GENETIC DISSECTION OF ARABIDOPSIS GENES GOVERNING IMMUNE GENE EXPRESSION

An Undergraduate Research Scholars Thesis

by

VINCENT E. PROVASEK

Submitted to Honors and Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

Approved by
Research Advisor: Dr. Ping He

May 2014

Major: Biochemistry
Genetics
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The plant innate immune system is built on a myriad of processes, many of which are uncharacterized, or incompletely identified. Infection of Arabidopsis thaliana with bacterium Pseudomonas syringae pv. tomato DC3000 carrying effector proteins AvrRpm1 and AvrRpt2, is known to elicit an effector-triggered immune response (ETI) via interaction with host receptor proteins RPM1 and RPS2, respectively. Recognition of pathogen-associated molecular patterns and pathogen effector proteins initiate intracellular signaling events causing physiological changes favoring the host preservation. To better characterize immune signaling networks, we examined effector-triggered immunity induction in mutagenized transgenic pWRKY46::LUC A. thaliana model organisms. Here, we identify a new putative immune gene (Aggie2015) associated with increased immune response upon infection with effector proteins AvrRpm1 and AvrRpt2. Identification of new immune genes associated with novel immune responses will help elucidate regulation and molecular components of immune signaling pathways. Understanding these pathways may lead to new possibilities in application research of disease and environmental stress resistant crops.
ACKNOWLEDGMENTS

I would like to acknowledge Drs. He and Shan for providing this valuable research opportunity and for their enduring patience and understanding as I learned the art of science research.

I would also like to acknowledge Wenwei Lin for his tremendous help and guidance as I worked through this study. Without his assistance, this would not have been possible.
## NOMENCLATURE

<table>
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<th>Abbreviation</th>
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<tr>
<td>PAMP</td>
<td>Pathogen Associated Molecular Pattern</td>
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<td>PTI</td>
<td>PAMP-triggered Immunity</td>
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<td>ETI</td>
<td>Effector-triggered Immunity</td>
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<tr>
<td>TF</td>
<td>Transcription Factor</td>
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<td>WRKY46</td>
<td>Early marker gene in ETI</td>
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Despite being sessile and lacking an adaptive immune system, plants are the earths longest surviving higher organism [1], and the plant innate immune system has certainly played a crucial role in this evolutionary success. For millions of years, plants have been locked in a perpetual battle with rapidly evolving pathogenic microbes. To maximize evolutionary fitness, plants co-evolved robust and complex defense mechanisms against pathogenic threats [27]. Plants perform the basic immune function of detecting self from non-self using evolutionarily conserved receptor proteins that perceive pathogens. Upon perception these proteins initiate signaling events leading to generalized immune responses designed to attenuate pathogenic activity. Most of the signaling pathways underlying these immune responses are unknown or only partially characterized. An effective means of elucidating pathway mechanisms is through the use of a forward genetics approach to identify associated immune genes followed by functional genomics studies to characterize the specific roles of the corresponding gene products within the overall process. Using methods to achieve widespread mutations across the genome, it is possible to identify organisms with novel phenotypes, and perform common gene-isolation techniques to localize and sequence the putative causal gene. By characterizing plant immune genes, it then becomes possible to demystify the vast and highly interconnected network of pathways constituting the plant innate immune system.

Plant Immunity

Like most organisms, plants are in constant contact with a great variety of microbes including bacteria, viruses, and fungi. While most of these microbes do not have the ability to cause disease, the relative few that do can have a substantial impact resulting in such events as widespread crop losses and species extinction. Interestingly, most plants show a resistance to infection by a majority of the microbes with which they interact, but the mechanisms underlying this phenomena are for the most part unknown. Understanding these mechanisms
holds the potential for advancing application research and development of disease and envi-
ronmental stress resistant plants by going beyond simple plant breeding to more advanced
transgenic approaches of plant species improvement.

The interaction between plants and microbes initiates a series of biochemical reactions that
ultimately determine the fate of both pathogen and host. Most plant pathogens are dis-
tributed passively throughout the environment, and come into contact with plant tissues
by chance. Evolutionary adaptations have permitted pathogens to improve their ability to
attach and infiltrate host tissues so as to increase the likelihood of finding nutrients and
habitat. It is at this point of infiltration that the direction of the relationship is determined.
The likelihood a plant will fall victim to disease is largely dependent on the abundance of
the pathogen, the genetically encoded virulence of the pathogen, the genetically encoded
immune potential of the host, and the transient environmental conditions at the time of
interaction. Each of these facets may impact if, and to what degree, a microbe will become
pathogenic relative to the plant host.

The plant-pathogen interaction is an ancient process marked by a series of attacks and
counter-attacks. Steady changes in the abilities and complexities of pathogen virulence and
plant host immunity have all culminated in the highly effective and complex interaction sys-
tem seen today. Plant immune responses are fundamentally based on maximizing pathogen
perception and minimizing time and energy expenditures associated with defense responses.
Alternatively, pathogenic activity has be centered around circumventing plant immunity to
achieve greater virulence.

The plant innate immune system can be viewed in terms of a three step process that parallels
an evolutionary time-line. The first step is the initial recognition and response to a pathogen.
The second step is the counter-attack of the pathogens by delivering effector proteins into
the host cell to disrupt the immune response. The third step is the plant counter-attack
by using cytosolic receptor proteins to detect the presence of pathogenic effectors so as to
elicit a separate immune response. This process repeats itself with each new counter-attack adding yet another level of complexity to the immune system signaling networks.

**PAMP-Triggered Immunity**

The first line of plant defense outside of the cell wall is the detection of non-self at the cell surface level. Embedded within the plant cell surface are pattern recognition proteins (PRRs) that physically interact with a relatively limited number of conserved molecular patterns common to the majority of microbes. If these microbe-associated molecular patterns (MAMPs) are associated with microbes shown to be potential infections agents to the plant, they are termed pathogen-associated molecular patterns, or PAMPs. Whether or not the microbe is pathogenic is independent of their possessing recognizable molecular features. PAMP-triggered immunity (PTI) is initiated by the physical interaction of the PAMP with a corresponding PRR. Cell surface PRRs function as receptor-like kinases, that when bound to PAMP ligands undergo a conformational change initiating intracellular signaling events, a process termed mitogen-activated protein kinase cascades (MAPKs) [7]. The PRRs involved in PTI typically possess a conserved extracellular leucine-rich repeat binding region responsible for the specific ligand binding of MAMPs/PAMPs. Interestingly, these PRRs resemble Toll-like receptors found in animal cell immune responses [3]. Generally, PTI occurs almost immediately after physical interaction between host and pathogen and has been considered the penultimate mechanism of disease resistance signaling due to its role in determining immune response specificity, duration, and severity by way of different MAPK cascades [3].

MAPKs function primarily to carry signals originating at the site of perception to the sites of activation and regulation in downstream targets. Moreover, MAPKs are not limited strictly to biotic triggers; they are virtually ubiquitous in their roles of amplifying and precisely regulating stimuli from biotic and abiotic stimuli [3]. Arabidopsis thaliana (A. thaliana) has 93 known MAPKs [3], each of which plays an integral role in different cell activities. Within immune response signaling, MAPKs mediate important downstream defense responses. Defense signaling MAPKs also display convergent behavior in that the same set of MAPKs are
activated by different bacterial triggers [5, 22, 26]. This implies multiple MAMP signaling pathways likely converge at a point upstream of the subsequent MAPK signaling cascade directly associated with initiating defense responses.

The cellular defense responses elicited by PTI have been shown to attenuate pathogen activity through a number of methods such as the hypersensitive response (HR) characterized by localized programmed cell death, systemic acquired resistance (SAR), generation of reactive oxygen species (ROS), regulation of plant hormones, and induction of pathogen-related (PR) gene expression.

PTI-mediated disease resistance varies slightly between host and non-host pathogens, however studies using PRR knock-out mutants have shown increased susceptibility to infection by a virulent pathogens such as *Pseudomonas syringae* pv tomato DC3000 [27]. Conversely, because most plants are non host to most potential pathogens, non host immunity is the most common form of plant defense. However, it is unclear as to whether non host resistance is PTI mediated defense dependent [22]. It is clear, however, that multiple layers of pre-formed and inducible defenses such as that of PTI-mediated defense explain how it is that species and non host resistance are so effective and result in disease resistance.

**Effector-Triggered Immunity**

The counter-response to PTI by bacterial pathogens resulted in successful microbes acquiring genes encoding effector proteins and advanced effector delivery systems to increase pathogen virulence [13]. Pathogenic effector proteins function to act within the host cell cytosol to interfere with defense response mechanisms and prevent PTI-mediated resistance. The current view on the role of effector proteins in the plant-pathogen interaction is their role in subverting intracellular immune response signaling to attenuate PTI [25]. If a pathogen evolves a novel effector and gains substantial fitness, it applies a strong selective pressure against plant host populations to evolve commensurate resistance genes. Over time, co-evolution resulted in successful plant acquisition of intracellular resistance (R) proteins to recognize cytosolic effectors and initiate separate signaling events [28]. There are 150 identified R proteins in
A. thaliana [20]. Typically, R proteins consists of a variable amino terminus followed by a nucleotide-binding site (NBS) domain followed by a leucine rich repeat (LRR) domain at the carboxyl terminus [20]. These NBS-LRR resistance proteins initiate immune signaling events that result in stronger and more prolonged versions of the same generalized immune responses as those initiated by PTI. General immune responses are typically characterized by varying degrees of ion fluxes, production of reactive oxygen species (ROS), changes in certain hormone levels, transcriptional reprogramming, and in some cases HR accompanied by programmed cell death (PCD) [13]. Because both ETI and PTI result in similar immune responses, it suggests there may be some degree of convergent signaling between the two immunity triggers [11].

Effector-triggered immunity is, perhaps, the latest in a long history of counter-moves devised by successful plants to avoid pathogen attack. Interestingly, there is a high degree of specificity surrounding the plant R protein-effector interaction. It has been shown that ETI is only induced for certain complementary interactions between specific R proteins and correlating pathogen effectors. This specific interaction was first identified as the Gene-for Gene hypothesis in 1942. It proposed each plant R gene (coding for R proteins) corresponds to a specific effector-coding gene within the pathogens genome [10]. However, functional genomic studies have shown pathogen effectors to be a highly diverse class of proteins in sequence and molecular function across different types of pathogens [24, 16, 4, 23]. It is unclear as to whether all R proteins function in a shared manner, or if the mechanisms vary. Interestingly, studies exploring the interaction between A. thaliana R proteins and pathogen effectors demonstrated a resistance to separate effectors of two evolutionary divergent pathogens [21], indicating host perception is likely based not on direct interaction with effectors, but recognition of effector-mediated activity in the cytosol. This view is expressed in the current plant-pathogen interaction model known as the Guard Hypothesis [13]. The model proposes virulent pathogen effectors target certain components within the immune signaling pathways and act to manipulate the targets such that the alteration contributes to pathogen success in susceptible host genomes. Furthermore, effector activity with host
targets generates pathogen-induced modified self molecular patterns that appear to specifically correlate with host R proteins, and upon interaction elicit ETI signaling events [15]. In effect, the model proposes R proteins act to guard specific areas of immune response signaling pathways, and by detecting metabolites from effector-mediated reactions, are able to indirectly recognize effectors independent of sequence or structure. This view explains how an organism such as A. thaliana with a limited genome size can recognize a large number of pathogen effectors. One of the best studied plant-pathogen models resulting in ETI is that between Pst DC3000 carrying unrelated effector proteins AvrRpm1 and AvrRpt2. In this interaction, Pst DC3000 utilizes a type III secretion system (T3SS) to directly introduce the effector proteins into the host cytosol. The effector proteins have been shown to interact directly with RPS1-Interacting protein 4 (RIN4), a well-known regulator of plant immunity [17] to inhibit PTI. Specifically, AvrRpm1 acts to induce phosphorylation of RIN4 [18] which is thought to activate the corresponding R protein, RPM1 leading to ETI. The AvrRpt2 effector interacts as a cysteine protease [2] by cleaving RIN4 at two sites, The resulting reaction metabolites are then perceived by R protein RPS2 to initiate ETI. Interestingly, AvrRpt2 has been shown to cleave in vitro other A. thalian proteins containing its consensus cleavage site [6] which indicates a possible mechanism where pathogen virulence is increased by manipulation of several different host targets. Because this interact model is the currently the best understood, it frequently used in experiments examining ETI. The general ETI process used in this studied may be visualized in Figure I.1.

Experimental Approaches

Integral to plant innate immunity are the immune genes responsible for encoding proteins that favor host preservation. Often times these immune genes encode transcription factors that mediate downstream transcription of PR proteins directly involved in the physiological changes necessary for self-preservation. The signaling events resulting from pathogen perception or R proteins have been characterized in large part through genetic studies of mutants with altered levels of defense response. Identification of genetic elements such as the
Fig. I.1. **Effector Triggered Immunity (ETI)** Bacteria have evolved type III secretion systems (T3SS) to introduce avirulence proteins (*AvrRpm1* and *AvrRpt2*) into the intracellular environment across the cell wall (CW) and plasma membrane (PM). Plants have responded with co-evolving intracellular resistance (R) gene proteins with a nucleotide-binding head and leucine-rich repeating domain and a variable N-terminus (NB-LRR). R-protein recognition of type III effectors could be direct or indirect and possible effects of R-protein activation include programmed cell death and/or early transcription of defense genes such as WRKY46; the signal transduction pathways leading to these effects are unknown. (NM: nuclear membrane)
Arabidopsis Genes Governing Immune Gene Expression (Aggie) have aided in characterizing major components of the signaling pathways underlying immunity. The Aggie genes are activated by unknown mechanisms and function to direct proceeding transcriptional activities responsible for immune responses [28]. It has been shown that up to 25% of all *A. thaliana* genes undergo altered transcript levels in response to pathogen perception [18, 2]. By understanding the regulatory components responsible for such drastic cellular changes it becomes possible to gain better understanding of how the immune response process functions, and how it may cross-communicate with signaling networks of other processes. Important to the defense response process are the vast number of transcription factors that have been shown to bind defense related gene promoter elements and regulate transcription[8]. One major transcription factor family in *A. thaliana* is the WRKY family identified by the highly conserved W-box binding sites located upstream of certain immune genes. Transcription factors such as those of the WRKY family have been implicated in regulation of immune response and mediation of specific responses such as systemic-acquired resistance and localized R protein resistance [19]. Such transcription factors have even been shown to help regulate hormone levels leading to regulation of separate processes, especially that of growth and development [9].

The transcription factor WRKY46 has been shown to be an early marker gene in ETI that is strongly activated by AvrRpm1 and AvrRpt2 [11]. Additionally, it does not appear to be strongly activated in response to PTI or general abiotic stressors [11] making it an ideal marker gene for studying the level of ETI induction upon pathogen infection. The level of ETI induction can by quantitatively described through the use of reporter genes fused to the TF WRKY46 promoter. In this study, firefly luciferase (LUC) was fused to the WRKY46 promoter region, and used to quantify ETI induction by measuring resulting luminescence after treatment with luciferin substrate (Fig.I.2).
Fig. I.2. Luciferase enzyme gene fused to WRKY46 promoter.

Map-based cloning, also referred to as positional cloning, for gene identification is a method that uses known locations of different genetic markers such as single-nucleotide polymorphisms (SNPs) or insertion/deletion (InDel) mutations between two genetic backgrounds to localize mutant genes within one background [14]. Currently, the two best studied ecotypes of A. thaliana are the Columbia (Col-0) and Landsberg (Ler). The genomes of both accessions are fully sequenced [12] which greatly facilitates the positional cloning process by removing the need to first identify the nature and position of genetic markers prior to using them in localizing the target mutation. Beginning with an unknown mutant, map-based cloning allows for the eventual identification of the causal gene underlying the observed phenotype. When combined with mutagenesis and high-throughput screening techniques, it essentially allows the plant to show the observer which genes are responsible for physiological processes of interest.

To ensure success of a forward genetic screen, it is important to have a number of mutations in the model genome numerous enough to increase the probability of a mutation occurring within the region of the genome responsible for the process of interest. For this reason, chemical mutagenesis is preferred over more specific methods such as T-DNA insertions or Fast Neutron Mutagenesis for creating candidates for positional cloning. Unlike these more specific methods, chemical mutagenesis can produce promoter or mis-sense mutations that may result in hypermorphic or hypomorphic gene knock-down rather than amorphic gene knock-out [8]. This effect increases the probability that a mutation will occur in the region of interest within the genome. Because the putative mutation behind observed phenotypes is restricted to one ecotype background, it can be located by following recombination frequency
when crossed to a different ecotype. By designing PCR primers based on the background sequence of the ecotype in which the mutation is located and the alternative background sequence of the ecotype with which the mutant was crossed, it is possible to evaluate relative recombination frequency between genetic markers at strategic locations within the genome using gel electrophoresis. It is then possible to progressively refine the region of the genome containing the gene of interest to only a few hundred kilo-base-pairs (kbp), a size small enough for practical application of Next Generation Sequencing (NGS). In this study, EMS mutagenesis was carried out on A. thaliana Col-0, and mutant candidates were crossed to wild-type accession Ler. We used the known Col0/Ler genetic markers flanking a region containing the putative immune gene responsible for increased ETI induction. We report the identification of a new immune gene of the family of Arabidopsis genes governing immune gene expression (Aggie) 2015. We anticipate to use Aggie2015 in future functional genomic studies to better understand its role in regulation of immune gene transcription in A. thaliana.

Hypothesis

We hypothesized that by using a forward genetics approach, we would identify causal mutations within the A. thaliana genome responsible for altered ETI induction upon infection with Pst carrying AvrRpt2, and by using positional cloning methods, localize the gene to a region of approximately 100 to 400 kbp. Understanding responses such as these holds the promise of a future capability to bioengineer plant varieties with innate resistance to pathogens. The consequences of this could be monumental, especially in the face of dire global issues such as world hunger, increasing demand for renewable energy, and carbon sequestration.
CHAPTER II
MATERIALS AND METHODS

Plant Material, Growth Conditions and Previous Work

Arabidopsis thaliana mutant plants were germinated in 108 cm³ pots containing soil (Metro-Mix 366) and raised in a growth chamber environment at 23°C, 60% relative humidity, and 75 µE m²s⁻¹ light with a photoperiod of 12 hours. Arabidopsis thaliana accession Columbia (Col–0) background was used to construct transgenic pWRKY46::LUC wildtype (WT) F1 generation plants; a resistance gene to herbicide glufosinate was also cloned into the genome. EMS (ethyl methanesulfonate) mutagenesis was carried out on 123 equal sized samples of approximately 2,000 WT transgenic pWRKY46::LUC seeds by treatment with 2.5% EMS for a 12 hour period to form the M1 generation seeds.

The M1 generation seeds were germinated to give an M2 progeny of approximately 50,000 mutant plants. After the initial genetic screen, seeds of selected candidate’s in the M2 generation were harvested and immediately dried at 37°C for a minimum of 48 hours. Seeds used to form the M3 generation were subjected to cold treatment at 7°C in 0.1% (w/v) agarose solution (Difco) for a minimum of 48 hours before direct germination. Candidates identified by the double confirmation screen were isolated and crossed to WT A. thaliana accession Ler to form the F2 generation. F2 seeds were germinated after cold treatment and backcrossed to WT parental pWRKY46::LUC. The F2 progeny were used to harvest tissue samples for DNA extraction and gel electrophoresis.

DNA extraction was performed by homogenizing one medium sized leaf (3-5 mg) in 500µL of CTAB buffer (2.0 g CTAB, 1 M Tris, pH 8.0, 0.5 M EDTA, pH 8.0, 5 M NaCl, to 100 mL using dH₂O and adjusted to pH 5.0 using 1 N HCl) followed by centrifugation at 12,000 RPM at room temperature for 5 minutes. To the decanted supernatant was added 250 µL of chloroform mixed by inversion followed by centrifugation at 13,000 RPM at room temperature for 1 minute. The aqueous phase was decanted to which 500 µL of cold
isopropanol was added followed by centrifugation at 13,000 RPM at 4°C for 45 minutes. The supernatant was discarded and the DNA pellet saved.

PCR amplification was carried out in 20 µL reactions consisting of 0.2 µM of each primer (Life Technologies), 1.5-3.0 mM MgCl₂, 0.5 units of Taq polymerase (Invitrogen), 0.2 mM of each dNTP (Promega) and 5 µL of DNA. Primer nucleotide sequences are available at ftp://ftp.arabidopsis.org/home/tair/Sequences/. PCR amplification was initiated at 94°C for 5 min, followed by 25 cycles with denaturation at 94°C for 30 s, and annealing at 50°C for 30 s followed by elongation time at 72°C for 10 min.

**Genetic Screen**

Transgenic *Pseudomonas syringae* pv Tomato DC3000 carrying effector protein AvrRpt2 was cultured using KB medium in the presence of 50 µg mL⁻¹ rifamycin and kanamycin and grown overnight at 28°C. To prepare Pst DC3000 for tissue inoculation, bacteria were pelleted by centrifugation at 6000 RPM (Beckman Microfuge 16 FX241.5P rotor) for 2 minutes followed by resuspension in 10 mM MgCl₂ to a final volume of 1 mL. This was repeated 2 - 3 times. The final resuspension concentration was diluted until an OD₆₀₀=0.01 was reached.

The high-throughput genetic screen was carried out on visibly healthy leaves of 4 week old plants. One leaf from each candidate was selected and hand inoculated using a needless syringe to introduce Pst DC3000 into the apoplast. Inoculated mutants were then incubated for 6 hours at normal growth chamber conditions. Following incubation, leaf tissue samples were removed and placed in a 36-well MicroPlate (Wallac) and treated with a luciferin substrate solution consisting of 40 µL of 0.1M Luciferin substrate (Sigma), 40µL Silwet L-77 (Lehel) to 20 mL dH₂O. The substrate solution was stored on ice away from light and applied by spray application to all areas of the MicroPlate. Treated tissues were incubated away from light for 10-15 minutes at room temperature before being analyzed using the GloMax Multi-Detection System (Promega) programmed to take 10 readings per well for a period of 1 minute and integration length of 0.5 sec.
Phenotypic Analysis and Disease Assay

Phenotypic analysis was conducted using WT pWRKY46::LUC, M3 generation mutant candidates, and corresponding F2 generation mutant candidates. To determine dominance or recessiveness of mutant phenotypes, each were inoculated with Pst DC3000 carrying Avr-Rpt2 and analyzed according to the same genetic screening protocol. Disease assays for F2 generation mutant candidates was performed by inoculating groups of 6 mutants with Pst carrying AvrRpt2, AvrRpm1 and AvrRps4. Leaf tissues were removed three days after inoculation to be ground in using mortar and pestle and suspended to an appropriate volume in dH2O. The suspension was cultured on KB plates in the presence of appropriate antibiotics and incubated overnight at 28°C. Bacterial colony forming units (cfu) were counted 0, 2, and 4 days post incubation.

Map-Based Cloning

The F2 populations for mapping the Aggie2015 mutation were derived from crossing mutants in the Col–0 background to wild-type plants in the Ler background. DNA from 24 Aggie2015xLer mutants was extracted and pooled into two samples that were amplified using PCR and visualized using a 4% agarose gel in 1X TAE buffer with ethidium bromide. Bulk segregation analysis was performed on the pools with InDel markers at 200 kbp, 4600 kbp, and 10600 kbp. DNA extraction was repeated to perform fine mapping using two InDel markers at 419 kbp and 4600 kbp.
CHAPTER III
RESULTS

Genetic Screen

The initial screen encompassed approximately 50,000 M2 generation plants. Less than 0.5% of the total population (243 candidates) exhibited increased ETI induction (high mutants), and less than 1.3% of the total population (675 candidates) exhibited reduced ETI induction (low mutants) (Fig. III.1). Double confirmation screening of the progeny from self-pollinated M2 generation candidates further refined the abnormal ETI induction phenotype populations to 15 high mutants and 20 low mutants (Fig. III.2). Additionally, the double confirmation screen revealed several mutant lines exhibiting altered ETI induction in addition to morphological abnormalities conserved from the previous generation. The high mutant identified by DCHM3 exhibited one of the most consistent phenotypes in the double confirmation screen with all six tissue samples exhibiting uniformly high luminescence readings. DCHM3 did not exhibit the highest luminescence value in the double confirmed population. Figure III.3 shows Aggie2015 ETI induction relative to control values.
Fig. III.1. Double Confirmation High Mutant Values vs. WRKY46 Controls

Fig. III.2. Double Confirmation Low Mutant Values vs. WRKY46 Controls
Fig. III.3. ETI Induction of *Aggie2015* vs WRKY46 Control

**Mutation Characterization**

The *Aggie2015* mutation was determined to be recessive by genetic screening data compared to WT *pWRKY46::LUC*. Figure III.4 shows the luciferase activity of *Aggie2015xLer* cross as approximately less than 50% of the self-pollinated, homozygous *Aggie2015* mutant compared to WT *pWRKY46::LUC* control. Disease assays (Fig. III.5) measured bacterial growth within separate leaf tissue samples 3 days after inoculation with *Pst* carrying effectors *AvrRpm1*, *AvrRps4* and *AvrRpt2* compared to WT controls; each leaf tissue sample was inoculated with *Pst* carrying only one of the three different effectors. In all three cases, pathogenic bacterial growth was attenuated in the *Aggie2015* mutant tissues.
Fig. III.4. **Phenotype Analysis**: the *Aggie2015* exhibits recessive behavior when crossed to wildtype *Ler*.

Fig. III.5. **Disease Assay**: the *Aggie2015* gene is involved with ETI induction initiated by three unrelated effector proteins, indicating it may be associated with general immune signaling networks.
Map-Based Cloning

The Aggie2015 mutant was crossed to background Ler and then backcrossed to WT parental pWRKY46::LUC to form the F2 population. Rough map-based cloning performed using 24 Aggie2015xLer high mutants (Fig. III.6). Using the relative intensities of bands in electrophoretic gels to determine recombination, the Aggie2015 mutation was mapped to chromosome III in the region flanked by markers at 10Mb and 0.2 Mb. Fine mapping further refined the region containing the causative Aggie2015 mutation between markers at 200 kbp and 4600 kbp (Fig. III.7). The third and fourth populations of F2 Aggie2015xLer mutants localized the mutation between markers at 419 kbp and 1354 kbp, then finally between markers at 943 kbp to 1354 kbp. This results in a 411 kbp region on chromosome III that likely contains the putative Aggie2015 causal mutation. These findings may be visualized in the map-based cloning diagram (Fig. III.8).
Fig. III.6. **Rough Mapping Electrophoretic Gels**: the *Aggie2015* gene was initially localized to chromosome III of the *A. thaliana* genome between markers at 10 Mb and 0.2 Mb.

![Rough Mapping Electrophoretic Gels](image)

Fig. III.7. **Fine Mapping Electrophoretic Gels**: the *Aggie2015* gene lies between the markers at 200 kbp and 4600 kbp.

![Fine Mapping Electrophoretic Gels](image)
Fig. III.8. **Mapping Diagram of chromosome III**: each iteration illustrates a progressively narrowed region of chromosome III carrying the putative *Aggie2015* mutation.
CHAPTER IV
DISCUSSION

Genetic Screen

Initial Screen

This high-throughput genetic screen was based on the known plant-pathogen interaction model of A. thaliana infected with Pst carrying AvrRpt2. The induction of known immune gene transcription factor TF WRKY46 was quantitatively determined by measuring the relative luminescence values of firefly luciferase reporter fused to the WRKY46 promoter region (Fig. I.2). The initial screening illustrates the necessity for such large M2 generation populations: the resulting mutant candidate pools were, in both cases, only a fraction of the initial M2 generation population. This result is expected when chemical mutagenesis methods are used. With larger populations, the number of mutations increases with respect to the constant size of the genome resulting in an increased probability of a mutation occurring within the region of interest. Additionally, during the M2 generation growth period, several plants exhibiting morphological abnormalities were observed. Undoubtedly these are a result of the random and widespread mutagenic effects of EMS treatment.

Double Confirmation Screen

The double confirmation screen permitted us to identify the most promising candidates from the initial screen mutant pools. We evaluated candidates in the double confirmation by groups of 6 plants, each of which was offspring from the same M2 generation mutant line. Extra consideration was given to uniformity of ETI induction across all the plants. The double confirmation screen assumes that each of the six plants from the same self-pollinated M2 candidate should exhibit similar, if not totally uniform, phenotype. From the results, it is clear that the probability of finding mutants with valuable phenotypes is rare. Only
35 out of 50,000 mutants initially screened exhibited a sustained and uniform high mutant phenotype with acceptable ETI induction compared to controls.

The double confirmation screen also revealed several mutants with sustained morphological abnormalities accompanied by abnormal immune response phenotypes. Previous studies have shown signaling mechanisms involved in immune response can overlap with signaling mechanisms underlying plant growth and development [27]. While it is not conclusive, these mutants do have some added value in that they may possess mutations of genes implicated in both immunity and development by modulating crosstalk activity between the respective signaling pathways. It is possible these genes may possess dual functionality and may provide important information regarding how plants differentiate and react to biotic and abiotic stressors, however this event is unlikely. We do not address this event here, but the data and mutant lines will saved for future study.

*Experimental Considerations*

There are a number of variables that were not controlled in the genetic screening process. Leaf tissue size analyzed in the genetic screen was not controlled. Previous work (unpublished data) indicated tissue size to not substantially impact luminescence values relative to control values where incubation, bacterial concentration, and tissue damage were not factors. The use of WRKY46 was a strategic decision due to its properties of reduced induction from abiotic stressors such as drought, intense or prolonged light exposure, and tissue damage caused by syringe inoculation. Other biotic stressors were also avoided, but may have contributed to unrecognized experimental error in the data. Due to its role in general immune response signaling, WRKY46 transcription levels may have been influenced by outside biotrophic pathogens commonly found in growth room environments.

*Mutation Characterization*

Our second genetic screen showed the *Aggie2015* mutation to cause an approximate threefold increase in ETI induction relative to the controls (Fig. III.3). The double confirmation
results show that *Aggie2015* was not the highest mutant, but was consistent over the 12 readings taken to give an average luminescence value of 20,195 RLU whereas the control average was 981 RLU. We also performed a genetic screen of the *Aggie2015, Aggie2015xLer*, and *pWRKY46::LUC* control lines to determine whether the mutation was dominant or recessive. The *Aggie2015* line is self-pollinating and should be homozygous for the causal mutant gene resulting in the highest ETI induction. The *Aggie2015xLer* cross should only differ from the self-pollinating line if the mutation is recessive. The data shows a reduced ETI induction in the *Aggie2015 x Ler* indicating the hypermorphic mutation to be recessive. With only one allele from the mutant parent, the mutant gene expression, and subsequent effect on ETI induction, is reduced. To better characterize *Aggie2015*, we performed three disease assays using the same infection system of *Pst* carrying a single effector. We measured bacterial growth within leaf tissues of 4 week old plants three days after inoculation with *Pst* carrying one of the three effectors. In Figure III.5, the vertical axis of each graph indicates the number of surviving bacteria within the leaf. This measure was made to evaluate the effect of host immune response on pathogen success given three different pathogenic threats in the form of three different and unrelated effector proteins. The data illustrates pathogen growth was attenuated independent of which effector was used indicating *Aggie2015* is likely involved in components of general ETI signaling, and is not confined to specific interaction with one type of host R protein or direct interaction with any one type of pathogen effector.

**Map-Based Cloning**

Our map-based cloning procedure used a total of four sets of F2 generation *Aggie2015xLer* cross mutants. Rough mapping used to identify the chromosome upon which the gene is located was performed using two pools of DNA extracted from a total of mutant crosses with primers designed for three pairs of genetic markers from each of the five chromosomes. We determined the general location to be on the third chromosome between markers at 10 Mb and 0.2 Mb. We compared the relative intensities of the bands as a measure of genetic recombination between the Col–0 background containing the *Aggie2015* mutant and the
wild-type *Ler* background. Bands of higher intensity reflect greater recombination, while bands of lower intensity reflect regions of lesser recombination. By applying the principal of genetic linkage, we know that genes with loci of closer mutual proximity are less likely to be separated during chromosomal crossover, thus by comparing the recombination frequencies, we identified progressively smaller regions of the chromosome containing the *Aggie2015* mutation. The rough mapping screen (Fig. III.6) shows three lanes: Lane 1, shows the recombination of the molecular marker at the highest position in the region; Lane 2, shows the recombination of molecular markers at the lowest position in the region evaluated; Lane 3, shows the recombination of *Aggie2015 x Ler* F1 generation DNA to serve as a control. The rough mapping data presents recombination data for three regions of chromosome III. The recombination observed within each region indicated the mutation was located downstream of 0.2 Mb. Furthermore, the results of the two other marker pairs show reduced recombination indicating the mutation is located in the general regions covered by the marker pairs. Using fine mapping data (Fig. III.7) of 29 samples taken from two pools of amplified mutant DNA with markers at 200 kbp and 4600 kbp we determined in which direction the mutations lies relative to the markers used in rough mapping. Lanes 7, 8, 9, 10, 14, and 26 show areas of heterozygous DNA and Col–0 marker which contrasts to the remaining majority of lanes in which the prominent bands belong to Col–0 markers only. The differences in the specified lanes indicates the mutation is localized within the region flanked by markers at 200 kbp and 4600 kbp. Using the same evaluation process, the third and fourth set of F2 generation mutant DNA revealed the *Aggie2015* mutation to be localized between markers at 1354 kbp and 943 kbp resulting in a region of 411 kbp. This region is small enough to be sequenced using Next Generation Sequencing technologies and analyzed by comparing the sequenced regions to published (www.arabidopsis.org) background sequences of the Col–0 and *Ler* accessions using computational techniques.
CHAPTER V
CONCLUSION

Our initial hypothesis was that by using a high throughput genetic screening method to analyze the ETI induction to a known plant-pathogen interaction model, we could identify mutant candidates containing genetic alterations of genes involved in plant ETI. Then, from these candidates we could identify 2 to 3 promising candidates with either high or low ETI induction, and by using a forward genetics approach, we could identify the putative causal mutation. Our results indicate that we have successfully identified a high ETI induction mutant, *Aggie2015*, and we have successfully used a forward genetics approach by way of map-based cloning procedures to firstly, identify the chromosome on which the mutation lies, and secondly, refine the location of the mutation to a region of approximately 300 kbp to 400 kbp. Using this data, we now are able to continue genetic characterization by using Next Generation Sequencing technologies to determine the exact sequence of the 411 kbp region. With this sequence we will be able to utilize the known genetic backgrounds of the Col–0 and Ler accessions to identify Col–0/Ler background polymorphisms to serve as genetic markers to pinpoint a highly refined region that will likely constitute the sequence of the *Aggie2015* mutation. With the known sequence, we can use gene properties such as gene length, position in the genome, and resulting amino acid sequence to characterize the protein products of the *Aggie2015* gene. The ultimate goal is to use this data to fit the *Aggie2015* gene and its role in plant innate immunity with that of already known immune genes and signaling pathways. We believe this will serve as a useful contribution to the understanding of plant innate immunity and how it might be deployed to more effectively control diseases. Additionally, elucidation of this ancient immune system will provide a better understanding of plant animal immune system evolution, which may contribute to new understandings of the mechanisms underlying our own immune systems.
REFERENCES


