EFFECTS OF MUTATIONS IN *GH3* GENES ON THE INTERACTION BETWEEN THE NECROTROPHIC FUNGUS *MACROPHOMINA PHASEOLINA* AND ITS PLANT HOST *ARABIDOPSIS THALIANA*

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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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DEDICATION

To my parents, family, and dear friends

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First and foremost I would like to thank my mentor, Dr. Bin Shuai, for her patience and guidance. This project would surely not have been possible without her wisdom and support. I would like to thank my labmates Tyler and Pryia for their technical support and Adam for his help and influence on me as a scientist and person. Evan and Khai have my utmost gratitude for the help they provided on this project. I would also like to thank my committee members Dr. James Beck for his help in phylogenetic analysis and Dr. Kandatege Wimalasena for helping to shape my understanding of biochemistry. Thanks is given to the Salk Institute Genomic Analysis Laboratory for kindly providing the sequence-indexed Arabidopsis T-DNA insertion mutants.

ABSTRACT

The necrotrophic soil-borne fungus *Macrophomina phaseolina* (Tassi) Goid (*M. phaseolina*) causes the disease charcoal rot in a wide range of plant species worldwide. Many of these species are agronomically important. Attempts to control the disease have been met with little success. Expression of auxin-regulating *GH3* genes have been shown to be up-regulated in Medicago plants upon infection with *M. phaseolina*. Other studies have shown that *GH3* knockout mutants have increased resistance to necrotrophic fungal pathogens such as *Botrytis cinerea*. Based on our previous research, we hypothesized that mutations in *GH3* genes in the plant host *Arabidopsis thaliana* (*A. thaliana*) would decrease the aggressiveness of *M. phaseolina*. The research presented here investigates this interaction in an attempt to elucidate the importance of *GH3* genes in the disease response pathway. To assess this, available insertion mutants for seven group II *GH3* genes of *A. thaliana* were included in this study. Since *GH3* is a multi-gene family and some genes may have functional redundancy, double mutants for two pairs of closely related genes were generated. Homozygous mutants for four of the seven insertion lines were established. Despite being prevalent in other research, homozygosity was unattainable for three of the mutant lines (*gh3.2*, *gh3.4*, and *gh3.6*). The *gh3.6* line was found to have an inverted tandem repeat of the T-DNA insert. Although obvious phenotypic differences were present for all the lines, three of the four homozygous lines produced their respective mRNA based on RT-PCR; therefore, they are most likely not a knockout of their perspective gene. The disease phenotypes of these mutant lines were compared to that of the wild type. With three repeats of inoculation, it was concluded that these lines showed no increased resistance to *M. phaseolina*. Given the data presented here, it is likely *GH3* genes play no major role in the pathogenicity of *M. phaseolina* on the model plant *A. thaliana*.

TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

LIST OF TABLES

LIST OF FIGURES

LIST OF ABREVIATIONS

°C	Degrees Centigrade
µL	Microliter
µM	Micromolar
ABRC	Arabidopsis Biological Resource Center
cDNA	Complementary DNA
dH_2O	Distilled Water
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide Triphosphate
DPI	Days Post Inoculation
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic Acid
EtOH	Ethanol
ET	Ethylene
g	Gram
gDNA	Genomic DNA
HCl	Hydrogen Chloride
Htz	Heterozygous
IAA	Indole-3-Acetic Acid
JA	Jasmonate
K	Thousand
Ler	Landsberg erecta
M	Molar

mg	Milligram
mL	Milliliter
mM	Millimolar
mQ	Milli-Q (water)
mRNA	Messenger Ribonucleic Acid
MS	Murashige and Skoog
NaCl	Sodium Chloride
NASC	Nottingham Arabidopsis Stock Center
PAMP	Pathogen Associated Molecular Pattern
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
RNA	Ribonucleic Acid
RNAi	RNA Interference
RNase	Ribonuclease
rpm	Revolutions Per Minute
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
SA	Salicylic Acid
SDS	Sodium Dodecyl Sulfate
T-DNA	Transfer DNA
Trp	Tryptophan
W/T	Wild-type

CHAPTER 1

INTRODUCTION: *MACROPHOMINA PHASEOLINA*, THE NECROTROPHIC PHYTOPHATOGEN

1.1 *Macrophomina phaseolina* and the Charcoal Rot Disease

Fungal pathogens infecting plants are of particular interest to us for many reasons. They destroy more than 125 million tons of the top five food crops (rice, wheat, maize, potatoes, and soybeans) each year [1]. More than 10,000 species of fungi are parasitic to living plants to varying degrees. Plant pathogens are separated into two broad categories based on their lifestyle. Biotrophic pathogens keep their host alive and feed off living tissue. This reduces the plants vigor and fruit yield. Necrotrophic pathogens on the other hand, create a concoction of enzymes and toxins that kill and then metabolize nutrients that are released by dying host cells [2]. Hemibiotrophic pathogens are biotrophic for the first part of their life and turn necrotrophic towards the end. *Macrophomina phaseolina* (Tassi) Goid (*M. phaseolina*) is a necrotrophic fungus that causes the disease charcoal rot in over 500 plant species worldwide [3]. Its major cultivated hosts include *Glycine max* (soybean), *Helianthus annuus* (sunflower), *Zea mays* (corn), *Sorghum bicolor* (sorghum), and *Gossypium hirsutum* (cotton) [4, 5]. Several clinical reports have established *M. phaseolina* as an intermittent human pathogen as well [6, 7]. Along with charcoal rot, *M. phaseolina* is the causal agent of numerous other diseases in plants including ashy stem blight, root rot, and stem rot [3, 8]. *M. phaseolina* is predominantly found in tropical, subtropical, and subtropical-temperate regions across North and South America, Europe, Africa, Southeast Asia, and China [9, 10]. Charcoal rot is among the top ten most important diseases affecting world production of soybean and ranked fifth in diseases reducing soybean yield in the United States [11]. In 2002, charcoal rot in soybean accounted for a total

yield loss of $175.2 million in the United States and reduced soybean yields in Kansas by 249,000 tons in 2003 [12-15]. These years were particularly bad due to favorable weather conditions for disease formation.

Symptoms of the disease appear during hot dry weather, when soil moisture is low and plants are stressed. *M. phaseolina* does have a sexual stage, but the asexual microsclerotia that it produces are the primary source of inoculum. Microsclerotia form from an aggregation of 50 to 200 individual hyphal cells. This structure consists of a thick outer rind, which serves as a protective layer against desiccation and irradiation, and an inner layer called the medulla that serves as a nutrient reserve and germinating tissue [16]. Microsclerotia are unable to survive in wet soils for more than 7-8 weeks, but under favorable conditions can survive for more than two years [17]. The fungus can infect seeds where it lies dormant until germination (or appears later under hot and dry conditions). *M. phaseolina* causes a wide range of symptoms in plants, all of which lead to premature senescence and plant death. Infected roots eventually turn dark brown to black. In older plants, leaves yellow and wilt, eventually turning brown. The disease becomes worse when host species are grown continuously in the same field due to increased soil populations of microsclerotia. Infection occurs through mechanical penetration of epidermal cells by appressoria, natural openings, or wounding sites. The fungus then grows inter- or intracellularly where it spreads to lateral and radial vascular bundles as well as cortical cells [18]. An established pathogen can be seen as small black speckles (microsclerotia) in these tissues [3]. *M. phaseolina* produces several phytotoxins, including phaseolinone, botryodiplodin, asperlin, isoasperlin, phomalactone, and patulin; which help increase the aggressiveness of the pathogen [19-21]. The catabolic enzymes endo- and exopolygalacturonase, polygalacturonate trans-

eliminase, galactosidase, cellulase, and pectin methyl esterase aid in the pathogenicity and aggressiveness as well [22, 23].

1.2 *M. phaseolina* Taxonomy and Gross Morphology

Byron Halsted first discovered *M. phaseolina* as a plant pathogen of sweet potato in 1890. Its wide host range along with its association with other plant pathogenic fungi caused much confusion and resulted in several names for the pathogen [18]. Other names for *M. phaseolina* in literature include *Macrophoma phaseoli* Maubl., *Botryodiploidia phaseoli* (Maubl.) Thirumalachar, *Rhizoctonia bataticola* (Taubenhaus) E.J. Butler, and *Sclerotium bataticola* Taubenhaus [24]. Traditionally fungi had two binomial names, one for the teleomorphic (sexual) stage and one for the anamorphic (asexual) stage. The classification system for fungi is slowly changing to a single naming system and *M. phaseolina* is now used to describe the teleomorphic stage where *Rhizoctonia bataicola* was once used [18]. *Macrophomina* is a monotypic genus of ascomycete within the Botryosphaeriaceae family. Figure 1 shows the current taxonomic classification of *M. phaseolina*.

Kingdom	*Fungi*
Phylum	*Ascomycota*
Subphylum	*Pezizomycotina*
Class	*Dothideomycetes*
Order	*Botryosphaeriales*
Family	*Botryosphaeriaceae*
Genus	*Macrophomina*
Specific epithet	*phaseolina*

Figure 1. Taxonomic classification of *M. phaseolina* (Information from NCBI Taxonomy Browser)

M. phaseolina tends to be variable in its hyphal pigmentation and microsclerotial size and shape. Hyphae have right-angle branching with a constriction and septa near the point of branching origin. Though present, acute branching is much less common. Microsclerotia can be spherical, oval, oblong, elliptical, curved, and even forked. At first, microsclerotia were mistaken for immature pycnidia, which are sexual reproductive structures. Pycnidia are globose to fusiform, grey turning to black with age. The size of pycnidia varies from about 23 μm^2 to 32 x 152 μm, and is dependent on the media on which the fungus is grown or the host organ from which it is isolated [18, 25, 26]. These structures form naturally on select hosts, such as *Glycine max*, *Helianthus annuus*, and jute (*Corchorus capsularis*) [27-29]. Colonies of *M. phaseolina* are grown on solid media at an optimal temperature of 28-35°C and form grey to black sclerotial mats.

1.3 Methods of Control

Efforts to control charcoal rot have been met with little success. Different types of tillage have little effect on the colonization of roots by *M. phaseolina* [30]. Unlike host specific phytopathogens, crop rotation achieves little when trying to control *M. phaseolina* due to its large host range. It has been shown that rotations with certain crops, especially cotton, can reduce the density of microsclerotia in the soil [31]. Chemical control has undesirable impacts on non-targeted beneficial microorganisms in the soil and often poses a hazard for human health [32]. The simplest, but not always feasible, management method is irrigation. This reduces stress on plants and increases mortality of microsclerotia. There have been several cases showing the effectiveness of biological controls against *M. phaseolina*. Several strains of fluorescent *Pseudomonas sp.* have been shown to inhibit the growth of the fungus. The disease

incidence of charcoal rot in broad bean (*Vicia faba* L.) inoculated with the fluorescent Pseudomonas strain RFP-36 was reduced by nearly half [33]. Maize (*Zea mayz*) treated with two bacilli isolates, MR-11(2) and MRF, showed similar results [34]. The *Bacillus subtilis* isolate BN1 was found to produce lytic enzymes that degrade the hyphae of *M. phaseolina* making it a good candidate for biological control [35]. Several beneficial bacteria produce the plant hormone indole-3-acetic acid (IAA), which could indirectly antagonize pathogens by promoting plant growth. Unlike chemical controls, biological controls perform unpredictably due to variable gene expression and poor root colonization, which makes them less practical for use in the field [36]. Attempts to find disease-resistant cultivars were only partially met by identifying a few disease-tolerant and moderately resistant cultivars of soybean [3]. It is thought that genetic engineering is the best alternative to develop a disease resistant system.

1.4 Plant Defense

Over time, plants have developed separate pathways for fighting off biotrophic and necrotrophic pathogens. There are several major phytohormones involved in plant defenses, including salicylic acid (SA), ethylene (ET), and jasmonates (JA). SA is generally involved in the activation of defense responses against biotrophic and hemi-biotrophic pathogens, whereas JA and ET are normally associated with defense against necrotrophic pathogens [37]. While fine-tuning of a plant's response to a single pathogen according to its infection strategy is essential for survival, it may be detrimental if the plant faces multiple pathogens with differing infection strategies. Temporal separation of activating SA and JA/ET pathways largely abolishes the antagonism between the two [38]. These hormones transmit information about an infecting organism to nearby and distal tissues, allowing the entire plant to aid in defense.

The first line of defense plants have is an innate, or basal, type of resistance that detects chemicals common to many microbes. These chemicals are called pathogen associated molecular patterns or PAMP's. Commonly known PAMP's include flagellar proteins, peptidoglycan and lipopolysaccharides components in cell wall, and elongation factors of bacteria, as well as chitin and ergosterols of fungi [39-43]. These molecular cues are detected by transmembrane receptor protein kinases (RPKs), and are responsible for triggering a cascade of events leading to a defense response. The manifestation of this response is the production of reactive oxygen species (ROS), antimicrobial phytoalexins, and catabolic enzymes followed by apoptosis or programmed cell death, which in turn kills or inhibits the growth of the pathogen. If a pathogen loses or mutates a PAMP, they can go undetected by their host and are pathogenic. Pathogens can also avoid triggering host defenses by inhibiting RPKs and downstream components of the defense cascade. Molecules with this type of function are termed effectors. As one might guess, effectors eventually became a new source of "PAMP's" for triggering plant defense responses. This process in which plant and pathogen are continuously gaining and losing genes to gain the upper hand is called the gene-for-gene model [44]. Plant resistance due to the detection of PAMP's and effectors that causes drastic phenotypic differences between susceptible and resistant cultivars is called qualitative resistance. Another type of resistance in plants is called quantitative resistance, and has a wide range of resistance levels compared to qualitative resistance. There are several theories as to its mechanism of action in quantitative resistance, including mutations of PAMP receptors, modifications in the defense cascade, and variable expression of phytoalexins [45]. Plant defense mechanisms have been hijacked by pathogens to work against the plant. For example, the biotrophic pathogen *Pseudomonas*

syringae pv. *tomato* activates the JA/ET defense pathway thereby suppressing the SA defense pathway, thus reducing the plants ability to eliminate the biotrophic pathogen [46].

As expected of a necrotroph, previous studies have shown that the JA/ET defense pathway is induced upon inoculating *Medicago truncatula* (*M. truncatula*) with *M. phaseolina* [47]. This infection also affects genes involved in auxin biosynthesis, metabolism and signaling, which can be due to the indirect effects of JA/ET pathways since JA and ET affect signaling, transport, and biosynthesis of auxin [48], or can also be due to the direct role of the pathogen on the auxin pathway. Expression analysis based on microarray data (Affymetrix Medicago Genome array) has suggested the suppression of auxin signaling by *M. phaseolina* [49]. Disturbing auxin homeostasis is a common virulence strategy for phytopathogens such as *Pseudomonas syringae*. This is achieved by the production of auxin-like molecules and other compounds that disrupt auxin signaling in the host plant. Loss of the ability to synthesize auxin-like molecules in this particular pathogen rendered it less virulent [46]. Furthermore, *A. thaliana* auxin signaling mutants have been shown to be more susceptible to the neurotropic pathogens *Plectosphaerella cucumerina* and *Botrytis cinerea*. [50].

CHAPTER 2

AUXIN, THE GROWTH HORMONE

2.1 Effects of Auxin on Plants

Auxins are involved in nearly every aspect of plant growth and development including cell division, tropic responses, vascular development, response to biotic and abiotic stimuli, apical dominance, fruit development, and induction of rooting [51, 52]. Auxins were the first class of plant hormone to be discovered. They were originally defined as a chemical that induces coleoptile curvature; but later, other morphological responses, such as the root initiation and cell division, were required for a chemical to be classified as an auxin. Despite being one of the most well studied plant hormones, information is lacking in its role in plant defenses. Auxin alters the expression of several groups of genes, namely *Aux/IAA, Gretchen Hagen* (*GH*), and *small auxin-up RNA* (*SAUR*) families [53]. Interestingly, *GH3.5* has been shown to modulate both SA and auxin signaling during pathogen infection [54]. It is possible that auxin has no direct involvement in plant defenses, but rather acts to modulate other hormone pathways. For instance, the SA and auxin signaling pathways are mutually antagonistic [55].

2.2 IAA Biosynthesis

Plants produce a variety of auxins, including indole-3-butyric acid (IBA), 4-chloroindole-3-aceetic acid (4-Cl-IAA), and even non-indole containing compounds (Phenylacetic acid). Of the known auxins, IAA produces the strongest effect. Like other hormones, only small amounts of IAA are needed for efficacy. IAA is found in picomolar amounts in most plant tissues [56].

Levels of IAA synthesis vary depending on tissue type and developmental stage of the plant. The highest levels of the hormone can be found in fertilized embryos and all parts of young seedlings [57]. At later stages of development, auxin is synthesized predominantly in

meristematic tissues and transported to target cells through the vascular system or intercellularly via the polar auxin transport (PAT) system [58]. Plants synthesize IAA through both tryptophan (Trp)-dependent and Trp-independent pathways. Several Trp-dependent pathways have been proposed, including indole-3-pyruvic acid, indole-3-acetamide, tryptamine, and indole-3-acetaldoxime pathways. The Trp-independent pathway was discovered by analyzing Trp biosynthetic mutants [53]. The multitude of pathways leading to the biosynthesis of IAA highlights its importance in plant development. Of the amount of information known on IAA synthesis, none of the pathways are yet completely defined to the level of knowing each gene, enzyme, and intermediate involved. Besides biosynthesis, the amount of active IAA is tightly regulated by an array of metabolic processes including transport, conjugation, de-conjugation, conversion and catabolism (Figure 2) [59].

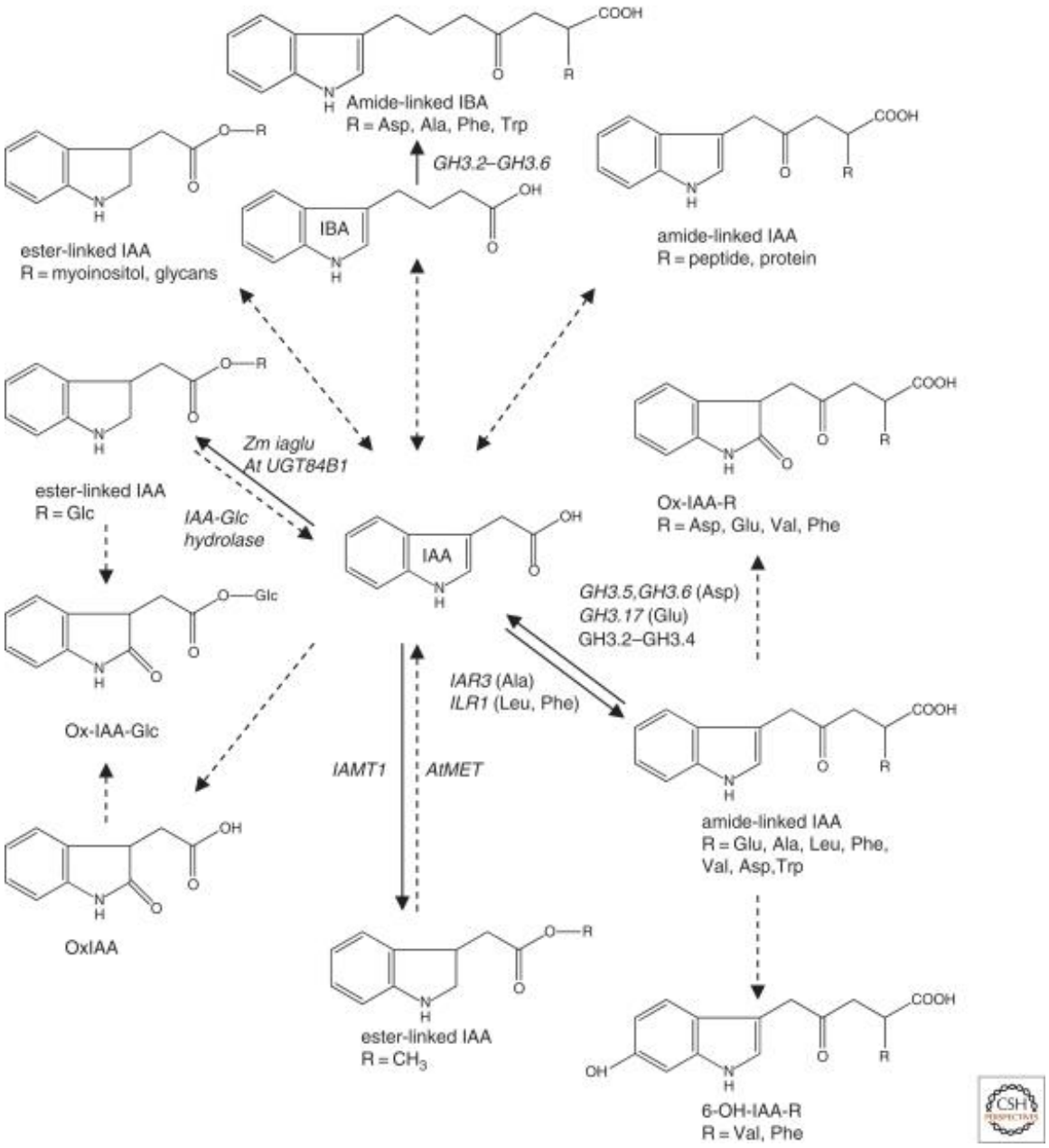

Figure 2. IAA metabolism in *A. thaliana*. IAA-amino acid conjugates formed by enzymes encoded by *GH3* genes serve as IAA storage or are degraded. Dashed lines indicate that either enzyme activity has been observed in vitro only, or that neither gene nor enzyme activity has been identified [59].

2.3 IAA Homeostasis and *GH3* Genes

Conjugation of IAA is a mechanism for regulating auxin homeostasis. Free IAA accounts for about 1% of the total IAA in plants [60]. Since hormonal effects of the conjugated

forms are greatly reduced, it is thought that they serve as a reservoir for active IAA. IAA-Ala and IAA-Leu act as storage conjugates whereas IAA-Asp and IAA-Glu are considered to be the starting point for IAA catabolism [53]. *GH3* genes are responsible for many of these conjugation reactions and belong to a multigene family. These genes are found in monocots, eudicots, as well as mosses [61].

There are three classes of *GH3* genes are found in , *GH1*, *GH3*, and *GH2/4*, all of which are up-regulated in the presence of auxin [62]. Little is known about *GH1* genes other than they share homology with *Aux22* and *Aux28* of soybean, *GH2/4* genes have homology to an assortment of genes, including heat shock and fungal inducible genes [63-65]. Based on studies in *Oryza sativa* (rice) and *A. thaliana*, *GH3* genes are categorized into three groups based on substrate specificity, sequence similarities, and splicing patterns. Group I *GH3* gene products use JA as a substrate, Group II gene products interact with IAA, and Group III *GH3* genes still await to be fully characterized [66]. Regulation of auxin-response genes, including some of the *GH3* genes, share the same mechanism. The protein Aux/IAA, a transcription repressor that binds to auxin response factor (ARF), inhibits transcription of auxin-responsive genes. Auxin binds to Aux/IAA when it is in high concentrations, which leads to the polyubiqitination of the repressor and ultimately its degradation via the proteasome. This frees up ARF as an activator, which binds to auxin response elements in the promoter of target genes to activate transcription.

Modulation of these auxin responsive genes through an unknown mechanism seems to be a common pathogenicity strategy for necrotrophic pathogens. In a recent study, the necrotrophic fungus *Botrytis cinerea* was shown to activate the transcription of *GH3.2*, consequently leading to the accumulation of IAA-Asp. Compared to wild-type (W/T) *A. thaliana*, *GH3.2* mutants were highly resistant to *B. cinerea*. Furthermore, exogenously applied IAA-Asp increased the

virulence of this pathogen in *A. thaliana* as well as in *Nicotiana benthamiana* and tomato plants [67]. There are several cases in which exogenously applied auxin resulted in reduced virulence of different necrotrophic pathogens [49, 68]. Previous work with *M. truncatula* shows an up-regulation of the auxin conjugating genes *GH3.3* and *UGT* during the initial infection stages of *M. phaseolina* [49]. Based on these studies, we hypothesize *M. phaseolina* increases its aggressiveness by disrupting auxin homeostasis by up-regulating *GH3* expression. The lack of mutant lines for *GH3* genes in *M. truncatula* prevents further research of their importance in the interaction with *M. phaseolina*. *Arabidopsis thaliana* on the other hand, has mutant lines for several *GH3* genes, providing a better tool to investigate the function of these genes in host disease responses to *M. phaseolina*.

CHAPTER 3

ARABIDOPSIS THALIANA AND REVERSE GENETICS

3.1 Arabidopsis, the Model Plant

A. thaliana is inarguably the most well studied plant in the world. It is a part of the flowering plant family Brassicaceae that includes several economically important species including *Brassica napus* (rapeseed), *Brassica oleracea* (broccoli, cabbage, kale, etc.), and *Brassica rapa* (turnip). The first mutant of *A. thaliana* was found in 1873, but it wasn't until the 1980's that the plant gained widespread popularity with the scientific community [69, 70]. This is due to several properties of this weedy plant. It has a rapid generation time, has the ability to generate large amounts of seeds by self-pollination, and its small size allows it to be grown at high densities in small spaces. A vast amount of genetic and genomic information is known about this plant as it was among the first organisms to have a fully sequenced/annotated genome [71]. Building on this, various databases with information about protein sequences and structure, polymorphisms, hormone pathways, and expression data were created. These information, along with visualization and alignment tools, have been consolidated through a single access point, arabidopsis.org, making research much less daunting. In addition to this, the plant is easily mutated via insertional mutation because of its high gene density [72]. While transformation of *A. thaliana* takes only a few months time, stable transformation of other plants can take up to a year, and requires exhaustive labor, materials, and laboratory space. *A. thaliana* research provides not only a theoretical framework but also direct methods for improving crops. For example, metabolic genes have been transferred from *A. thaliana* to *Nicotiana tabacum* (Tobacco), *Solanum tuberosum* (potato), and rice to produce crops with more in biomass [73-75]. Resistance genes have been transferred from *A. thaliana* to rapeseed and plants outside the

Brassicaceae family to confer resistance to bacteria and fungi [76]. Lastly, many pathosystems between necrotrophic fungi and *A. thaliana* are well established [37, 77].

3.2 Application of Insertion Mutants in Reverse Genetics Studies

Loss-of-function mutations are useful tools for studying the function of gene products. RNA interference (RNAi) and insertional mutations are two common ways by which this is achieved. RNAi silences the expression of target genes post-transcriptionally by targeting mRNAs for degradation. This is done without altering the sequence of the gene of interest. Knocking-down gene expression using RNAi can be advantageous in cases where insertional mutation results in lethality. RNAi is particularly useful in knocking down the expression of multiple genes with sequence homology, such as those present in polyploids, and of large gene families [78]. Despite its advantages, there are several limitations to this technique. RNAi can have variable levels of gene knock-down, its application is limited to organisms that exhibit RNAi, and can have unstable transmission to succeeding generations [79]. In contrast, insertion mutations disrupt genes by inserting exogenous genetic material into the genome thereby preventing transcription, maturation of mRNA, or the proper functioning of a gene product. Once generated, the insertion will be stably transmitted over generations. It represents the fastest and most straightforward way of knocking out a gene. These types of mutants have been widely used in reverse genetic studies to identify the function of many genes. Despite its convenience and widespread usage in the research community, this loss-of-function technique has limitations as well. The insertion of exogenous material can affect the expression of native and transgenes as well as cause deletions, interchromosomal rearrangements, and insertion of random genetic material [80-83]. Furthermore, the most commonly practiced methods of producing insertion mutants is a random process and therefore knockouts for every gene are not available.

3.3 Generating Insertion Mutants

Insertion mutants were mostly due to insertion of either transfer-DNA (T-DNA) or transposon into the target gene . T-DNA is a small part of the tumor inducing (Ti) plasmid found in the soil bacterium *Agrobacterium tumefaciens* (*A. tumefaciens*). One of the benefits of using T-DNA as opposed to other vectors is that much of the original T-DNA can be replaced with foreign DNA. This ensures that a minimal amount of vector sequence, only about 25 bp flanking each side of the payload, is inserted into the plants genome along with the desired payload [84]. *A. thaliana* plants can be easily transformed by dipping either their roots or immature floral parts into a solution of *A. tumefaciens* containing the desired Ti plasmid [85, 86]. Engineered T-DNA typically contains an antibiotic resistant gene that helps select for transformed seed or tissue, which can be grown into mature plants.

T-DNA insertion is largely a random process. To prevent redundancy within the scientific community, independent institutions submit their sequenced insertion lines to stock centers to be maintained and distributed to others. Several large collections of T-DNA insertion lines include Salk, SAIL, WiscDsLox (WISC), GABI, FLAG, and Saskatoon (SK) collections. These lines are available through the Arabidopsis Biological Resource Center (ABRC) and Nottingham Arabidopsis Stock Center (NASC). With the number of insertion lines generated currently, one would expect knockouts for more than 99% of the genes in *A. thaliana*. However, about 9% of protein-coding genes have yet to be knocked out [87]. This discrepancy could be due to T-DNA insertion favoring regions of open chromatin.

Unlike T-DNA insertion mutants, transposon insertion uses a transposon to disrupt target genes. One advantage over T-DNA mutants is that transposons can be remobilized, resulting in the original gene sequence, to confirm that a particular phenotype is caused by the transposon

insertion [88]. In addition, certain transposon systems show a preference for proximal transposition, thus analogous polymorphisms can be generated [89]. The transposon insertional mutants used in this experiment were generated from the *Landsberg erecta* ecotype that were transformed with maize Activator (Ac)/Dissociation (Ds) transposable elements [85]. The Ac/Ds system is well understood, as it was the first transposable element system to be discovered [90]. Ac type elements confer transposase, which is required for transposition of both Ac and Ds elements. Ds elements on the other hand, do not encode transposase, and are therefore relatively stable in the absence of Ac elements. The inverted repeats of the Ac element can be modified to prevent transposition of this element. Both elements are inserted into cells via T-DNA vector; homozygous Ac and Ds lines are then crossed allowing for transposition of the Ds element. The somewhat random nature of transposition events allows for the generation of a large number of independent insertion lines. Further transposition of the newly transposed Ds element is avoided by eliminating the Ac element through outcrossing or selfing. Although W/T *A. thaliana* has several Ac-like transposases, there is no evidence that they confer transposition of *Zea maize* Ds elements [91]. A unique double selection scheme was used to identify plants that have a transposed Ds element but no Ac element. The Ds element carries *NPTII*, which confers resistance to the antibiotic kanamycin. The immobilized Ac element and T-DNA donor site encode for the indole acetic acid hydrolase (IAAH) enzyme for counter selection of these two features. Transgenic *IAAH* in *A. thaliana* elevates naphthalene acetic acid (NAA) to toxic levels due to the presence of exogenous naphalene acetamide (NAM) [92]. Along with being one of its advantages, proximal transposition is a disadvantage for the Ac/Ds system, in that more lines need to be generated and assessed to cover the entire genome [93]. Also, the presence of multiple copies of Ds elements are particularly prone to chromosomal breaks, resulting in

instability in the transgenic lines [94]. Aside from this, the Ac/Ds system is favored over other transposon systems due to its low copy number [95]. The number and location of inserts in T-DNA and transposon lines are determined through similar techniques.

A common drawback of T-DNA and transposon lines is the tendency for having multiple insertions per line. Salk lines have about 1.5 insertions on average per line, while only 5-10 percent of Ds transposon lines have multiple inserts [96, 97]. The percent of double inserts was found to be even higher in other T-DNA collections [98]. Un-indexed inserts in these types of lines can lead to erroneous conclusions based on unknown genotypes. This is a particular problem when inserts are proximal to one another and segregate together.

3.4 Locating Insertion Sites via PCR Amplification and Sequencing

There are two popular methods for determining the random insertion site of T-DNA and transposons. Both methods involve amplification of the regions flanking the transgenic material. This region is known as the flanking sequence tag (FST). The first method is thermal asymmetric interlace PCR (TAIL-PCR), which uses nested primers specific to the transgene sequence along with random degenerate primers to selectively amplify the FSTs. Three consecutive PCR reactions are run in this procedure. For each consecutive run, a nested transgene-specific primer that is predicted to produce a shorter amplicon is used to ensure amplification of the insert and flanking sequence only. The other method is adapter ligation mediated PCR, which involves restriction enzyme digestion and adapter ligation. After restriction digestion, adapters are ligated to transgene and the flanking sequence at restriction sites. This method uses insert and adapter specific primers. The adapter is asymmetric and has a cryptic primer binding site that is insufficient for primer binding until synthesis of its complementary strand via insert specific primer. This increases specificity ensuring only the

FST-trangene is amplified. Adapter ligation mediated PCR fails to identify insertions for genes that have no proximal restriction sites. This method was used in determining the FST of Salk lines in this experiment, while TAIL-PCR was used to determine transposon FSTs [85, 87]. After amplification of the transgene, amplicons are sequenced via Sanger or next-generation sequencing techniques to determine the location of the insertion. Information regarding insertion lines is available at The Arabidopsis Information Resource (www.arabidopsis.org) and the Salk Institute Genomic Analysis Laboratory (signal.salk.edu)

CHAPTER 4

MATERIALS AND METHODS

4.1 Plant Materials

Transposon lines were obtained from NASC and include,

SGT4174 (*gh3.1*, no longer distributed)

SGT5949 (*gh3.2*, no longer distributed).

T-DNA insertion lines were obtained from ABRC and include,

SALK_202887 (*gh3.3*)

SALK_102549 (*gh3.4*)

SALK_014376 (*gh3.5*)

SALK_133707 (*gh3.6*)

SALK_050597 (*gh3.17*)

Transposon lines were generated by Parinov et al. [99] and T-DNA mutants generated by the Eker lab of the Salk Institute [100]. To confirm the insertion in each line and isolate homozygous mutants, plants were grown in soil and genotyped. Seeds of homozygous plants were collected and grown on full strength MS media (see below). Pooled DNA from twenty 10-day-old seedlings was genotyped to confirm the absence of a W/T allele.

4.2 Plant Sterilization and Growth Conditions

The amount of seed to be used was estimated and seeds placed in a 1.5 mL microfuge tube. Seeds were sterilized first with 500 µL of 95% EtOH for five minutes (min). The EtOH was removed and seeds were rinsed with sterile H_2O. Seeds were then treated with 500 µL of freshly prepared 20% bleach soln' (4.0 mL bleach, 16 mL dH_2O, and 1 drop Tween-20) for five min. Samples were then rinsed five times with sterile H_2O. A small amount of water was left in

each microfuge tube and the samples were placed in the dark at 4°C to ensure uniform germination. After three days of incubation at 4°C, seeds were sown onto full strength Murashige and Skoog (MS) media (4.32g MS salt (Sigma), 10.0g sucrose, and 7.0g agar per 1,000 mL dH_2O). The seeds were left to germinate/grow under a short-day photoperiod for vegetative growth (8 hr day/16 hr night) or a long-day photoperiod (16 hr day/8 hr night) to minimize . After ten days on MS media the seedlings were transferred to sterile potting soil and grown under the same conditions as before. Potting soil was prepared by loosely filling pots with dry potting soil and letting pots stand in fertilizer-containing water (~15 mL 20:20:20 fertilizer to 1.5 L tap-water) overnight. Plants were watered twice a week by placing pots in tap water for ten min.

4.3 Plant Crosses

Flowers to be used from female parents were selected before anthers began shedding pollen onto the stigma. The flowers of the female parent were then emasculated by removing sepals, petals, and anthers. The pistil was then observed to determine if self fertilization had taken place. If pollen grains were present, the flower was removed to prevent seed contamination. Emasculated flowers were given 24 hours to mature. Pollen bearing anthers were collected from the male parent. The mature anthers were rubbed against the stigma of the emasculated flowers [101]. Crossed flowers were marked with sewing thread for later identification. To generate enough seed for later analysis, three to five healthy flowers were crossed for each plant.

4.4 Preparation of Fungal Inoculum and Inoculation Procedure

The fungal inoculum was maintained on potato dextrose agarose (PDA) plates by inoculating with agar plugs (approx. 1.5 cm^2) from previous inoculum grown on PDA. Plates

were then incubated in the dark at 27°C for three days. After incubation, PDA plates were stored at 4°C for later use. For large scale production, agar plugs from PDA plates containing freshly grown *M. phaseolina* were used to inoculate potato dextrose broth (PDB). Beakers containing the PDB were allowed to incubate at room temperature for two weeks. The fungal mats that grew in the beakers were collected and dried at room temperature for 24 hours. The fungal mats were then ground into a fine powder using a mortar and pestle to produce fungal inoculum.

For each set of inoculations, 48 wild type plants (Ler or Col, depending on the mutant line) and 48 plants of each mutant line were planted. To ensure successful infection, plants were not watered three days before inoculation. Five-week-old plants were removed from pots and the soil gently removed from the roots by hand. Remaining debris on roots was removed by briefly rinsing the roots in dH_2O. The roots of negative control plants were mock inoculated with 0.015% agarose soln' for 30 sec. The roots of plants to be infected with *M. phaseolina* were placed in fungal inoculum for 30 sec. The fungal inoculum consisted of 1g of grounded sclerotia and 10 mL 0.015% agarose soln'. Care was taken to ensure roots were uniformly coated with the inoculum. New inoculum was made for every 12 plants to be infected. Of the plants used in each replicate, half were used as a negative control while the other half were used as the experimental group. Immediately after inoculation, plants were repotted with minimally watered autoclaved soil. The normal watering scheme was resumed 24 hr after inoculation.

4.5 Infection Assessment

Twenty-four hours after inoculation, plants were photographed and scored based on the following matrix depending on whichever symptom gave the greatest score. This was repeated everyday at the same time, until infected plants were dead.

0 – No detectable symptoms

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

6 – Dead

4.6 DNA Isolation

Two to four green leaves were excised from each mature plant and collected in a 1.5 mL microfuge tube. The tubes were then flash frozen in liquid nitrogen. A mini-pestle was used to grind up the frozen tissue. 500 µL of extraction buffer (200 mM Tris-Cl [pH 8.0], 250 mM NaCl, 25 mM EDTA, and 0.5% SDS) was added to each sample. Samples were macerated again to homogenize the tissue. Samples were then vortexed for 5 sec and centrifuged for 2 min at 14K rpm to pellet cellular debris. 450 µL of the supernatant was then transferred to a new microfuge tube, to which equal volume of isopropanol was added. After inverting the tubes several times to mix, the samples were left at room temperature for 2 min to precipitate DNA. The tubes were then centrifuged for 2 min at 14K rpm. The supernatant was discarded and the DNA pellet was washed with 1 mL of 70% ethanol (EtOH). EtOH was removed after centrifugation for 1 min at 14K. After air-drying for 2 min, the DNA pellets were re-suspended in 50-100 µL elution buffer (10 mM Tris-HCl [pH 8.0]). A pipette tip was used to help break up the DNA pellet. Samples were then centrifuged for 1 min at 14K rpm to pellet remaining contaminants. Supernatant was transferred to a new microfuge tube with care taken to avoid any pellet present. DNA samples were stored at -20° C.

4.7 PCR

Each PCR rxn' consisted of 12 µL sterile Milli-Q (mQ) water, 2.0 µL 10X ThermoPol buffer (New England Biolabs), 2.0 µL dNTP soln' (2 mM for each of the four dNTPs), 1.0 µL Taq DNA polymerase (1unit μL^{-1}), 1.0 µL forward and 1.0 µL reverse primers (10 µM each), and 1.0 µL of genomic DNA (gDNA). *GH3* specific forward and reverse primers were used to test for W/T alleles (Appendix A). The mutant allele, generated by transposon- or T-DNA insertion, was confirmed by using DS3a-1 (for transposon insertion) or LBa1 (for T-DNA insertion) primer combined with one of the *GH3* gene-specific primers. These reagents were added to a thin-walled PCR tube. PCR rxn's were run in a MJ Research PTC-200 thermocycler (Bio-RAD). The PCR program consisted of three min at 95°C for initial denaturing, followed by 30 cycles of denaturing at 95°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 2 min. A five min 72°C extension step was added after the 30 cycles to complete extension of unfinished amplicons. Amplification of Actin 7 was done to confirm successful isolation of gDNA, in this case a 57°C annealing temperature step was used instead of 55°C.

4.8 RNA Isolation

Eighteen to twenty 10-day-old *A. thaliana* seedlings were placed into an RNase AWAY (Molecular Bio-Products, San Diego, CA) treated and autoclaved mortar that was pre-chilled with liquid nitrogen. More liquid nitrogen was added to the mortar to flash-freeze the seedlings. Petri plates in which the plants grew were weighed before and after removing the plants to determine the amount of sample collected. For every 50-100 mg of plant tissue, 1 mL of TRIzol (Life Technologies, Carlsbad, CA) was used. Tissue was homogenized in TRIzol with a pestle that was pre-treated with liquid nitrogen. The resulting homogenate was transferred into an RNase-free 1.5 mL microfuge tube. The tube was then centrifuged at 12,000 g for 10 min at 4°C. The RNA containing supernatant was then transferred to a new microfuge tube by

pipetting. The supernatant was incubated at room temperature for 5 min to allow RNAs to dissociate from ribonuclear protein complexes. 200 µL of chloroform was added per 1 mL of TRIzol used. This soln' was mixed vigorously by shaking for about 30 sec and incubated at room temperature for 3 min. The sample was then centrifuged at 12,000 g for 10 min at 4°C for phase separation. The top colorless aqueous phase was transferred to a clean microfuge tube by pipetting. An isopropanol/high salt soln' (1:1) was added in equal volume as aqueous phase recovered to precipitate RNA. The high salt component (0.8 M sodium citrate, 1.2 M NaCl) was used to help remove polysaccharides. The sample was left to incubate at room temperature for 10 min and then centrifuged at 12,000 g for 10 min at 4°C. The supernatant was poured out and the RNA pellet was washed with 1 mL of 75% RNase-free EtOH. After vortexing and centrifugation at 7,500 g for 5 min at 4°C, the EtOH was poured off and any excess pipetted away. The pellets were then air-dried for 2 min and then resuspended in 10.0 µL of RNase-free H_2O. Samples with un-dissolved RNA were heated to 55-66°C for 5 min to help solubilize the pellet. Quality and quantity of RNA were checked using a Nanodrop spectrophotometer and gel electrophoresis. The A_{260}, A_{260}/A_{280}, and A_{260}/A_{230} measurements were recorded from the Nanodrop. For gel electrophoresis, 1.0 µL of RNA sample was mixed with 4.0 µL H_2O and 1.0 µL 6X loading dye and loaded in a 2% agarose gel containing EtBr. The gel was run at 100V for 20 min. The gel was then exposed to UV light and the image recorded with a gel documentation system from FotoDyne.

4.9 RT-PCR

Two µg of total RNA was added to a 0.2 mL thin-wall PCR tube with 1.0µL of oligo(dT) (20µM) (Life Technologies) and enough RNase-free water to bring the total volume to 12 µL. This was then heated to 65°C for 5 min in a thermocycler and put on ice afterwards. To each

reaction a master mix containing 4.0 µL 5X 1st strand buffer, 2.0 µL DTT (100 mM), 1.0 µL dNTPs (10 mM each dNTPs), and 1.0 µL SuperScript III reverse transcriptase (Life Technologies) was added. A negative RT control was run for each RNA sample in which RNase-free H_2O replaced the SuperScript III reverse transcriptase. The RT rxn was done at 50°C for one hour followed by 70°C for 5 min for enzyme denaturation. One microliter of cDNA was used for PCR with gene-specific primers. The PCR cycling conditions are the same as that described in the PCR section. Gel electrophoresis was then done to confirm the presence/absence of cDNA.

CHAPTER 5

RESULTS AND DISCUSSION

5.1 Genotyping Insertion Lines

To test effects of *GH3* gene products on the interaction between *A. thaliana* and *M. phaseolina*, a reverse genetics approach was adopted. Homozygous mutants were established for four of the seven *gh3* insertion lines (*gh3.1*, *gh3.3*, *gh3.5*, and *gh3.17*) (Figure 3). Heterozygous plants from the remaining insertion lines were self-pollinated and their progeny genotyped in an attempt to find homozygous individuals. All individuals genotyped for the un-established lines were heterozygous. This includes 27 plants for *gh3.2*, 107 plants for *gh3.4*, and 135 plants for *gh3.6* (Figures 4-6). Using the Chi-square goodness of fit test it was determined, with a confidence level of 0.001 or less, that the genotype inheritance for these lines is not in the 1:2:1 ratio found in the expected Mendelian inheritance.

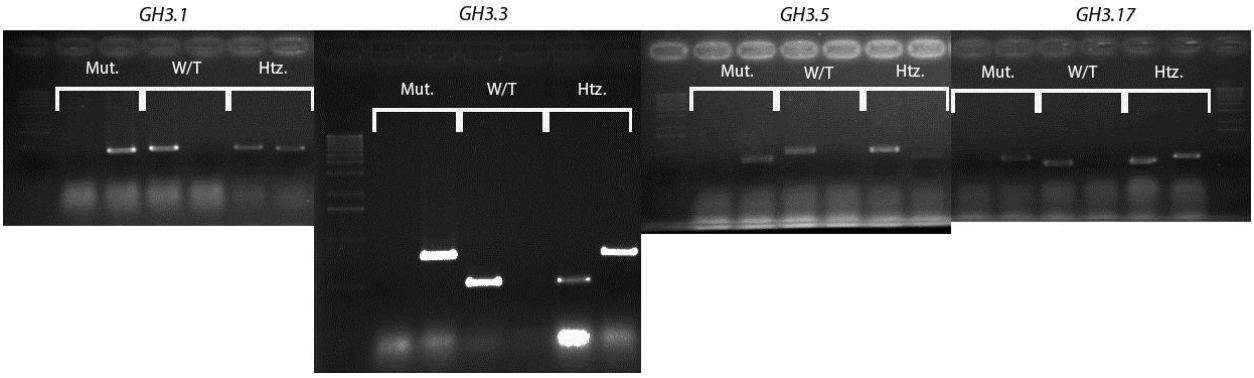

Figure 3. Genotyping using pooled gDNA from 20 progeny of homozygous mutant parents. The first sample in each pair is for the W/T amplicon and the second is for the insert amplicon. DNA from wild type or heterozygous plants were used as controls.

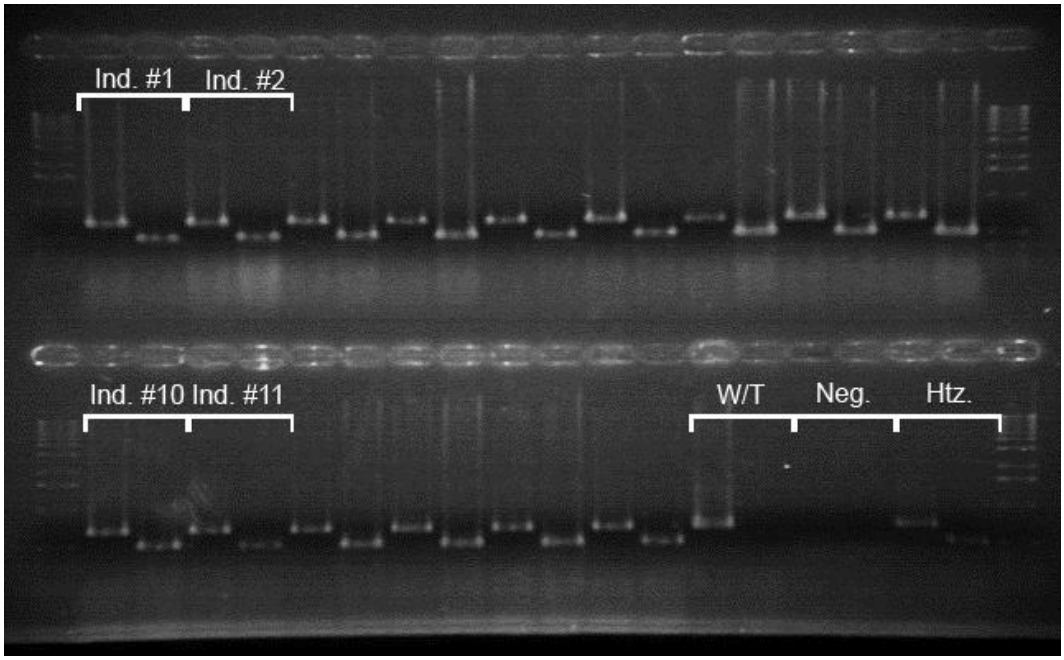

Figure 4. Genotyping of *gh3.2* plants by PCR. The first sample in each pair is the W/T amplicon and the second is for the insert amplicon. The genotying results showed that all 15 individuals were heterozygous. Conrtol PCRs using wild type DNA, no template control or heterozygous DNA were included.

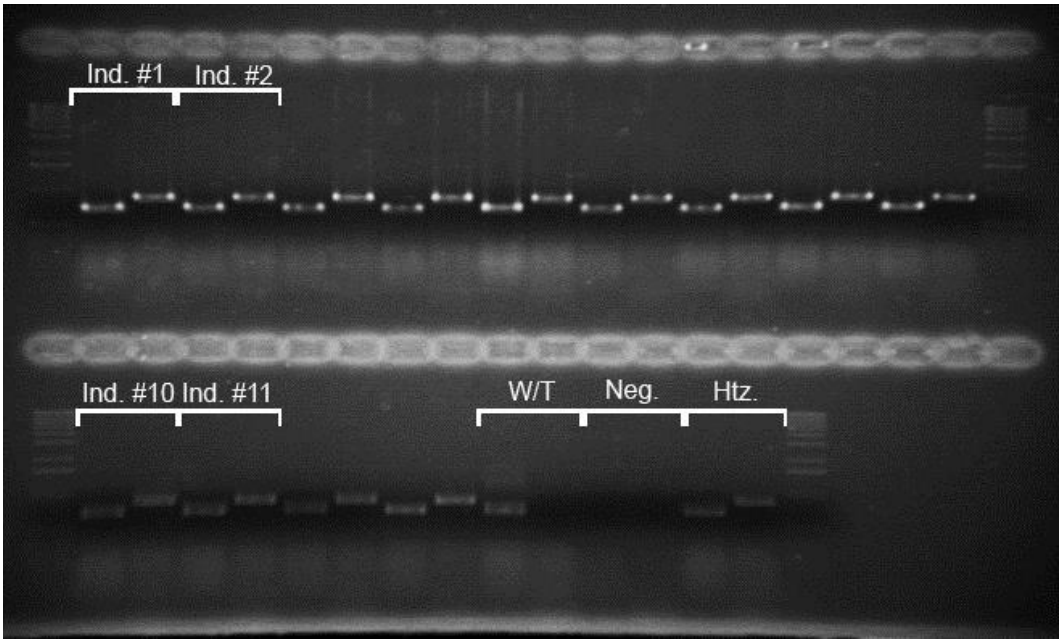

Figure 5. Genotyping of *gh3.4* plants by PCR. The first sample in each pair is for the W/T amplicon and the second is for the insert amplicon. The genotying results showed all 13 individuals were heterozygous. Conrtol PCRs using wild type DNA, no template control or heterozygous DNA were included.

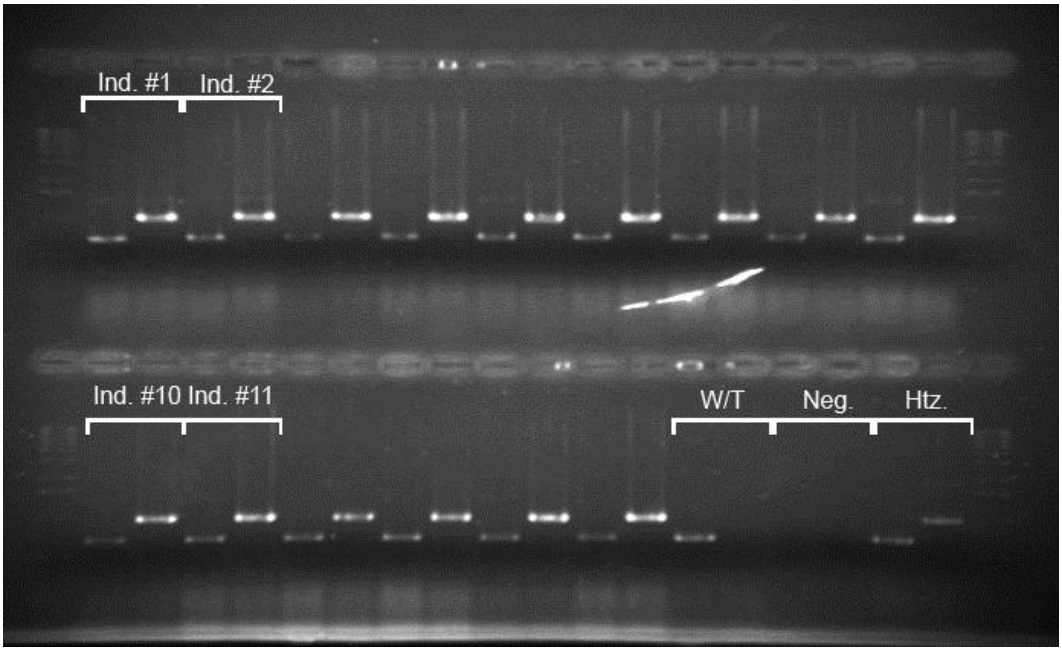

Figure 6. Genotyping of *gh3.6* plants by PCR. The first sample in each pair is for the W/T amplicon and the second is for the insert amplicon. The genotying results showed all 15 individuals were heterozygous. Conrtol PCRs using wild type DNA, no template control or heterozygous DNA were included.

There are several possible explanations for these observations. Although not reported to be an essential gene, a homozygous knockout of this locus could be lethal. Opposing this idea is the fact that homozygous mutants of these genes can be found in literature, including several of the lines used here [67, 102, 103]. However, ambiguity and lax methods have led to questionable conclusions in some cases. In Gutierrez's [103] research, homozygous mutants are determined by confirming the insertion is present in at least twelve progeny, with the idea that probability would be against a selfing heterozygote having this number of offspring with T-DNA. This is clearly an insufficient method of determining homozygosity, especially taking into account the data presented here. Since insertion lines tend to have more than one insert, there could be a second, closely linked, T-DNA insert into an actual lethal allele. The probability of

this happening is quite low however, as only about 1.5% of loci in the *A. thaliana* genome are essential [104, 105]. Homozygous lethality in these lines could be caused by a mechanism other than direct loss of function. For example, instability of inverted tandem repeats like those found in the *gh3.6* line could cause instability of the chromosome [106]. To complicate the matter, there is report of double T-DNA mutants resulting in translocation between chromosomes, which is applicable to all T-DNA lines as they may or may not contain an extra copy or copies of T-DNA. The researchers of these findings suggest checking for chromosome aberrations anytime there is anomalous segregation in T-DNA mutants, especially in generating double mutant lines [107]. The possibility of cross-pollination between mutant lines could also lead to extra copies of T-DNA or transposons. One study shows a high proportion, around 13%, of homologous double mutants resulting lethality [108].

Another potential problem that could lead to the observed segregation pattern is non-specific amplification. *GH3* gene products perform the same function, and so, have similar nucleic acid sequences. Primes with low stringency could result in non-specific binding to paralogous genes, even when there is no W/T target gene present. Of the primers used in this experiment, *GH3.2* and *GH3.4* primer pairs had the lowest stringency, with only one mismatch each compared to non-specific *GH3* genes. The effectiveness of these mismatches were maximized via their 3' end location [109]. Nevertheless, the quantitative amplification remained constant (band intensity) across samples where probability favored homozygous mutants of the target gene (Figures 3-5). In an attempt to rectify the potential problem, annealing temperatures were gradually increased only to result in complete abolishment of amplification. Non-specific binding is often easily dismissed, as band size is typically different from those of the desired product. Using Primer BLAST, predicted sizes of non-specific products for *GH3.2* and *GH3.4*

differ by 10-15 bp and would not be resolvable on the gels used in this experiment. This could explain the lack of mutant homozgosity in the lines used here, but it does not explain the lack W/T progeny from heterozygous parents. Non-specific amplification could result in the lack of W/T progeny if contamination between mutant lines was an issue. In a case where contamination between knockout lines was an issue, W/T progeny should still appear, but at lower ratios. For example, if a knockout line was contaminated by the pollen of another, one would expect $1/16^{th}$ of the progeny to have no T-DNA. The number of plants genotyped for *gh3.4* and *gh3.6* lines clearly show that this is not the case.

5.2 Generating Double Mutants

In order to better understand the role of *GH3* in the disease progression of *A. thaliana* by *M. phaseolina*, double mutants of highly similar *GH3* mutants were generated. This was done to ensure the loss of function of one *GH3* gene, due to insetional mutation, was not rescued by the over-expression of another. Phylogenetic analysis was done to determine *GH3* genes with highest similarity. Initially protein sequences were used for this analysis, but later nucleic acid sequences were later used as these sequences contain more evolutionary information [110]. Sequences from Appendix B were pre-aligned in ClustalX2.1 in a pairwise manner using a International Union of Biochemistry (IUB) weighted matrix [111]. The aligned sequences were then used to construct a phylogenetic tree using a TREEFINDER [112] (Figure 7). Here *GH3.2* and *GH3.4*, as well as *GH3.5* and *GH3.6* genes form clades of their own with bootstrap values of 100% out of 1000 iterations. The log likelihood of this tree is -17546.03, with the Hasegawa, Kishino, and Yano (HKY) substitution model being used [113]. The presence of these genes on differing chromosomes allowed for ease of crossing as they should observe independent

assortment. Homozygous double mutants of the F_2 could be expected to be found in a 1:16 ratio depending on normal Mendelian inheritance.

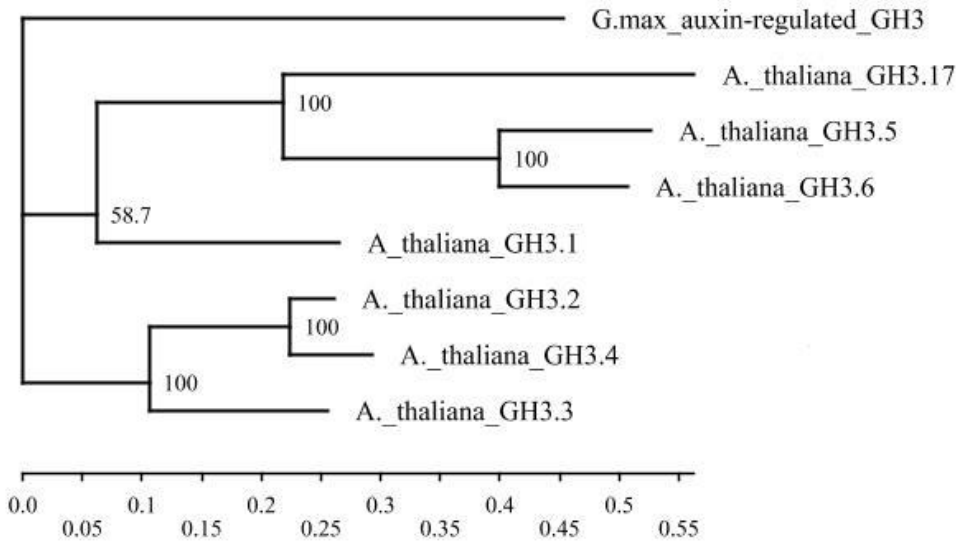

Figure 7. Phylogenetic tree of *A. thaliana GH3* genes. A predicted *Glycine max GH3* gene was used as an outgroup.

Crossing of mutant plants was started at the same time as genotyping for single mutants to save time. Genotyping results for the F_2 generation of these crosses are presented in Table 1. Insertion mutant plants were successfully crossed, but failed to yield double homozygous mutants. These two crosses show non-Mendelian inheritance with a significance level of 0.005% or less. This data is consistent with the absence of homozygous mutants in *gh3.2*, *gh3.4*, and *gh3.6*. Furthermore, *gh3.5* segregates in the expected 1:2:1 ratio. However, the W/T genotypes for *GH3.2*, *GH3.4*, and *GH3.6* are not consistant with the heterozygous only genotypes found for the single mutant lines. One possibility that could explain the W/T genotypes found here and the heterozygous only genotypes for the un-established single mutant lines is non-specific amplification of paralogous *GH3* genes combined with *GH3.2*, *GH3.4*, and *GH3.6* lines starting out as homozygous mutant. Under these conditions, all progeny of the

single mutant lines would consistently result in a heterozygous genotype. Furthermore, introduction of a W/T allele at the homozygous mutant loci, due to cross pollination of lines, could result in a W/T genotype in the F_2 generation of a double mutant cross.

TABLE 1

RESULTING F_2 GENOTYPES OF MUTANT CROSSES

gh3.2* x *gh3.4	Observed	***gh3.5* x *gh3.6***	Observed
GH3.2/GH3.2 *GH3.4/GH3.4*	1	*GH3.5/GH3.5* *GH3.6/GH3.6*	1
GH3.2/GH3.2 *GH3.4/gh3.4*	11	*GH3.5/GH3.5* *GH3.6/gh3.6*	12
GH3.2/GH3.2 *gh3.4/gh3.4*	0	*GH3.5/GH3.5* *gh3.6/gh3.6*	0
GH3.2/gh3.2 *GH3.4/GH3.4*	9	*GH3.5/gh3.5* *GH3.6/GH3.6*	4
GH3.2/gh3.2 *GH3.4/gh3.4*	21	*GH3.5/gh3.5* *GH3.6/gh3.6*	10
GH3.2/gh3.2 *gh3.4/gh3.4*	0	*GH3.5/gh3.5* *gh3.6/gh3.6*	0
gh3.2/gh3.2 *GH3.4/GH3.4*	0	*gh3.5/gh3.5* *GH3.6/GH3.6*	3
gh3.2/gh3.2 *GH3.4/gh3.4*	0	*gh3.5/gh3.5* *GH3.6/gh3.6*	6
gh3.2/gh3.2 *gh3.4/gh3.4*	0	*gh3.5/gh3.5* *gh3.6/gh3.6*	0

Genotype results of *gh3.2* x *gh3.6* and *gh3.5* x *gh3.6* crosses. Uppercase letters represent W/T alleles while lowercase letters represent mutant alleles.

5.3 Assessment of the Charcoal Rot Disease

Established homozygous lines were planted and inoculated with *M. phaseolina*. Plants were scored according to the matrix given in Section 4.5. Inoculated W/T plants and mutant plants showed no difference in the progression of disease symptoms (Figures 8-11). The amount

of time it took for plants to reach mortality was compared to determine if there were any significant differences. A one-way ANOVA showed no significant difference among lines at a significance level of 0.05 for F (5,12) = 0.85 and p = 0.538. Basic statistical information used for the ANOVA is in Table 2.

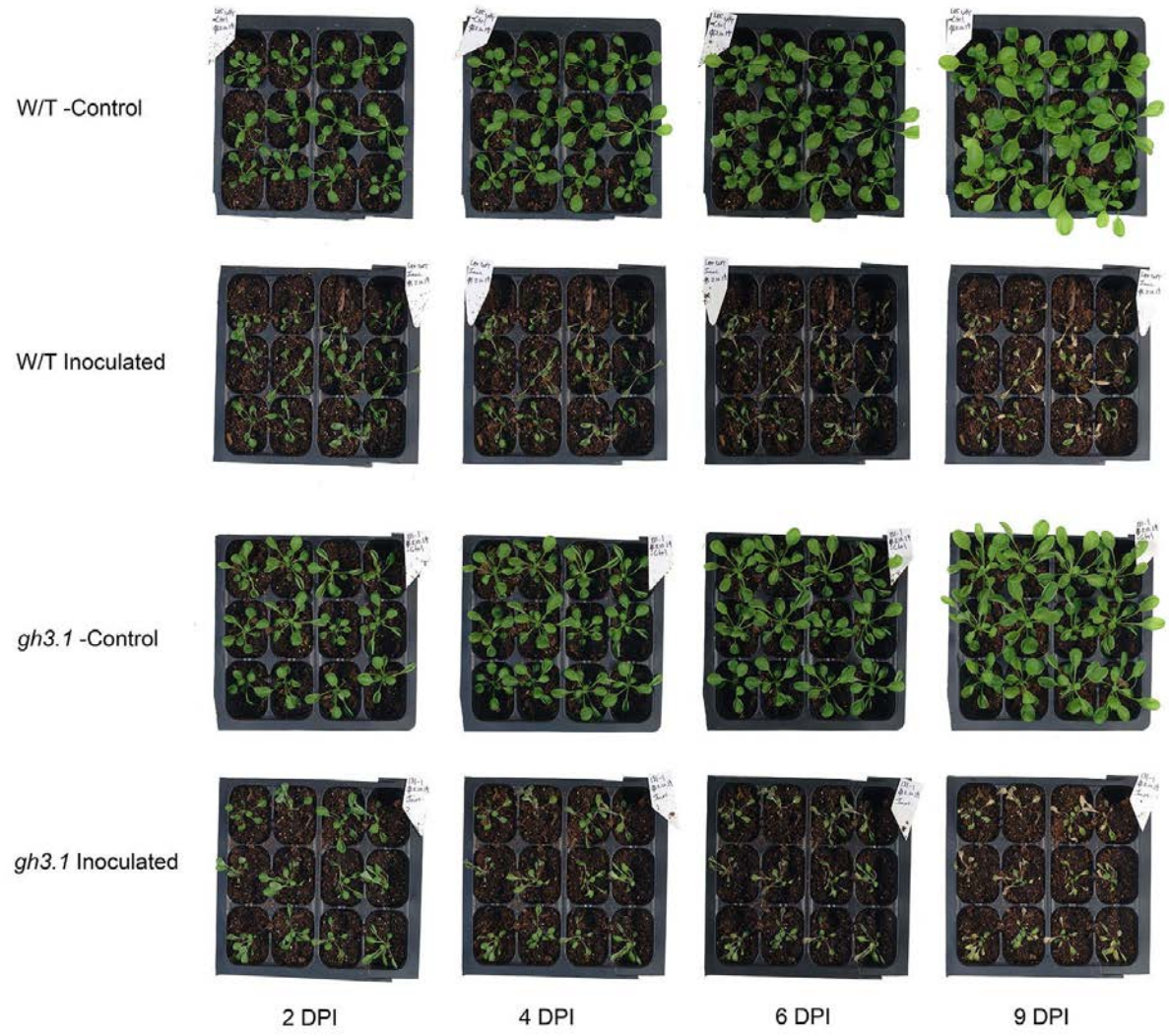

Figure 8. Progression of disease symptoms for *gh3.1* from 2 DPI to 9 DPI. Wild type and mutant plants were inoculated with fungal sclerotia (Inoculated) or agarose solution (-Control).

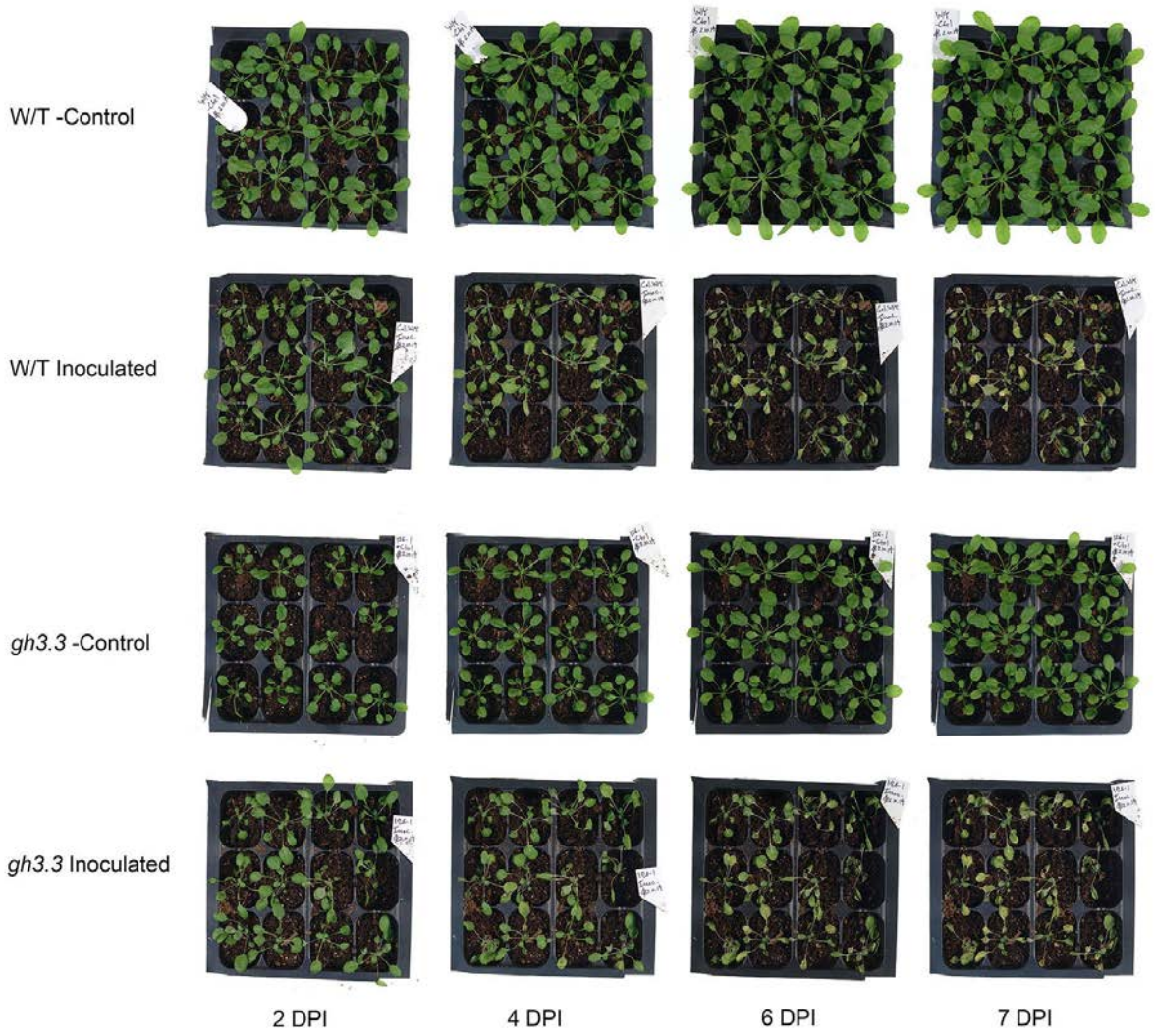

Figure 9. Progression of disease symptoms for *gh3.3* from 2 DPI to 7 DPI. Wild type and mutant plants were inoculated with fungal sclerotia (Inoculated) or agarose solution (-Control).

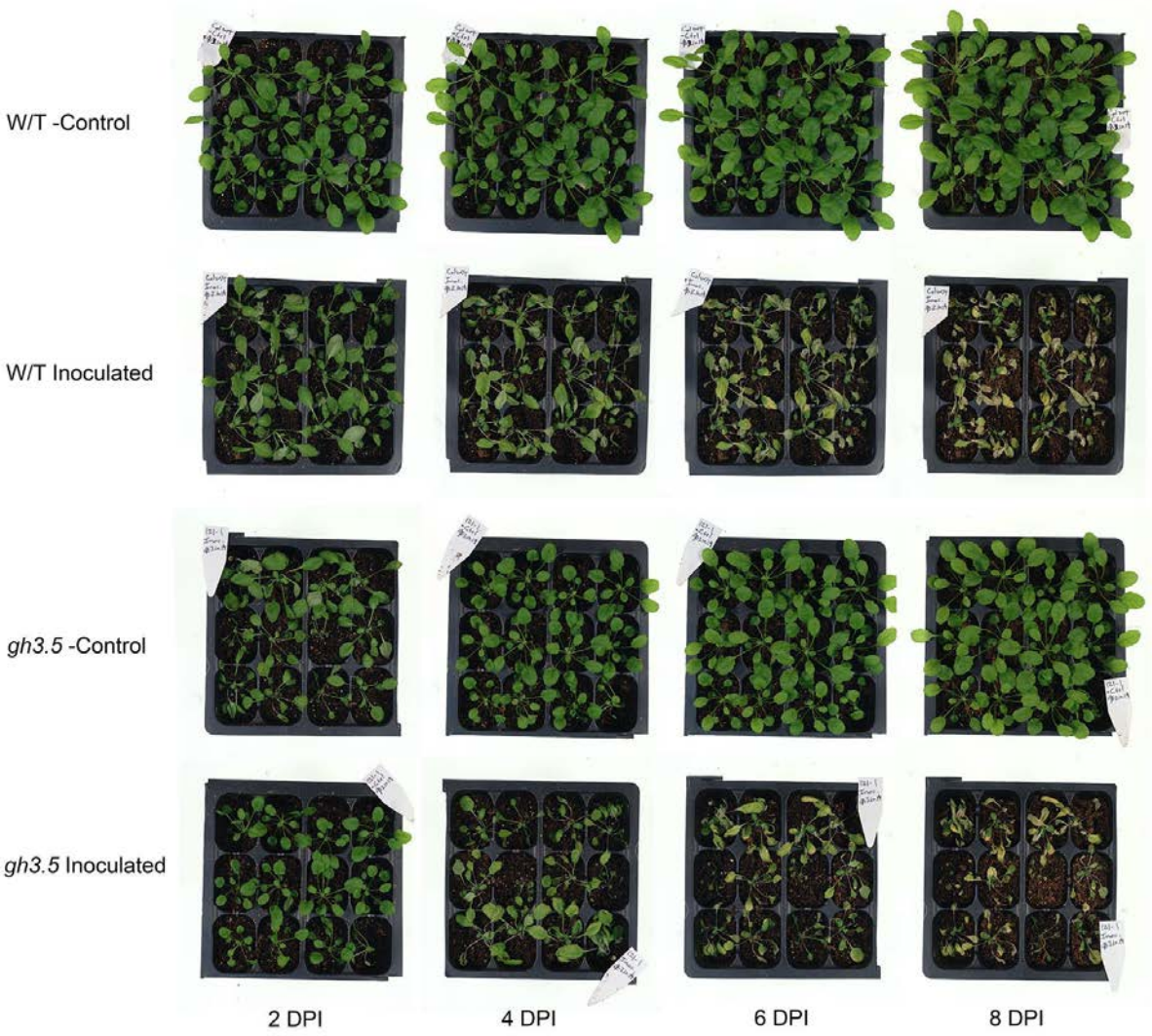

Figure 10. Progression of disease symptoms for *gh3.5* from 2 DPI to 8 DPI. Wild type and mutant plants were inoculated with fungal sclerotia (Inoculated) or agarose solution (-Control).

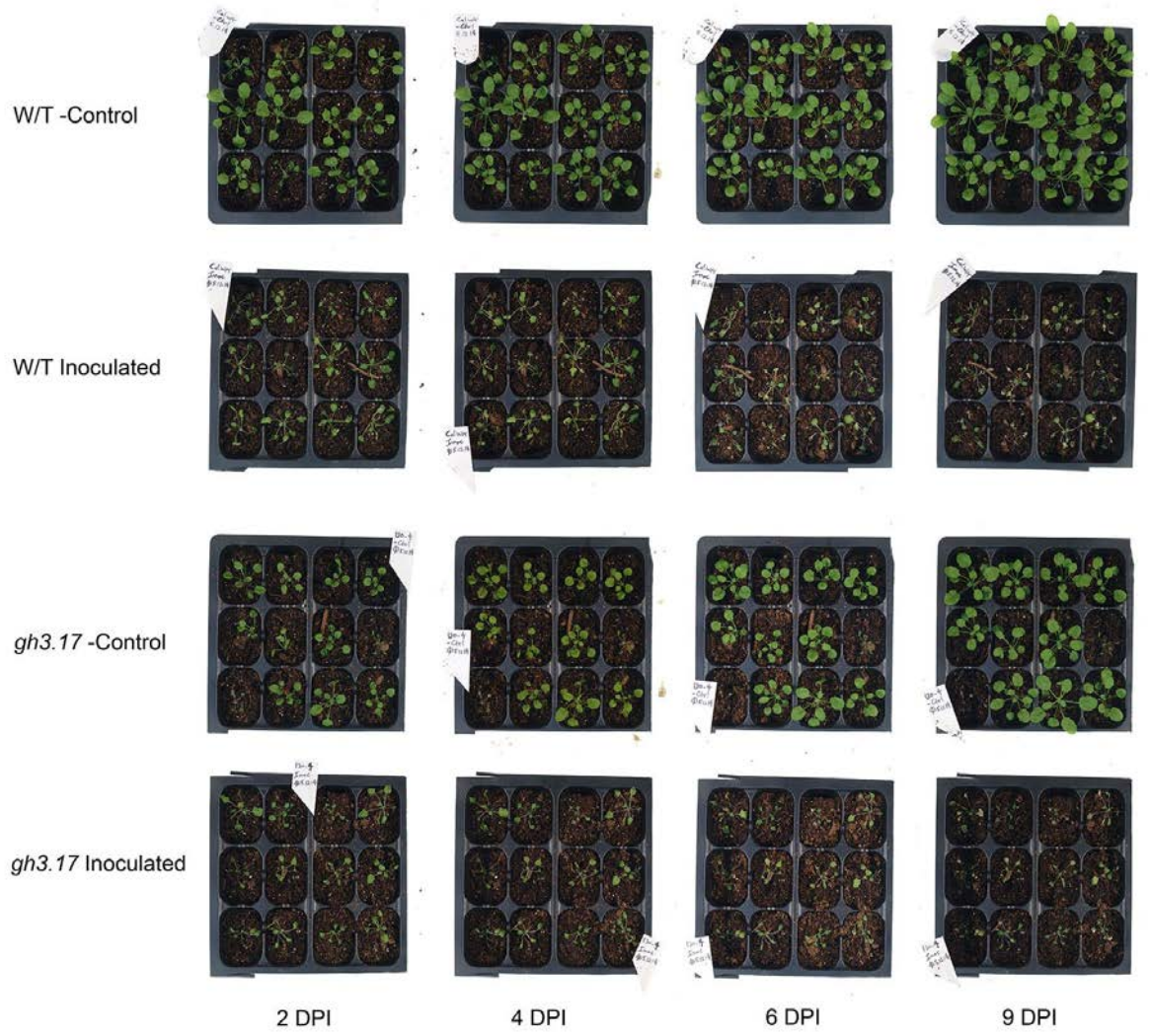

Figure 11. Progression of disease symptoms for *gh3.17* from 2DPI to 9 DPI. Wild type and mutant plants were inoculated with fungal sclerotia (Inoculated) or agarose solution (-Control).

TABLE 2

Basic Statistics of Plant Lines to Reach Mortality (DPI)

Plant Line	Mean	Standard Deviation
Ler	8.0	1.4
gh3.1	8.0	1.4
Col	8.3	1.1
gh3.3	7.0	0.0
gh3.5	9.0	1.4
gh3.17	8.7	0.58

The possibility for increased resistance due to mutations in *GH3* genes still exists. Extreme levels of infection could have resulted from the gross inoculation procedure used in this experiment. During inoculation, the root surfaces were entirely covered by microsclerotia. Diluted inoculum may reveal subtle differences in low levels of quantitative resistance. More sensitive methods of measuring disease symptoms could also reveal differences in resistance. This would include quantitative methods of determining the amount of fungus present, such as counting microsclerotia per certain length of root or using qPCR to measure the fungal biomass.

5.4 Confirmation of Loss of Function in Insertion Lines

To confirm abolishment of *GH3* gene products, RT-PCR was done to check for the absence of the mRNA in each homozygous line. Despite a clear dwarfed phenotype in *GH3* mutant plants, three of the four knockout lines showed amplification of mRNA (Figure 12 and 13). Three replications of RNA isolation and RT-PCR reactions resulted in the same findings. Primers designed to amplify cDNA in this part of the experiment span introns. This allows for gDNA contamination to be ruled out, as amplification would lead to amplicons of considerably larger size. The T-DNA insertion for the *GH3.5* line is found in the promoter of the gene. Although this type of insertion does not result in complete knockout of the gene a majority of the time, gene knockdown is very common. As for *gh3.3* and *gh3.17* lines, T-DNA positioned in exons can still lead to mRNA transcription [114]. The resulting proteins, if produced at all, would more than likely lead to nonfunctional products. To check for altered cDNA sequences for these lines, PCR reactions with a T-DNA specific primer or using primers that span the insert can be run (Figure 13).

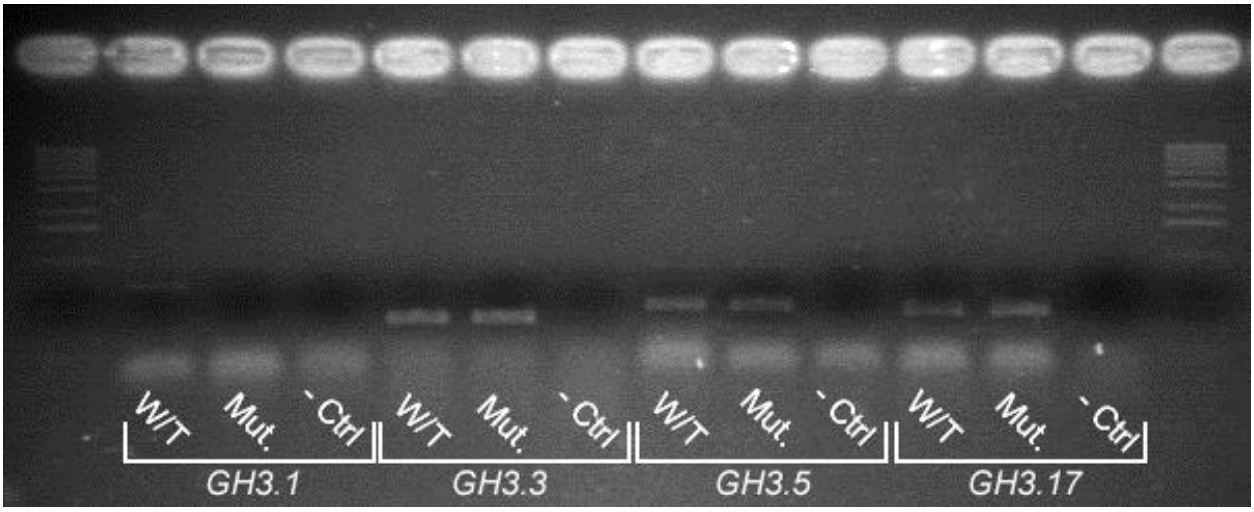

Figure 12. Presence/absence of mRNAs for each *GH3* gene was checked in corresponding homozygous mutant line by RT-PCR.

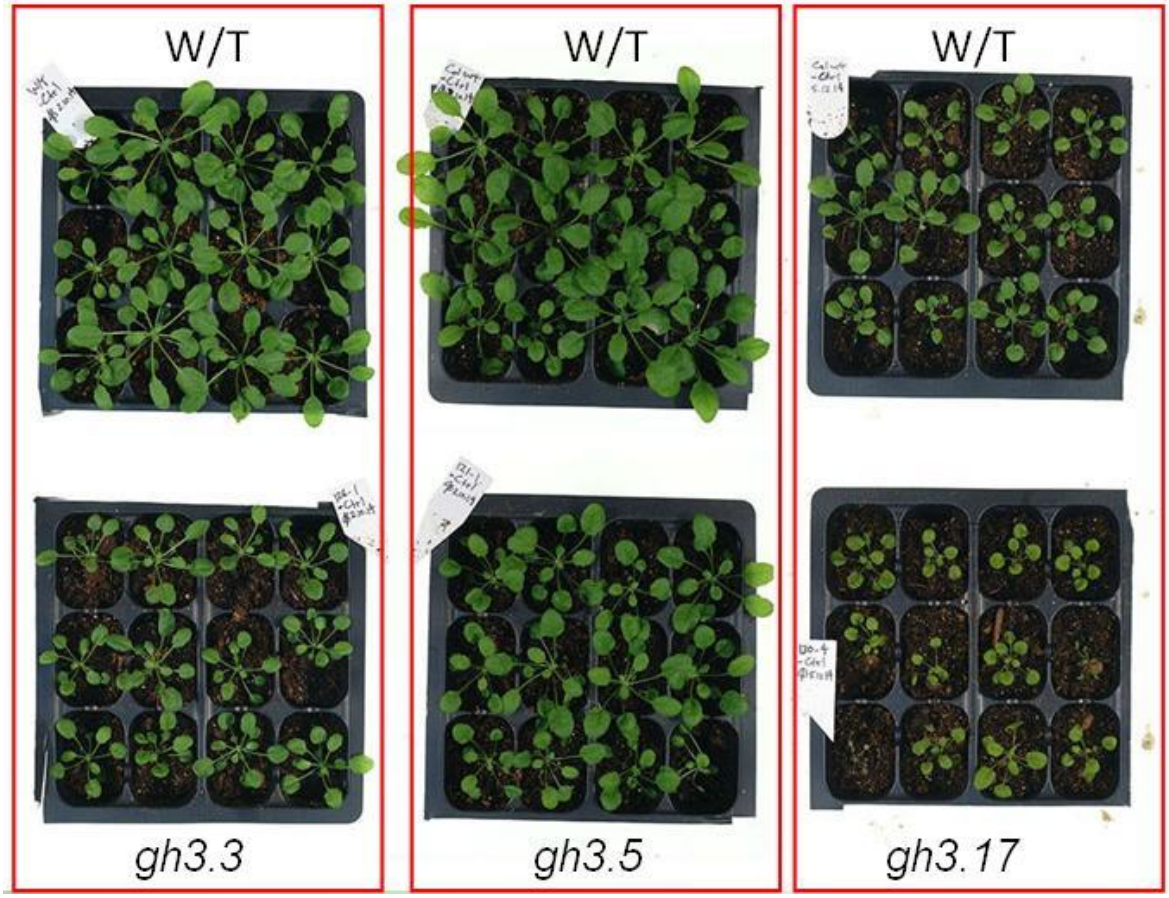

Figure 13. Dwarfed phenotype of homozygous mutant lines compared to W/T lines. Plants are 37 days old.

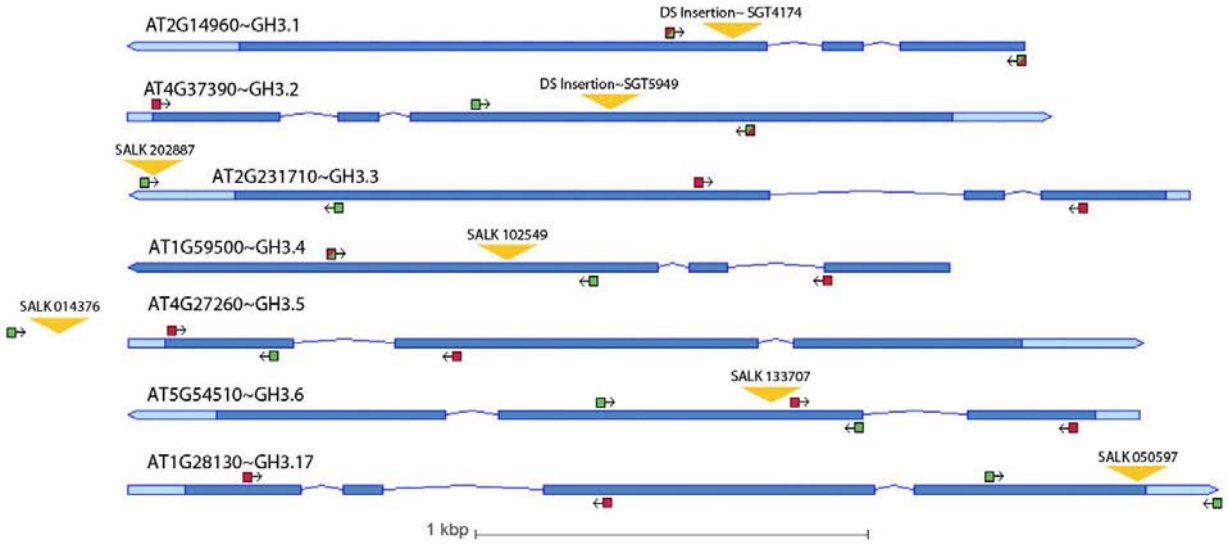

Figure 14. T-DNA or Ds insertion sites in each mutant line and the locations of primers used in genotyping and RT-PCR. Yellow triangles represent insertion site of transposons or T-DNA, green and red boxes represent binding sites for primers used in genotyping and RT-PCR respectively.

5.5 Conclusion

While expression of *GH3* genes have been shown to be modulated by *M. phaseolina* in *M. truncatula* and confer resistance to other necrotrophic pathogens when mutated, data presented here suggests altered *GH3* genes have no significant effect on the interaction between *M. phaseolina* and *A. thaliana*. To confirm these results, different mutant lines of the same *GH3* genes can be used, as well as using RNAi technology to reduce the expression of all *GH3* genes. A better assay for assessing disease progression may also help to reveal subtle differences between different lines. This experiment highlights the complexity still present in reverse genetics research. It offers the possibility of gaining a better understanding on the efficiency of insertional mutations and begs to question if such insertions alter the expression of closely related genes.

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APPENDICES

APPENDIX A

PRIMERS USED IN EXPERIMENT

Primer	Gene of Interest	Sequence (5' to 3')	Procedure	Predicted size (bp)
AtGH3.1-F	*GH3.1*	GCGGTAGACTCCAACCTCTCTTC	Genotyping	941
			RT-PCR	702
			Ds3'-1a	~925
AtGH3.1-R	*GH3.1*	GAGGAGACCAGAGGCAAAGA	Genotyping	941
			RT-PCR	702
			Ds5'-1a	~925
AtGH3.2-F	*GH3.2*	ATGGCCGTTGATTCACCTC		
AtGH3.2-R	*GH3.2*	CTAACGACGTCGTTCTGG		
AtGH3.2-F2	*GH3.2*	TAACGAAGCCATCCTCTGCT	Genotyping	698
			Ds5'-1a	~550
AtGH3.2-R2	*GH3.2*	AATTATGGAACCCCGTGACA	Genotyping	698
			Ds3'-1a	~550
AtGH3.3-F	*GH3.3*	TGCTTGGAAATGGAGGAGTC	Genotyping	514
			LBa1	~750
AtGH3.3-R	*GH3.3*	CATCGACGCTTAGTACATTTGC	Genotyping	514
AtGH3.3-F2	*GH3.3*	GACCAAAGTTCCGGTGGTTA	RT-PCR	405
AtGH3.3-R2	*GH3.3*	TCTGGACAAAGGATGGCTTC	RT-PCR	405
AtGH3.4-F	*GH3.4*	AGCCATCCTCTGCTGTGACT	Genotyping	688
AtGH3.4-R	*GH3.4*	TGGAGCGGAATTATGAAACC	Genotyping	688
			RT-PCR	983
			LBa1	~900
AtGH3.4-F2	*GH3.4*	CTCATCCCATCACCGAGTTT	RT-PCR	983
AtGH3.5-F	*GH3.5*	CGGAAAGAGAGAAAA		
AtGH3.5-F2	*GH3.5*	TGACGACCAAATGTCTCGAA	Genotyping	950
			LBa1	~850
AtGH3.5-F3	*GH3.5*	CCTGAGGCACCAAAGAAAGA	RT-PCR	499
AtGH3.5-R	*GH3.5*	CGATCCTGTTGATCTCAGGC	Genotyping	950
AtGH3.5-R2	*GH3.5*	CTCCTGGAGTCTTGGATTCG	RT-PCR	499
AtGH3.6-F	*GH3.6*	GTTCTGGGACTTCTGGTGGA	Genotyping	683
AtGH3.6-R	*GH3.6*	TGTGCAGACAAGAGGCAAAC	Genotyping	683
			LBa1	~950
AtGH3.6-F2	*GH3.6*	GAGAGCCTCGCTGAGAAGAA		
AtGH3.6-R2	*GH3.6*	GACCACCTGGTGTCTTGGAT		
AtGH3.17-F	*GH3.17*	TTCAACATCCTTCAAGCCTC	Genotyping	631
			LBa1	~750
AtGH3.17-R	*GH3.17*	CGAAAAAGAGAGGGAGACAAAG	Genotyping	631
AtGH3.17-R2	*GH3.17*	GAATGGTCTGGTCAGGGCTA	RT-PCR	441
AtGH3.17-F2	*GH3.17*	GAGCATTTCTGGACGGAGAA	RT-PCR	441
Ds3'-1a	Transposon	GGTTCCCGTCCGATTTCGACT	Genotyping	
Ds5'-1a	Transposon	ACGGTCGGGAAACTAGCTCTAC	Genotyping	
LBa1	T-DNA	TGGTTCACGTAGTGGGCCATCG	Genotyping	
ACT7-F	*Actin 7*	AAAATGGCCGATGGTGAGG	gDNA confirm.	1450
			cDNAconfirm.	1172

ACT7-R	*Actin 7*	ACTCACCACCACGAACCAG	gDNA confirm.	1450
			cDNA confirm	1172

Ds3'-1a and Ds5'-1a used in combination with GH3.1 and GH3.2 primers; LBa1 used with the primer designated insertion conf. for each GOI.

APPENDIX B

NUCLEIC ACID SEQUENCES USED TO DETERMINE PHYLOGENETIC RELATIONS BETWEEN INDOLE-3-ACETIC ACID AMIDO SYNTHETASES

>gi|30679281|ref|NM_127059.2| Arabidopsis thaliana putative indole-3-acetic acid-amido synthetase GH3.1 mRNA, complete cds

ATGGCGGTAGACTCCAACCTCTCTTCGCCCTTGGGACCTCCGGCGTGTGAGAAGGAC
GCAAAAGCCCTTCGTTTCATCGAGGAAATGACCCGAAACGCCGACACCGTTCAAGA
AAACCTTTTGGCGGAGATTCTCGCTCGCAACGCTGACACCGAGTACCTCCGCCGCTT
CAACCTATGCGGCGCCACCGACCGTGATACCTTTAAAACGAAGATTCCAGTCATTAC
ATACGAAGATCTCCAGCCAGAGATTCAACGCATTGCTGAGGAGACCGCTCTCCCATC
TTATCTGCCCATCCCATCTCTGAGTTCCTCACTAGCTCTGGAACATCAGCCGGAGAG
AGGAAACTTATGCCGACCATTAAAGAAGAGCTCGATCGTCGCCAGCTTCTTTACAGT
CTCCTCATGCCCGTAATGAATTTGTATGTGCCGGGTCTAGATAAAGGAAAGGGAATG
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AGTTTGGTAACTTCTGTTTTGTGTTTATATTCATGTGCATGTTGTTACATTACACAGA
TACGTGACTGATATGGACAAGGGTAAGGCTCTCATCTTCCTGTTCACCAAAGCCGAG
CAGAAAACTCCGAGTGGCTTAGTGGCACGTCCAGTGTCGGCTAGCATGTACAAGAG
CGACCAGTTCAAAAACAGACCCTACGACCCATACAACGTGTACACAAGCCCAGACG
AAGCAATCCTCTGCCCCGATTCCTTCCAAAGCATGTACACCCAAATGCTATGCGGCC
TCATCATGCGCCATCAAGTCCTCAGAGTTGGTGCAAATTTCGCCTCCGGCCTTCTCC
GATCCATCCACTTGCTCCAGCTCAATTGGGCCCAACTATCCCACGACATCTCCACCG
GAACCCTAAACCCCAAAATCACTGACCCTGCCATCAAACAACGCATGACCCAAATC
CTCAAACCCGACCCAGAACTTGCTGAATTCATTGTGAAAGAATGCTCAGGAGAAAA
CTGGGAACGCATAATCCCCAGAATCTGGCCAAACACCAAGTATGTAGAGGTGGTTG
TGACCGGTGCCATGGCGCAGTATGTTCCAACTCTTGATTATTACAGCGGAGGCCTAC
CTCTCGCTTCCAACATATACGGATCCTCCGAGTGCTTCTTTGGGATTAACCTCAACCC
ATTTTGCAACCCCTCTGACGTGTCATACACCATCATGCCAAACATGGGTTACTTCGA
GTTCTTGCCTCAGGATCACGATGATGCTTCTTCGTCTTCAGGTTCCAGTTTCACTCTG
TCGCGTTTAATCGACCTTGATGATGTTGAACTCGGAAAATCCTACGAAATTGTTGTG
ACCACGTACTCCGGTTTATGCCGTTACCGTGTGGGCGACATTCTCCGAGTGACGGGC
TTCCACAACACCGCCCCGCAGTTCAGCTTCGTGAGGAGAAAAAACGTGTTGCTGAG
CATCGACTCGGACAAAACGGACGAAGCGGAGTTACAAAACGCCGTGGAGAAGGCC
TCCGTGCTGTTGAAGGAGTTCAAGACGAGCGTGGTGGAGTACACGAGCTTTGCGGA
CACGAAGTCGATCCCGGGGCATTACGTGATTTACTGGGAGCTCTTGATGAAGGACTC

TTCCAACGCTCCAACTACTGAAGCGTTGGAGCAGTGTTGTTTAACAATGGAAGAATC
GCTGAACGCCGTTTATCGACAAGGTAGGGTTGCGGACCATTCTATTGGACCGTTAGA
GATTCGTGTGGTGAAGAATGGAACCTTTGAGGAGCTTATGGACTACGCTATCTCACG
TGGCGCGTCTATTAGCCAGTACAAGGTTCCACGGTGCGTGACCTTCACGCCCATAAC
GGAGTTGCTCGACTCTAGGGTTGAGTCCGTTCATTTTAGCCCCTCTGAACCACACTG
GACCCCGGAACGACGTCGTTGAAGGGTAACTACACATACCTGCGTGAAGTCATGCG
CTCAATAATTAAAATAAAGAGATTGAAAGGAATGTGCCTTTTCATTTTGGCCGTGCT
TTTTAAAGTTTATGTGTTATGTTTTAGATTTGTGTCTCTGAAAAATATGGTCAAAAGG
AATTGTCCTAGACATCTTTGTCTATCCTATGTAGGAAACCATCATGTGCACACGTTTA
ATTAGTCGATCTATTTATAATGTTAATACATTATAATTTTAATATCCTTCACGTGCAT
GTAACTTTCATGGGGCCAAAAGTCTGAGAAACGGTTGTCTTTTCTCTTCTTTTTCGTG
TAAAGTTAATTACCCGTATATAACATGAATTTGAGTTGAATTAATAAAGTCGTTATA
ATGTTTCAGATGTGAAACTCAAACTCCAAACA

APPENDIX C

DISEASE PROGRESSION DATA

Results for 131-1 (*gh3.1*); Feb. 10, 2014 inoculation

	W/T –Ctrl	W/T Exp	131-1 –Ctrl	131-1 Exp
1 DPI	1	1	1	1
2 DPI	0	1	0	1
3 DPI	0	2	0	2
4 DPI	0	3	0	3
5 DPI	0	4	0	4
6 DPI	0	5	0	5
7 DPI	0	5	0	5
8 DPI	0	5	0	5
9 DPI	0	6	0	6

Results for 131-1 (*gh3.1*); May 15, 2014 inoculation

	W/T –Ctrl	W/T Exp	131-1 –Ctrl	131-1 Exp
1 DPI	1	1	1	1
2 DPI	0	1	0	1
3 DPI	0	2	0	2
4 DPI	0	4	0	4
5 DPI	0	5	0	5
6 DPI	0	5	0	5
7 DPI	0	6	0	6

Results for 126-1 (*gh3.3*); Feb. 10, 2014 inoculation

	W/T –Ctrl	W/T Exp	126-1 –Ctrl	126-1 Exp
1 DPI	0	0	0	0
2 DPI	0	1	0	1
3 DPI	0	1	0	1
4 DPI	0	3	0	3
5 DPI	0	4	0	4
6 DPI	0	5	0	5
7 DPI	0	6	0	6

Results for 126-1 (*gh3.3*); March 12, 2014 inoculation

	W/T –Ctrl	W/T Exp	126-1 –Ctrl	126-1 Exp
1 DPI	0	0	0	0
2 DPI	0	1	0	1
3 DPI	0	2	0	2
4 DPI	0	2	0	2
5 DPI	0	3	0	3
6 DPI	0	5	0	5
7 DPI	0	6	0	6

Results for 121-1 (*gh3.5*); August 1, 2013 inoculation

	W/T –Ctrl	W/T Exp	121-1 –Ctrl	121-1 Exp
1 DPI	1	1	1	1
2 DPI	0	1	0	1
3 DPI	0	2	0	2
4 DPI	0	2	0	2
5 DPI	0	3	0	3
6 DPI	0	3	0	3
7 DPI	0	4	0	4
8 DPI	0	4	0	4
9 DPI	0	5	0	5
10 DPI	0	6	0	6

Results for 121-1 (*gh3.5*); March 10, 2014 inoculation

	W/T –Ctrl	W/T Exp	121-1 –Ctrl	121-1 Exp
1 DPI	1	1	1	1
2 DPI	0	1	0	1
3 DPI	0	2	0	2
4 DPI	0	3	0	3
5 DPI	0	3	0	3
6 DPI	0	4	0	4
7 DPI	0	5	0	5
8 DPI	0	6	0	6

Results for 130-1 (gh3.17); July 12, 2013 inoculation

	W/T –Ctrl	W/T Exp	130-1 –Ctrl	130-1 Exp
1 DPI	1	1	1	1
2 DPI	0	1	0	1
3 DPI	0	2	0	2
4 DPI	0	3	0	3
5 DPI	0	4	0	4
6 DPI	0	5	0	5
7 DPI	0	5	0	5
8 DPI	0	6	0	6

Results for 130-4 (*gh3.17*); March 10, 2014 inoculation

	W/T –Ctrl	W/T Exp	130-4 –Ctrl	130-4 Exp
1 DPI	1	1	1	1
2 DPI	0	1	0	1
3 DPI	0	1	0	1
4 DPI	0	2	0	2
5 DPI	0	3	0	3
6 DPI	0	4	0	4
7 DPI	0	4	0	4
8 DPI	0	5	0	5
9 DPI	0	6	0	6

Results for 130-4 (*gh3.17*); May 12, 2014 inoculation

	W/T –Ctrl	W/T Exp	130-4 –Ctrl	130-4 Exp
1 DPI	1	1	1	1
2 DPI	0	1	0	1
3 DPI	0	2	0	2
4 DPI	0	4	0	4
5 DPI	0	4	0	4
6 DPI	0	5	0	5
7 DPI	0	5	0	5
8 DPI	0	5	0	5
9 DPI	0	6	0	6