002). Infection with necrotrophic fungus *Botrytis cinerea* induced expression of *ERF* and over-expression of *ERF* confers resistance to necrotrophic fungi *B. cinerea* and *Plectosphaerella cucumerina* (Berrocal-Lobo et al., 2002). *ERF* can be activated by either ET or JA or synergistically by both (Lorenzo, Piqueras, Sánchez-serrano, & Solano, 2003). Responsive to ET, *Chit IV* expression increased after infection with necrotrophic fungi *Fusarium solani* f. sp. *phaseoli* and *Phytophthora megasperma* f. sp. *medicaginis* in *M. truncatula* (Salzer et al., 2000). Chitinases are hydrolytic enzymes that cleave bonds between residues in the polysaccharide chitin, a primary structural component of the walls

of true fungi (Salzer et al., 2000). *HEL* is a defense related gene that is also responsive to both JA and ET (Van Oosten et al., 2008). Because mRNA for the *HEL* gene accumulated after ET application it is considered an ET response gene. However, it is also commonly used to monitor JA-dependent defense responses (Anderson, Lichtenzveig, Gleason, Oliver, & Singh, 2010; Kunkel & Brooks, 2002; Potter et al., 1993).

3.2 Results and Discussion

While it is clear that both JA and ET play a role in plant defense and that there is substantial crosstalk between both pathways, the extent of their roles and the specifics of their crosstalk remains foggy. Hormonal crosstalk is considered an essential process in order to fine-tune the plant's defense responses (Spoel & Dong, 2008). Plant defenses are custom tailored to the infection strategy of a pathogen and may be specific to not only plant families and species but also to varying genotypes within a species (Hanks et al., 2005; Samac et al., 2011; Spoel & Dong, 2008). Therefore, it would not be farfetched to propose that a reason for the difference in disease progression observed between A17 and R108 after JA and ET treatment is due to variation in the hormonal pathways involved in plant defense. By comparing expression of downstream genes indicative of JA and ET pathways at different time points of *M. phaseolina* infection process, pathway regulation can be analyzed. To do this, A17 and R108 seedlings were grown in Magenta boxes and inoculated with *M. phaseolina* after 14 days. Root tissue was harvested at 24, and 48 hour time points. Mock inoculated control plants were harvested at 24 hours. RNA was extracted from the root tissue and used to prepare cDNA. The cDNA was then used to monitor expression of individual genes using real time quantitative PCR (RT-qPCR) to compare the relative abundance of each gene in control vs. *M. phaseolina* infected samples.

Genes representative of the JA pathway are *CHS*, *PR10*, and *Def1*. In A17, both *CHS* and *PR10* showed a general trend of upregulation at 24 and 48 hour time points (Figure 6). *CHS*

showed an approximate 5-fold increase at 24 hr and a 6.5-fold increase at 48 hr. *PR10* had an approximate increase of about 14.5 at both 24 and 48 hr. In R108, both genes exhibited delayed responses where upregulation did not occur until 48 hr. *CHS* had a 4.5-fold increase and *PR10* had a 2.25-fold increase at 48 hr, which was less than that observed in A17 samples. *Def1* in A17 was strongly up regulated with a 6-fold increase at 24 hr and a 13-fold increase at 48 hr. In R108 however, *Def1* was slightly down regulated approximately 70% and 40% of expression in 24 hr and 48 hr samples compared to controls (Figure 7). Based on expression of marker genes *CHS* and *PR10,* it appears that the JA pathway was induced quicker and stronger in A17 than in R108, while according to *Def1*, the pathway was suppressed in R108 while induced in A17. In

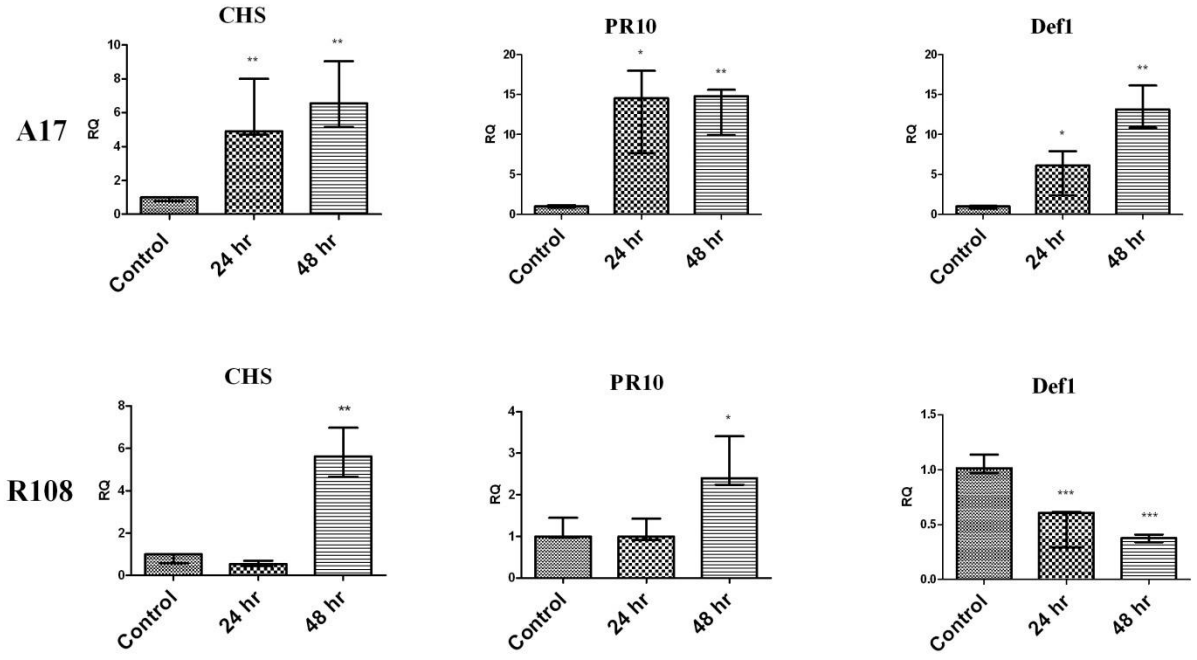

Figure 7. Relative abundance of JA marker genes in *M. truncatula* genotyps A17 and R108 after *M. phaseolina* inoculation. RQ (relative abundance) of each gene at 24 hr or 48 hr after *M. phaseolina* inoculation was calculated using comparative Ct method. *UBQ* was used as endogenous control , and control samples were used as the calibrator. RQ for control was arbitrarily set as 1. ***, P <0.001, **, P < 0.01, *, P < 0.05 *CHS* , *CHALCONE SYNTHASE; PR10, PATHOGENESIS RELATED 10, DEF1, DEFENSIN1.*

both patterns, the JA pathway did not respond similarly between the genotypes and these alterations could play a part in the lack of JA-induced resistance to *M. phaseolina* in R108 compared to A17.

As stated before, *CHS* represents the first committed step in flavonoid biosynthesis (Samac et al., 2011). Among their many roles, flavonoids help protect plants against microbe invasion (Harborne & Williams, 2000). Certain flavonoids, usually isoflavonoids, flavans, or flavanones, have antifungal capacity (Harborne & Williams, 2000). In certain tree legumes, isoflavonoid maackiain (3-hydroxy-8,9-methyle-nedioxypterocarpan) is a constitutive antifungal agent (Harborne & Williams, 2000). Specifically, in *Cicer bijugum*, maackiain was not only constitutively present but also enhanced in the presence of fungal pathogen *Botrytis cinerea* (Harborne & Williams, 2000). Both *Defl* and *PR10* also have antifungal properties. Plant defensins in general are mostly antifungal (Thomma, Cammue, & Thevissen, 2002). Upregulation of *PR10* genes in *M. truncatula* was observed in response to several fungal and bacterial pathogens and showed antifungal properties *in vitro* in peanut (Samac et al., 2011). Given the general antifungal characteristics of these genes, their delayed and suppressed expression in R108 is plausibly linked to the difference in disease progression between the two genotypes.

Expression of genes representing the ET pathway was not as clear cut as those for JA. In A17, *ERF* was slightly upregulated at 24 hr with a 4-fold increase and then strongly upregulated at 48 hr with an 18-fold increase. In R108, *ERF* was upregulated by 7-fold at 24 hr but only 2.8-fold at 48 hr indicating down regulation from 24 to 48 hr (Figure 8). *Chit IV* was upregulated by approximately 5-fold at 24 hr and 4.6-fold at 48 hr in A17 while only a slight 1.4-fold increase was observed at 24 hr and no significant change at 48 hr in R108. *HEL* in both A17 and R108 was upregulated at 48 hr however it was more strongly upregulated in A17 (6.3-fold) than R108 (1.5-fold). In fact, when both genotypes showed upregulation in ET and JA responsive genes,

A17 had higher fold changes than R108. *Chit IV* and *HEL* both showed general trends of upregulation in both genotypes with A17 having stronger upregulation. *ERF* showed upregulation in both genotypes as well. However in A17, there was slight induction at 24 hr followed by a high induction at 48 hr while in R108 the higher induction was observed only at 24 hr.

While evidence might not be as compelling as the JA pathway, the stronger induction noticed in A17 compared to R108 could play a role in different disease progression between these two genotypes. This might especially be the case given the difference in 48 hr expression of *ERF* where the level decreased in R108 but dramatically increased in A17. *ERF* is a

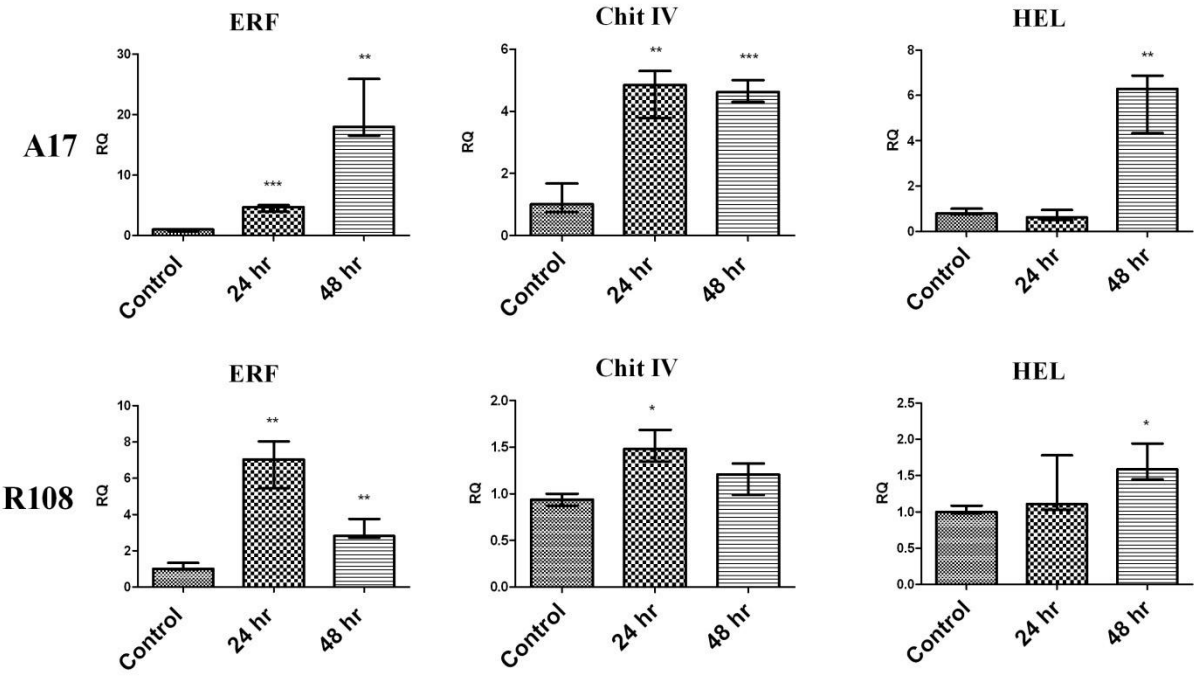

Figure 8. Relative abundance of ET marker genes in *M. truncatula* genotyps A17 and R108 after *M. phaseolina* inoculation. RQ (relative abundance) of each gene at 24 hr or 48 hr after *M. phaseolina* inoculation was calculated using comparative Ct method. *UBQ* was used as endogenous control , and control samples were used as the calibrator. RQ for control was arbitrarily set as 1.***, P <0.001, **, P < 0.01, *, P < 0.05. *ERF, ETHYLENE RESPONSE FACTOR; CHIT IV, CHITINASE IV; HEL, HEVEIN LIKE PROTEIN.*

transcription factor that is usually attributed to the ET pathway (hence its name ethylene response factor) but also responds to JA (Lorenzo et al., 2003). In Arabidopsis, *ERF* induces

defense genes further downstream including the plant defensin gene *PDF1.2* and a basic chitinase gene b-CHI (Kidd, Aitken, Schenk, Manners, & Kazan, 2010). In *M. truncatula*, over expression of ethylene response factor *MtERF1-1* in roots increased the plants resistance to pathogenic fungus *Rhizoctonia solani* and oomycete *P. medicaginis* (Anderson et al., 2010). The roles of chitinases in fungal defense is self evident given that walls of true fungi contain chitin. *Chit IV* was strongly induced in A17 in response to several fungal pathogens including *Fusarium solani* f. sp. *phaseoli* and *Phytophthora megasperma* f. sp. *medicaginis* (Salzer et al., 2000). *HEL* is part of the *PR4* genes suggested to have antifungal activity due to their N-terminal cysteine-rich chitin-binding domain (hevein domain) (Broekaert, Lee, Kush, Chua, & Raikhel, 1990). In rubber tree (*Hevea brasiliensis*), hevein has chitin binding properties and inhibits growth in several chitin-containing fungi (Parijs et al., 1991). Given their direct antifungal roles and regulation of defense responses including genes associated with antifungal properties, the stronger upregulation of ET pathway genes in A17 could be part of the reason for its increased resistance to *M. phaseolina* compared to R108.

3.3 Summary

In numerous plant families, JA and ET pathways induce defense responses that are often provoked by pathogen attack (Avanci et al., 2010; Yoo et al., 2011). In *M. truncatula*, exogenous application of JA and ET produced delayed disease progression after infection by *M. phaseolina* in A17 but not in R108. To investigate this observation, we performed expression analysis of marker genes representing both pathways. RT-qPCR analysis revealed that induction of genes indicative of the JA signaling pathway such as *CHS* and *PR10,* was faster and stronger in A17 than in R108. In addition, *Def1* was up regulated in A17 while it was down regulated in R108. Expression of genes downstream of the ET pathway was less clear but a general trend of strong upregulation was noticed in A17 compared to R108. Many of the marker genes used in

this study are directly related to fungal defense and, considering the JA and ET pathways that regulate genes involved in plant defense including those with antifungal properties, it is plausible that the difference in the induction of JA and ET response genes contributes to the different hormonal responses between A17 and R108. The activation of these hormonal pathways and downstream genes by exogenous JA or ET in these two genotypes should further be tested to follow up the findings reported here.

CHAPTER 4

MATERIALS AND METHODS

4.1 Fungal material

Fungal material used for inoculations was *Macrophomina phaseolina* isolate #210 provided by Dr. Nancy Brooker (Pittsburg State University, Pittsburg, KS, USA). Fungus was propagated on potato dextrose agar (PDA) plates at 27-30°C. For root dip inoculum pitchers of approximately ¼ liter potato dextrose broth (PDB) was inoculated with 3-5, ~2 cm squares of 3–day-old *M. phaseolina* infested PDA and kept at 25-30°C. A sclerotia mat formed on the surface and was harvested after 1 ½ -2 weeks. The mat was dried in a hood for 3 days and then finely ground with mortar and pestle and stored at 4°C.

4.2 Plant material

Seeds were scarified using concentrated sulfuric acid for approximately 8 minutes (time varied for ecotype seeds) and rinsed 3-5 times with sterile water. Seeds for Magenta box preparation were also surface sterilized with 20% bleach for 10 minutes and rinsed 3-5 with sterile water. Seeds were germinated for 2-3 days on ½ Murashige and Skoog (MS) plates in a dark, room temperature location (covered with tray on the bench top). Jemalong A17 and R108 seeds were grown and harvested in house with original lines from the Samuel Roberts Nobel Foundation (provided by Dr. Srinivasa Rao Uppalapati). Seeds for ecotypes were obtained from U.S National Plant Germplasm System (NPGS - http://www.ars-grin.gov/npgs/). Plants for the ecotype screen were grown in wet, sterilized soil (Sunshine Professional Grow Mix, Sun Gro Horticulture, Bellevue, WA, USA) under a 12 hour cycle at 32^{o}C day, 30^{o}C night and 44% of relative humidity. Plants used for gene expression analysis were grown in ½ MS media with 1% sucrose in Magenta boxes under a 12 hour cycle at 32^{o}C day and 30^{o}C night.

4.3 Inoculation procedures

4 to 5-week-old ecotype plants were unpotted and roots were rinsed clean with tap water. Roots were dipped in 0.015% agarose for 30 seconds for control plants or *M. phaseolina* suspension (1 gram of ground sclerotia per 10 ml of 0.015% agarose) for treatment plants. Plants were repotted in 2/3 original soil and 1/3 dry, sterilized soil. Plants were watered no sooner than 24 hours post inoculation and then as needed until they died. Plants were scored everyday starting 1 day-post-inoculation (dpi) until death according to the following matrix: 0: no detectable symptom; 1: 1-10% chlorotic or 1-5% necrotic; 2: 10-20% chlorotic or 5-10% necrotic; 3: 20-40% chlorotic or 10-20% necrotic; 4: 40-60% chlorotic or 20-40% necrotic; 5: 60-80% chlorotic or 40-60% necrotic; and 6: plant dead.

For magenta box inoculations, sterilized wheat seed in an Erlenmeyer flask was inoculated with 3, ~2 cm squares of 3 day old *M. phaseolina* infested PDA and was incubated at 27-30°C for 1-2 weeks with occasional hand shaking and disturbance until seeds were uniformly covered with *M. phaseolina* and stored at 4°C. Plants were inoculated by placing an infested seed at the base of the root. Control inoculations were done with a sterile seed placed at the base of the root.

4.4 Sample preparation and RNA isolation

Two-week-old R108 and A17 plants were inoculated with *M. phaseolina*-covered wheat seeds. Roots were harvested at time points 24 and 48 hours after inoculation and quickly frozen in liquid nitrogen and stored at -80°C until RNA isolation. Control plants were harvested at 24 hours. RNA was isolated using TRIzol (Invitrogen) reagent. Approximately 200mg of root tissue was frozen with liquid nitrogen and finely ground using sterilized mortar and pestle. 2ml of TRIzol was added and tissue was homogenized in the solution. Contents were transferred to 2 RNAse free microcentrifuge tubes and centrifuged at 12,000g for 10 min at 4°C. The upper

phase was transferred to a new tube and incubated at room temperature for 5 min in order to let RNAs dissociate from ribonuclear protein complexes. 200 µl of chloroform was added to each tube and the tube was vigorously shaken for 15 seconds and incubated at room temperature for 3 min. Tubes were then centrifuged again at 12,000g for 10 min at 4°C in order to separate phases. The top, colorless phase was transferred to new centrifuge tubes. RNA was precipitated by adding 250 µl of high-salt solution (0.8M sodium citrate, 1.2M sodium chloride) and 250 µl of isopropanol. Tubes were inverted and incubated at room temperature for 10 min followed by centrifugation at 12,000g for 10 min at 4°C. The supernatant was removed and the RNA pellet was washed with 1 ml of 75% ethanol. Tubes were vortexed and centrifuged at 7,500g for 5 min at 4°C. Up to 2 quick spins and pipetting after decantation was performed to thoroughly remove the supernatant. Pellet was air dried for 1-2 minutes and contents were consolidated into one tube by resuspension in 20µl of RNase free water. RNA quality was assessed using a spectrophotometer and confirmed using gel electrophoresis.

RNA samples were DNase treated with TURBO DNase (Ambion). Reactions were set up using 5µl of 10X TURBO DNase buffer, 1µl of TURBO DNase and 10µg of RNA. Reaction volume was brought up to 50µl using RNase free water and incubated at 37°C for 30 minutes. To deactivate the enzyme a phenol-chloroform extraction was preformed. The sample volume was brought up to 200µl using RNase free water. 200µl of 25:24:1, (Phenol:Chloroform:Isoamyl alcohol) (Fisher Scientific) was added and the total contents was vortexed briefly and incubated at room temperature for 1 minute. Contents were then centrifuged at maximum speed for 2 minutes for phase separation and the aqueous phase was carefully recovered and placed into a new tube. 1/3 volume high-salt solution and 2/3 volume of isopropanol of estimated volume of recovered aqueous phase were added. Contents were incubated at room temperature for 10 minutes and the tube was centrifuged at 12,000g for 10 minutes at 4°C. The supernatant was decanted and the remaining pellet was washed with 1ml 75% ethanol prepared with DEPC water.

Contents were vortexed briefly and centrifuged at 7,500g for 5 minutes at 4°C. Supernatant was removed in the same manner as the RNA isolation procedure and the pellet was let air dry in the tube for 1-2 minutes and resuspended in 20µl of RNase free water.

4.5 RT-qPCR

Complementary (c)DNA was made from the DNase-treated RNA using reverse transcription (RT). 2µg of total RNAs were combined with 1µl OligodT primer (20µM) and the reaction volume was brought up to 12µl using RNase free water in a 0.2ml thin-wall PCR tube. Samples were heated at 65°C for 5 minutes and then chilled on ice. 1µl of Superscript III Reverse Transcriptase (Invitrogen), 2µl DTT (100mM), 1µl dNTPs (10mM) and 4µl 5X First strand buffer were added and contents were incubated at 42°C for an hour. The enzyme was then denatured by a 70°C incubation for 15 minutes.

Quality and confirmation of cDNA synthesis was performed using polymerase chain reaction (PCR) with constitutively expressed gene *tubulin* and gel electrophoresis. The PCR was prepared with 2 µl 10x Thermolpol buffer New England Biolabs (NEB), 2 µl dNTPs (2 mM), 4 µl primer mix (1µM of forward and reverse primer), 0.2 µl Taq polymerase (NEB), 1 µl cDNA and 10.8 µl RNAse free water. Reactions were carried out in a 0.2ml thin-wall PCR tube and run according to the following cycle: 1) initial denaturation for 3 minutes at 90°C, 2) 30 cycles of 30 seconds at 90°C for denaturation, 30 seconds at 57°C for annealing, 1 minute of extension at 72°C and 3) final elongation for 5 minutes at 72°C.

Real time quantitative PCR (RT-qPCR) reactions were set up as 10 µl reactions containing 5 µl of Power SYBR Green PCR master mix (Applied Biosystems), 1 µl of gene specific primers (1 µM) and 1 µl diluted cDNA sample. Reactions were performed in a StepOnePlus real-time PCR machine with 96-wells (Applied Biosystems). *Ubiquitin* (*UBQ*) was

used as the endogenous control gene and the control (0 hr) was used as the calibrator. Sequences for the *UBQ* and gene specific primers are listed in appendix A.

Relative quantity (RQ) of each gene was calculated using the comparative C_T method as shown below, where C_T = cycle threshold (the number of cycles required for the fluorescent signal to cross the background level):

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

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

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

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

The C_T values were normalized to endogenous housekeeping gene *UBQ* and values at time points 24 and 48 hour were compared to that of 0 hour samples (used as the calibrator where RQ was set to 1) in order to graph relative abundance. Each time point had three biological replicates, and each biological replicate had triplicate technical reactions. RT-qPCR results were analyzed by StepOne Plus software, and the data were exported to GraphPad Prism (GraphPad Software, Inc.) to generate the graphs and perform the student t-test.

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APPENDICES

APPENDIX A

Primers used in the study for PCR and RT-qPCR. F = forward, R = reverse.

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

APPENDIX B

Scores for screened ecotype lines. 5s* indicates lines where shoots remained. AID=Alternative Identficiation. In lines PI 244285, PI 469100, PI 535619 and W6 6071 (bottom of appendix) 1 of the plant replicates did not exhibit similar disease progression to the others in its group. Replicates were grouped and recorded according to their score. For example, in W6 6071, 3 treatment plants were screened. At day 6, 2 were at the 5s* stage and 1 was at the 3 stage. This was recorded as 2-5*s, 1-3.

Line #	1 dpi	2 dpi	3 dpi	4 dpi	5 dpi	6 dpi	7 dpi	8 dpi	COUNTRY
PI 190084	0	2	5	-	6				
PI 190086	0	3	5	-	6				
PI 190087	0	3	5	-	6				Australia
PI 190089	0	3	5	-	6				Australia
PI 283661	0	2	3	4	6				
PI 384645	1	-	4	5	6				Morocco
PI 384648	0	2	3	4	6				Morocco
PI 384649	0	3	4	5	5	5	6		Morocco
PI 384650	0	2	4	5	6				Morocco
PI 384655	0	1	5	5	5	6			Morocco
PI 384656	0	-	3	5	6				Morocco
PI 384659	0	0	2	3	5	6			Morocco
PI 384660	0	1	5	5	6				Morocco
PI 384662	0	2	4	5	6				Morocco
PI 384664	0	3	4	5	5	6			Morocco
PI 464816	0	2	4	5	5	6			Turkey
PI 469099	0	2	4	5	6				
PI 493295	1	2	4	6					Portugal
PI 493296	0	1	3	5	6				Portugal
PI 493297	0	2	3	5	6				Portugal
PI 505437	1	3	4	5	6				
PI 516923	0	0	3	4	5	6			Morocco
PI 516926	0	1	2	5	6				Morocco
PI 516928	1	2	3	4	4	6			Morocco
PI 516939	0	3	5	5	5	5	6		Morocco
PI 516944	0	1	4	5	5	5	5	6	Morocco
PI 535622	0	4	5	6					Tunisia
PI 535649	0	1	5	5	6				Tunisia
PI 535650	0	1	4	5	6				Tunisia
PI 535652	0	1	4	5	6				Tunisia
PI 566886	0	3	5	5	5	6			Morocco
PI 566888	0	2	4	5	6				Greece
PI 577601	0	3	5	5	5	5	5	6	Italy

PI 577605	1	2	4	5	6				Greece
PI 577609	1	3	5	6					Greece
PI 577610	0	1	3	5	6				Cyprus
PI 577611	1	4	6						Cyprus
PI 577615	1	3	5	6					France
PI 577616	1	4	5	6					
PI 577623	0	1	5	5	6				Sweden
PI 577648	0	2	5	6					Italy
PI 641409	1	4	5	5	6				
PI 641410	0	1	1	4	6				Greece
PI 641411	0	1	2	5	6				Greece
PI 641412	0	1	2	4	4	5	6		Tunisia
W6 5990	0	1	3	5	6				Algeria
W6 5991	0	1	2	4	5	6			Algeria
W6 5992	0	1	3	5	6				
W6 5997	0	2	3	5	6				Morocco
W6 5998	0	1	3	5	6				Algeria
W6 6000	0	3	4	5	6				Algeria
W6 6001	0	2	4	5	6				Spain
W6 6019	0	2	5	6					Italy
W6 6065	0	2	4	5	6				Italy
W6 6066	0	1	3	5	6				Italy
W6 6067	0	2	4	5	6				Greece
W6 6069	0	1	2	4	5	6			France
W6 6072	0	3	5	6					Sweden
W6 6073	0	3	5	6					
W6 6076	0	1	3	5	6				Germany
W6 6078	0	1	2	4	6				Tunisia
W6 6079	1	2	3	4	5	6			
W6 6080	0	1	2	4	6				Morocco
W6 6081	1	2	4	5	6				
W6 6082	0	1	3	5	6				Cyprus
W6 6084	0	1	4	5	6				Malta
W6 6107	0	1	2	4	6				Italy
W6 6108	0	1	2	4	6				Italy
W6 6113	0	2	3	5	6				Italy
W6 6114	1	2	3	4	5	6			Italy
W6 6115	0	1	3	4	5	6			Italy
W6 6116	0	2	4	5	5	6			Italy
W6 6117	0	1	3	4	6				Greece
PI 190082	0	1	3	5	5	5s*			
PI 190083	0	2	4	5s*					
PI 190090	0	1	3	4	5	5s*			Australia
PI 190091	0	1	4	5	5	5s*			

PI 239877	0	1	3	5	5s*				Morocco
PI 239878	0	1	3	4	4	5s*			Morocco
PI 243884	0	1	3	4	5	5s*			
PI 283662	0	2	5	5s*					
PI 284123	0	1	3	5	5	5s*			
PI 292434	0	1	4	5	5	5s*			
PI 384647	0	1	3	5	5s*				Morocco
PI 384665	0	1	4	4	5	5s			Morocco
PI 442892	0	1	4	5	5	5s*			
PI 442895	0	2	4	5	5s*				
PI 442896	0	1	4	5	5s*				
PI 464815	0	0	3	4	5	5	5s		Turkey
PI 469102	0	1	4	5	5	5s*			
PI 505438	0	1	3	4	5	5	5s*		
PI 516924	0	1	4	5	5	5s*			Morocco
PI 516925	0	2	4	5	5	5s*			Morocco
PI 516927	0	1	4	5	5	5s*			Morocco
PI 516929	0	1	3	5	5s*				Morocco
PI 516938	0	3	4	5	5s*				Morocco
PI 516940	1	2	4	5	5	5s*			Morocco
PI 516941	0	1	3	4	5	5	5	5s*	Morocco
PI 516942	0	1	4	5	5	5s*			Morocco
PI 516943	0	1	4	5	5	5s*			Morocco
PI 516945	0	3	4	5	5s*				Morocco
PI 535616	0	2	3	4	4	4	5	5s*	Tunisia
PI 535618	0	3	5	5	5s*				Tunisia
PI 535648	0	2	4	4	5s*				Tunisia
PI 535651	0	2	3	4	5	5	5s*		Tunisia
PI 535739	0	2	4	4	5	5	5s*		Libya
PI 535752	0		0	3	5	5s*			Morocco
PI 537150	0	1	4	5	5	5s*			Italy
PI 537194	0	1	5	5	5	5s*			Malta
PI 537213	0	1	4	5	5	5	5s*		Tunisia
PI 564941	0	1	3	4	5	5	5s*		
PI 566887	0	1	3	4	5s*				Italy
PI 566889	0	2	4	5	5s*				Australia
PI 566890	0	3	5	5	5s*				
PI 577434	0	2	4	5	5	5s*			Turkey
PI 577597	0	1	4	5	5	5s*			Greece
PI 577598	0	1	3	5	5	5s*			Tunisia
PI 577599	0	1	3	4	5s*				Italy
PI 577603	0	3	4	4	5	5s*			
PI 577604	0	2	3	4	5	5s*			Greece
PI 577607	0	3	5	5	5s*				Greece

PI 577612	0	1	4	4	5	5s*			Cyprus
PI 577613	0	1	4	5	5	5s*			
PI 577614	0	1	3	3	5	5s*			Lebanon
PI 577642	0	1	3	4	5s*				United States
PI 577643	0	1	3	4	5s*				
PI 577645	0	3	5	5	5s*				Germany
PI 577646	0	1	3	4	5s*				Malta
PI 641406	0	2	3	5	5	5s*			Malta
PI 641407	0	1	2	4	4	5s*			Italy
PI 641408	0	2	3	5	5	5s*			Australia
PI 641413	0	4	5	5	5s*				Algeria
W6 4980	0	2	4	5	5s*				
W6 4996	0	1	4	5	5	5s*			Greece
W6 4998	1	2	4	5	5	5s*			Algeria
W6 5999	0	2	3	4	5	5s*			
W6 6068	1	1	3	4	4	5s*			Cyprus
W6 6074	0	0	3	5	5	5s*			United States
W6 6075	0	2	4	5	5s*				Australia
W6 6077	0	1	4	4	4	5	5s*		Malta
W6 6118	0	1	4	5	5	5s*			Italy
PI 244285	0	1	4	5	5	2-5s*, 1-4			
PI 469100	0	1	4	5	2-6, 1-3	2-6, 1-3	2-6, 1-3		
PI 535619	0	1	2	3	4-5s,1-1	4-5s*,1-1			Tunisia
W6 6071	0	1	3	3	2-4,1-3	2-5s*,1-3			Spain