Identification, Validation, and Mapping of *Phytophthora sojae* and *Soybean Mosaic Virus* Resistance Genes in Soybean

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Abstract (Academic)

Estimated at approximately $43 billion annually, the cultivated soybean *Glycine max* (L.) Merr., is the second most valuable crop in the United States. Soybeans account for 57% of the world oil-seed production and are utilized as a protein source in products such as animal feed. The value of a soybean crop, measured in seed quality and quantity, is negatively affected by biotic and abiotic stresses. This research is focused on resistance to biotic disease stress in soybean. In particular, we are working on the *Phytophthora soja* (*P. sojae*) and *Soybean Mosaic Virus* (SMV) systems. For each of these diseases, we are working to develop superior soybean germplasm that is resistant to the devastating economic impacts of pathogens. The majority of this research is focused on screening for novel sources of *P. sojae* resistance with core effectors to identify resistance genes (*R*-genes) that will be durable under field conditions. Four segregating populations and two recombinant inbred line (RIL) populations have been screened with core effectors. Effector-based screening methods were combined with pathogen-based phenotyping in the form of a mycelium-based trifoliate screening assay. One RIL population has been screened with virulent *P. sojae* mycelium. Disease phenotyping has generated a preliminary genetic map for resistance in soybean accession PI408132. The identification of novel *R*-genes will allow for stacking of resistance loci into elite *G. max* cultivars. The second project covered in this dissertation describes the validation of the SMV resistance gene *Rsv3*. Utilizing a

combination of transient expression and homology modeling; we provide evidence that Glyma14g38533 encodes *Rsv3*.

Abstract (General Audience)

Estimated at approximately $43 billion annually, the cultivated soybean *Glycine max* (L.) Merr., is the second most valuable crop in the United States. Soybeans account for 57% of the world oil-seed production and are utilized as a protein source in products such as animal feed. The value of a soybean crop, measured in seed quality and quantity, is negatively affected by pathogens and other stressors. This research is focused on resistance to pathogen disease stress in soybean. In particular, we are working on the *Phytophthora soja* (*P. sojae*) and *Soybean Mosaic Virus* (SMV) systems. For each of these diseases, we are working to develop superior soybean lines that are resistant to the devastating economic impacts of these pathogens. The majority of this research is focused on screening for new sources of *P. sojae* resistance, using certain pathogen virulence proteins called core effectors, to identify resistance genes (*R*-genes) that will be durable under field conditions. Four segregating populations and two recombinant inbred line (RIL) populations have been screened with core effectors. Effector-based screening methods were combined with pathogen-based phenotyping in the form of an assay that involved the use of *P. sojae* to infect detached soybean leaves. One RIL population has been screened with virulent *P. sojae*. Disease screening has generated a preliminary genetic map for resistance in soybean accession PI408132. The identification of novel *R*-genes will allow for stacking of resistance genes into elite *G. max* cultivars that can be grown by farmers. The second project covered in this dissertation describes the validation of the SMV resistance gene *Rsv3*. Utilizing a combination of a molecular assay and protein prediction software; we provide evidence that the soybean gene Glyma14g38533 encodes *Rsv3*.

Dedicated to the memory of my grandfather,

Virgil Wayne Russell

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Table of Contents

List of Figures

List of Tables

List of Abbreviations

SMV: Soybean mosaic virus

HR: Hypersensitive response

Rsv3: SMV resistance gene against strains G5-G7

QTL: Quantitative trait loci

SCN: Soybean cyst nematode

PRR: Pattern recognition receptor

PAMP: Pathogen-associated molecular pattern

PTI: Pathogen-associated molecular pattern (PAMP) triggered immunity

ETS: Effector triggered susceptibility

ETI: Effector triggered immunity

ROS: Reactive oxygen species

NBD-LRR/NLR: Nucleotide-binding domain leucine-rich repeat

Avr: Avirulence, usually is reference to a gene

Rps: Resistance to *Phytophthora sojae*, describes a gene

RIL: Recombinant inbred line

SSR: Simple sequence repeat

SNP: Single nucleotide polymorphism

T3SS: Type-3 secretion system

Psg: Pseudomonas syringae pathovar *glycinea*

CI: Cytosolic inclusion

L29: SMV G7 resistant line containing *Rsv3*

W82: Williams 82, SMV G7 susceptible line

DEX: Dexamethasone, chemical to induce promoter

pEDV6: The effector detector vector

RxLR-DEER: Conserved amino acid sequence in oomycete effectors

Gm326: *G. max* population derived from cross of PI408132 and Williams

Gm320: *G. max* population derived from cross of PI408097 and Williams

Gs2292: *G. soja* population derived from cross of PI407076 and Williams

Gm32: *G. max* population derived from cross of PI567139B and Williams

Attribution

All manuscripts in this dissertation have multiple authors, which includes all chapters with the exception of Chapter 6. The contributions of all co-authors are detailed below.

- **Dr. M. A. Saghai Maroof:** Principal investigator, Department of Crop and Soil Environmental Science at Virginia Tech. Participated in the experimental design, coordination, and revision of all manuscripts in this dissertation.
- **Dr. John M. McDowell:** Principal investigator, Department of Plant Pathology, Physiology, and Weed Science at Virginia Tech. Participated in the experimental design, coordination, and revision of all manuscripts in this dissertation.
- **Dr. Michael G. Fedkenheuer:** Postdoctoral Researcher, Department of Plant Pathology, Physiology, and Weed Science at Virginia Tech. Participated in the experimental design and implementation of experiments described in *Phytophthora* manuscripts.
- **Dr. Kevin E. Fedkenheuer:** Postdoctoral Researcher, Department of Plant Pathology, Physiology, and Weed Science at Virginia Tech. Participated in the experimental design and implementation of experiments described in *Phytophthora* manuscripts.
- **Dr. Neelam R. Redekar:** Postdoctoral Researcher, Department of Crop and Soil Environmental Science at Virginia Tech. Participated in the experimental design/implementation, and review of *Rsv3* manuscripts. She also participated in experimental implementation of Chapter 2 *Phytophthora* manuscript.
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- **Dr. Ruslan M. Biyashev:** Research Manager, Department of Crop and Soil Environmental Science at Virginia Tech. Participated in the experimental design/implementation for all experiment-based manuscripts.
- **Dr. Sue A. Tolin:** Professor Emerita, Department of Plant Pathology, Physiology, and Weed Science at Virginia Tech. Participated in the experimental design of the *Rsv3* manuscript.
- **Dr. Brett M. Tyler:** Principal Investigator, Department of Botany and Plant Pathology, Oregon State University. Participated in experimental design, coordination, and review of *Phytophthora* manuscripts.
- **Nick A. Dietz:** Undergraduate Researcher, Department of Plant Pathology, Physiology, and Weed Science at Virginia Tech. Participated in the experimental implementation of *Phytophthora* manuscripts.

Chapter 1: Molecular Function of Genes Underlying Host Resistance against Root and Seedling Diseases of Soybean

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Abstract

Root and seedling diseases can be devastating to commercial soybean production. To better defend against the pathogens that cause these diseases, we must understand the complex molecular mechanisms that underlie host resistance. To become a successful pathogen, an organism must overcome the multi-layered molecular defense network potential host plants use to defend themselves. Evidence points to an evolutionary molecular arms race between plant pathogens and their hosts in which pathogens suppress or evade host immunity while host plants evolve more sophisticated surveillance systems. Host-adapted pathogens utilize effector proteins that are secreted to the outside and inside of plant cells to disrupt the innate defenses of plant species. However, plants utilize nucleotide-binding domain containing leucine-rich repeat intracellular proteins to detect pathogen effectors and initiate an immune response. This molecular model explains many instances of gene-for-gene resistance. In this article we summarize what is currently known about the molecular mechanisms of host resistance provided by gene-for-gene resistance and quantitative trait loci against root and seedling diseases of soybean. We discuss the current state of soybean genetic resistance against three significant pathogens and strategies for increased protection against these pathogens in the future. We hope that an understanding of the interactions between plant pathogens and their hosts will allow breeders to more readily develop disease resistance cultivars.

Introduction

The cultivated soybean *Glycine max* (L.) Merr., is the second most valuable crop in the United States, estimated at approximately $43.2 billion, annually. Soybeans account for 57% of the world oil-seed production and are utilized as a protein source in products such as animal feed [1]. The value of a soybean crop, measured in seed quality and quantity, is negatively affected by biotic and abiotic stress. Plants must constantly defend themselves against numerous biotic stressors in the form of microbes and pests including bacteria, fungi, oomycetes, nematodes, insects, and viruses. A multi-layered molecular defense network prevents the majority of microbes and pests from becoming pathogenic by recognizing invaders and quickly responding to prevent their spread. A basal level of immunity protects plants from the majority of would-be pathogens, but this defense can be subverted by secreted pathogen virulence proteins known as effectors. Immune surveillance genes, known as disease *Resistance* (*R*) genes, encode proteins that are able to recognize pathogen effectors and activate defense responses. In addition to *R*-genes, host resistance can be encoded by quantitative trait loci (QTLs) in which multiple genes together, provide various degrees of partial resistance to plant pathogens. Root and seedling pathogens, including soybean cyst nematode and oomycete pathogens, such as *Phytophthora sojae* and species within the *Pythium* genus, are among the most devastating causes of disease in soybean. In this review, we highlight the current molecular concepts underlying resistance strategies against three soybean pathogens with diverse lifestyles. We summarize the current state of soybean disease resistance against three diverse root and seedling pathogens, and describe strategies to improve upon this resistance. We highlight the importance of a pathogen's lifestyle and the molecular mechanisms underlying host resistance when working to develop superior soybean germplasm.

Phytophthora sojae and Pythium

Oomycetes, which are often referred to as "water molds", share common features with fungi such as filamentous growth, but are not closely related. Taxonomically, *Phytophthora* and *Pythium* are genera within the same family of oomycetes [2]. *Phytophthora* and *Pythium* share similar life cycles. Dormant oospores overwinter in dry plant tissue until conditions promote the oospores to form sporangia. Sporangia then release swimming zoospores that are attracted to the roots of soybean plants. The zoospores encyst and colonize the root cortex of the plant using hyphae that grow within the intra-cellular space and form haustoria, which are feeding structures that sequester nutrients from the host. Oomycetes eventually forming more sporangia for secondary infection or dormant oospores for a long-term strategy [3]. However, *Phytophthora* and *Pythium* use different nutrient acquisition strategies in Soybean. *Phytophthora sojae* (*P. sojae*), which is the only *Phytophthora* species that infects soybean, is a hemibiotroph, but the many species of *Pythium* that can infect Soybean are necrotrophs [4]. Biotrophic pathogens require a living host to complete their lifecycle while necrotrophic pathogens feed by destroying host cells [5]. Hemibiotrophs complete part of their life cycle as biotrophs in conjunction with their host and eventually switching to necrotrophy, while necrotrophs have a very limited or non-existent biotrophic phase [6]. *P. sojae*, which can result in up to 100% yield loss, has a host range that is limited to the *Glycine* species. *Pythium* species tend to have wide host ranges and soybean yield losses as a result are not well documented. Both *Phytophthora sojae* and *Pythium* species have potential to cause seedling damping-off in poorly drained fields, and must be closely managed to mitigate the associated damage.

Soybean Cyst Nematode

Heterodera glycines, also known as the Soybean Cyst Nematode (SCN) is the most detrimental soybean disease in the United States causing an estimated $1.2 billion of crop damage annually [7]. First reported in Japan over a century ago, SCN has spread worldwide and is present virtually everywhere Soybean is grown [8]. SCN is managed through a combination of non-host crop rotation strategies, nematicides, and genetic resistance [9]. Commercial Soybean resistance to SCN is primarily derived from two only Soybean lines, Peking and PI88788 [10]. The lack of genetic diversity has allowed for an increase in SCN populations that can reproduce on "resistant" cultivars [11, 12]. SCN is an obligate, sedentary, parasite. The SCN lifecycle consists of six stages including egg, 4 juvenile stages (J1-J4) and the adult stage. After hatching in the soil, stage-two juveniles (J2) are attracted to soybean root exudates. After reaching a root, the SCN J2s penetrate the outer cell layers using their stylet and secreted digestive enzymes [9, 12]. The parasite will then select a root cell near the vascular system to form its feeding structure known as a syncytium, which grows as the parasite disrupts the cell walls near the syncytium thus increasing the availability of nutrients for the pathogen. Examinations of SCN resistant soybean lines have shown no change in the ability of J2 nematodes to penetrate the roots, but the development of mature female parasites is disrupted [13]. SCN females will remain here for the remainder of their lifecycle, while SCN males will remain sedentary until they are ready for reproduction. After reproduction, adult female nematodes form protective cysts around their eggs that can survive in the soil for nearly a decade [14]. The entire SCN lifecycle can take place in 3-4 weeks and therefore multiple generations can develop within a single soybean growing season [15] In addition to soybean, the host range for SCN includes other legumes, and therefore Soybean should be rotated with non-host crops such as corn.

Layers of Plant Defense

For successful parasitism, potential pathogens must overcome a multi-layered molecular defense network that protects host plants. Pre-formed barriers, such as the waxy leaf surface and hairs, make up the first layer of host defense against a potential pathogen or pest [16]. In the second layer, basal resistance can be conferred against a wide variety of pathogens via pattern-recognition receptors (PRRs). PRRs recognize conserved pathogen motifs, also known as pathogen-associated molecular patterns (PAMPs), and elicit a general defense response which includes callose deposition at the cell walls, an increase in extracellular pH, and an increase in reactive oxygen species (ROS) which together provide PAMP-triggered immunity (PTI) [17, 18]. PTI provides resistance against potential pathogens that are not specifically adapted to the host [19]. PAMPs are conserved, essential, components that are conserved within an entire class of microbes [19]. Notable PAMPs include bacterial flagellin, elongation factor Tu, and fungal chitin. For example, the *Arabidopsis* PRR *FLS2,* which is a leucine-rich receptor kinase, recognizes a specific 22 amino acid flagellin peptide known as *flg22* and activates immune responses that substantially impede bacterial colonization[20, 21].

A potential pathogen must either suppress or evade host PTI to become successful [18]. Evasion of PTI can be achieved if the pathogen removes or alters the corresponding PAMPs. However, the conserved, critical, pathogen components that PRRs often target can be difficult to alter without a loss of virulence [22]. This feature of PRRs may drive more pathogens towards suppression as a strategy for overcoming PTI. Suppression primarily occurs when the plant pathogen secretes virulence proteins, known as effectors, to disrupt signaling downstream from the PRR and prevent immune response activation [19, 23]. Effectors from host-adapted

pathogens can result in effector-triggered susceptibility (ETS). Oomycete effectors are delivered into host cytosol via specialized structures called haustoria [19]. Suppression of PTI results in vulnerable host plants despite the presence of PRRs.

To combat the suppression of PTI by pathogen effectors, plants have evolved *R*-genes that mediate a third layer of host defense known as effector-triggered immunity or ETI [17]. Unlike PTI, ETI provides resistance against pathogens specifically adapted to the host [24]. Most *R*-genes encode for nucleotide-binding domain containing leucine-rich repeat (NBD-LRR or NLR) surveillance proteins that recognize pathogen effectors and activate ETI [24, 25]. Based on Flor's gene-for-gene model of disease resistance, an *R*-gene can only activate ETI in the presence of an effector that is specifically recognized by that *R*-gene [26]. Effectors recognized by *R*-genes are referred to as avirulence (*avr*) genes and their recognition leads to a robust host defense response [22, 27]. This defense response is often characterized by localized cell death also known as the hypersensitive response (HR) which is designed to prevent pathogen spread from the area of infection [19]. NLR gene activation is associated with a significant fitness penalty in the absence of a recognized pathogen as resources are unnecessarily allocated to defense instead of growth. The HR response is an extreme measure by which the host plant sacrifices some cells to protect the greater host and therefore it must be tightly regulated. Host plants must balance the strength and sensitivity of their defense responses in an environment where specific pathogens may not always be present [25]. However, effectors are often redundant and are structurally variable making many of them dispensable. The selection pressure generated by *R*-genes on pathogens leads to an "evolutionary arms race" as the host develops more sophisticated surveillance networks and the pathogen seeks to avoid or undermine the

current host surveillance system [23]. Structurally similar NLRs have been found in both plant and animal species, but are hypothesized to have developed independently through convergent evolution, reinforcing the utility of NLRs as a surveillance mechanism against host-adapted pathogens [24, 28]. An overview of PTI, ETS, and ETI is provided in Figure 1.1.

NLR Structure

NLR genes are divided into two subgroups based on their amino-terminal motif. The Toll Interleukin 1 receptor-like NBD-LRRs comprise the first subgroup and the most common in plants. Non-TIR-NBD-LRRs, which are primarily Coiled-Coil NBD-LRRs, make up the second subgroup of *R*-proteins [18, 29]. NLRs act as molecular switches that remain auto-inhibited until they are "turned on" via a conformational change resulting from protein-protein interactions [24, 30]. The recognition of a pathogen effector by an NLR allows for bound ADP in the nucleotide binding domain to be replaced with ATP which converts the switch from "off" to "on". The protein can then be inactivated via hydrolysis of ATP to ADP [28]. NLR proteins are auto-inhibited by the LRR domain which negatively regulates the NB domain and prevents activation. The LRR protein domain, which tends to form a horseshoe-shaped structure, is highly sequence variable and extremely flexible due to limited secondary structure [31]. Dimerization of NLR proteins is also believed to play an important role in the initiation of downstream defense signaling. Proteins encoded by NLR genes have been shown to cooperate in the recognition of pathogen effectors by forming heterodimers. In *Arabidopsis,* the RPS4/RRS1 protein pair provides disease resistance against multiple fungal and bacterial pathogens. RPS4 and RRS1 are linked within the *Arabidopsis* genome and would therefore likely be inherited together. It is

hypothesized that the use of NLR protein pairs would increase effector specificity in addition to reducing autoimmunity [25].

Effector Recognition

NLR-mediated recognition of a pathogen effector can either be direct or indirect. Direct interaction is specific binding of an NLR protein and a pathogen effector. For example, the rice NLR protein Pi-ta binds to the *Magnaporthe grisea AvrPita* and shows high levels of diversifying selection likely due to the antagonistic evolutionary relationship between the NLR and effector [19, 30]. However, direct interaction between NLR protein and effectors can be disrupted through effector mutations and conformational changes that prevent interaction with a cognate NLR protein. Indirect recognition is more common and is summarized using the guard model and the decoy model of effector recognition. According to the guard model, NLR proteins monitor defense signaling host proteins for modification by pathogen effectors. Effectors are targeted to components of the plant immune system and therefore NLRs "guard" these components [32]. In this way, the presence of a relatively small number of NLR genes can protect against a diverse repertoire of effectors [27]. *Arabidopsis* enhanced disease susceptibility 1 (EDS1) and RPM1 interacting protein 4 (RIN4) are examples of effector targets in plants that are guarded by NLR proteins. EDS1 is hypothesized to link NLR proteins to downstream responses while RIN4 is a negative regulator of PTI and ETI that is degraded in the presence of a pathogen [33].

The decoy model of effector recognition is similar to the guard model in that an effector target is monitored for evidence of manipulation. However, the decoy model uses a mimic of host proteins targeted by effectors that do not have a role in plant immunity. In *Arabidopsis*, a

homolog of the receptor-like kinase BIK1 called PBL2 has been shown to serve as a decoy to detect the *Xanthomonas campestris* pv. *campestris* (*Xcc*) effector protein AvrAC [34]. An NLR protein called ZAR1, which is also known to recognize the *Pseudomonas syringae* effector Hop1A, acts in tandem with the pseudo kinase RKS1 to recognize AvrAC and produce a defense response [34]. The use of a decoy is thought to allow for co-evolution with intermediaries and NLR genes to increase the specificity and expand the flexibility of the plant immune system.

Binding of the NLR protein to the guardee/decoy has been shown to occur through two mechanisms. The guardee/decoy can either be constitutively bound to the NLR protein or it can associate only after binding a pathogen effector [29]. However, indirect recognition that is based on constitutive association of the decoy/guardee to the NLR may be susceptible to autoimmunity as a result of allelic variation between two unlinked genes. This issue, which is known as hybrid necrosis, points to a potential difficulty for breeders attempting to develop resistant populations. If an *R*-gene that is based on a constitutive association with a decoy or guardee is introgressed into a population that lacks the respective decoy/guardee, necrosis would result as the *R*-gene is constitutively activated.

To avoid the issue of hybrid necrosis, decoys and their corresponding NLRs are sometimes encoded by the same gene or linked genes to ensure that both components are inherited together [32, 35]. Also known as the integrated decoy model, this mechanism of linked NLR-decoy genes is the next step in the evolutionary arms race between the host and host-adapted pathogens. In *Arabidopsis* the NLR protein DM1 triggers autoimmunity when expressed with certain unlinked DM2 alleles [28, 36]. Unlinked *R-gene*s may also functionally suppress each other when expressed together and is an obstacle for breeders who attempt to stack *R*-genes

for agronomic use [25]. The presence of decoy surveillance systems allows for the possibility of customized NLR genes in which decoy domains can be engineered to further enhance the surveillance capabilities of available NLR genes. A summary of the mechanisms of effector recognition by NLR proteins is summarized in Figure 1.2.

The specific details of the downstream mechanism that leads from NLR activation to ETI are not well understood. Some NLR genes have shown evidence of nuclear-cytoplasmic trafficking after activation and have been associated with WRKY transcription factors. This indicates that at least some NLR proteins can function as scaffolds for gene regulation [28]. The accumulation of salicylic acid (SA), which is a phytohormone often associated with host immunity, increases in response to recognition of an effector by most NLR proteins. A reactive oxygen species (ROS) burst also follows effector recognition, ultimately leading to a hypersensitive response and impeding pathogen growth. In *Arabidopsis*, the up-regulation of genes by NLR proteins can lead to accumulation of SA and in turn elicit an HR [25]. Tight control of genes involved in SA accumulation is necessary, but SA up-regulation could also provide an increase in disease resistance. ROS has also been hypothesized to enhance the strength of the immune response [28, 37].

Soybean Resistance Genes against Oomycetes and Nematodes

Recognition of an effector by a resistance gene results in an HR that prevents pathogen proliferation. Currently 24 Resistance against *Phytophthora sojae* (*Rps*) *R*-genes have been identified, with 6 of these having been deployed in commercial soybean cultivars (*Rps*1a, *Rps*1b, *Rps*1c, *Rps*1k, *Rps*3a, *Rps*6) [3]. The *Rps1* locus, which includes the commercially deployed *R*-genes *Rps1a*, *Rps1b*, *Rps1c*, and *Rps1k*, maps to chromosome 3 of soybean [38, 39]. The identity

of *Rps1k*, which is among the most widely deployed *Rps* genes, has been narrowed down to two candidate genes, both of which encode NLR proteins [40]. The *Rps3* locus, including *Rps3a*, is either linked or allelic to *Rps8*, and both map to chromosome 13 [39, 41]. *Rps6* is located on chromosome 18 and is linked with *Rps4* and *Rps5 [42]*. However, none of the identified *R*-genes is able to protect against all strains of *Phytophthora sojae* and the pathogen has begun to evolve to overcome known *Rps* genes [3, 43].

Over 118 sources of either dominant or recessive host resistance against SCN have been identified. However, sources of major resistance used in commercial production are not provided by NLR genes within mapped resistance loci [9]. *Resistance to Heterodera glycines* 1 (*Rhg1*) and *Rhg4* are currently the two major sources of SCN resistance [10]. *Rhg1* and *Rhg4* have been cloned and functionally validated [44-46].

Major gene-for-gene resistance is not well studied for *Pythium* species in soybean. The exception is the *Pythium* and *P. sojae* resistant cultivar "Archer" [47]. Initially, the *Pythium* resistance in Archer was associated with *P. sojae R*-genes *Rps6* and *Rps1k*, but it was later discovered that the genes were independent of each other [48, 49]. Eventually, a single *R*-gene conferring complete resistance against *Pythium aphanidermatum* was characterized and mapped in Archer. The *Pythium R*-gene *Rpa1* was mapped to linkage group F (MLG F) on chromosome 13 of the soybean genome which includes many other soybean *R*-genes such as *Rps3* of *P.sojae* and *Rsv1* of *Soybean Mosaic Virus* [50]. The target of *Rpa1* remains unknown along with the downstream mechanism of resistance. While cell-death HRs may provide resistance against a biotrophic or hemibiotrophic pathogen such as *P. sojae* and SCN, they may lead to increased susceptibility to a necrotrophic pathogen such as *Pythium* species [51].

The majority of known *P. sojae* resistance genes recognize pathogen effectors with conserved RxLR amino acid motifs. The RxLR motif was initially identified based on its conserved presence in highly evolutionarily divergent oomycete effectors [52]. Some evidence suggests the RxLR motif is important for effector entry into the host soybean cell [53]. Rapid evolution of RxLR effectors to overcome *Rps* genes is achieved through a variety of mechanisms. Many effectors are functionally redundant and can be lost or mutated to avoid recognition by an *R*-gene without a fitness penalty. *Phytophthora* genes encoding RxLR effectors have been shown to be concentrated in genomic regions with many transposons while genes that support essential biological functions are in much more stable genomic regions [54]. This points to a "two-speed" genome, in which the effector repertoire of a pathogen can rapidly evolve to avoid host resistance without disruption of essential genes [55, 56]. RxLR effectors, however, are not necessary for virulence for all oomycetes. Necrotrophic oomycete pathogens, such as *Pythium* species, do not have RxLR effectors. For example, *Pythium ultimum* has been shown to completely lack RxLR effectors and have very few Crinkler effectors [4].

Quantitative Trait Loci

Resistance quantitative trait loci are genomic regions in which multiple genes together provide varying degrees of partial resistance [57, 58]. QTLs can provide major or minor resistance, and when stacked together can be an effective disease resistance strategy. In contrast to *R*-genes, QTLs are more likely to provide resistance to multiple pathogens or pathogen isolates and provide a broad spectrum disease resistance phenotype [57]. Intermediate disease responses, not just binary "resistant" or "susceptible" phenotypes are seen as QTLs only provide partial disease resistance [57, 58].

One drawback of QTL-based resistance is that breeding for QTLs is difficult as multiple genetic factors need to be introgressed. Ideal genotypic data for QTL mapping would be a large recombinant inbred line (RIL) population resulting from a cross of a resistant parent with a susceptible line. Additionally, markers such as simple sequence repeat (SSR) markers or single nucleotide polymorphism (SNP) markers are required genotype data for QTL mapping. The development of the soybean 50kSNP chip has made it easy to identify many markers for use in QTL mapping [59].

Approximately two dozen QTLs against *P. sojae* have been identified in soybean. The majority of these have been mapped in the resistant cultivar "Conrad" [57]. However, only three plant QTLs, which encode proteins with diverse molecular functions, have been cloned to date and none are within soybean. QTLs, unlike *R*-genes, do not conform to a standardized function. QTLs can include proteins involved in cellular signaling such as MAPKKK, hormone regulation, and defense compounds [57]. When comparing high levels of partial resistance and *R*-gene mediated resistance to *P. sojae,* it has been shown that *P. sojae* hyphae are able to penetrate further into the soybean root cortex of lines with *R*-gene mediated resistance [60]. For QTLs against *P. sojae* the genes and gene functions involved are highly variable and often unknown.

Rhg1 and *Rhg4*, the two major sources of SCN disease resistance in commercial soybean cultivars are QTLs [10]. *Rhg1* contains tandem repeats of four genes, of which three are implicated to provide resistance. The molecular mechanisms underlying resistance for *Rhg1* genes are not known, but it has been shown that any of the three genes alone is not sufficient to provide resistance to SCN [44, 61]. *Rhg1* has been shown to be essential for PI88788-type SCN resistance and is present in high copy number. *Rhg1* is also present in Peking-type resistance but

in low copy number and *Rhg4* is also required [12, 62]. *Rhg4* encodes an enzyme that is required for folate synthesis. The mechanism for *Rhg4*-mediated resistance is not known, however it is hypothesized that reducing folate available at SCN feeding sites and causing HR-mediated cell death [46, 63].

There are few documented cases of partial resistance against *Pythium* species in soybean, however six QTLs have been identified against *Pythium irregulare* (*P. irregulare*) [60]. Preliminary data suggest the presence of additional QTLs against various *Pythium* species, but remains unconfirmed [64]. Fine mapping and the determination of additional QTL molecular functions for root and seedling diseases will likely reveal genes of diverse structure and function. The identification of additional QTL, elucidation of QTL mechanisms, and attempting to pyramid known QTLs in elite *Glycine max* lines has the potential to yield durable and broad spectrum resistance against genetically diverse root rot pathogens.

Conclusions

Our understanding of NLR genes has surpassed the gene-for-gene model as we continue to discover new NLR functions and characteristics. Plants employ highly specific sophisticated surveillance systems that protect key features of the host immune system. The downstream specifics of NLR activation are still being determined, but it is clear that tight regulation of NLR genes will be essential for any plants used in an agricultural system. Questions as to the events downstream of resistance gene activation, the molecular dynamics that underpin NLR activation, and the non-canonical functions of NLR proteins are still to be answered. Partial resistance provided by QTLs, unlike NLR genes, defies canonical form thus far. Perhaps the identification

and characterization of additional plant QTLs in the future will allow for patterns to be identified.

Quantitative and qualitative host resistance provides our primary sources of disease resistance against a diverse repertoire of soybean pathogens. A better understanding of plant pathogens from a molecular and epidemiological perspective, allows researchers to utilize and even design resistance against specific pathogens or strains. Host plants are constantly under pressure from numerous pathogens to develop superior surveillance mechanisms while pathogens fight to avoid detection. This interaction points to an ongoing evolutionary battle in which no single resistance source will provide a permanent management solution and no pathogen can avoid detection indefinitely. Utilizing many resistance genes of diverse structure and function will yield the most consistent durable resistance.

Figures and Tables

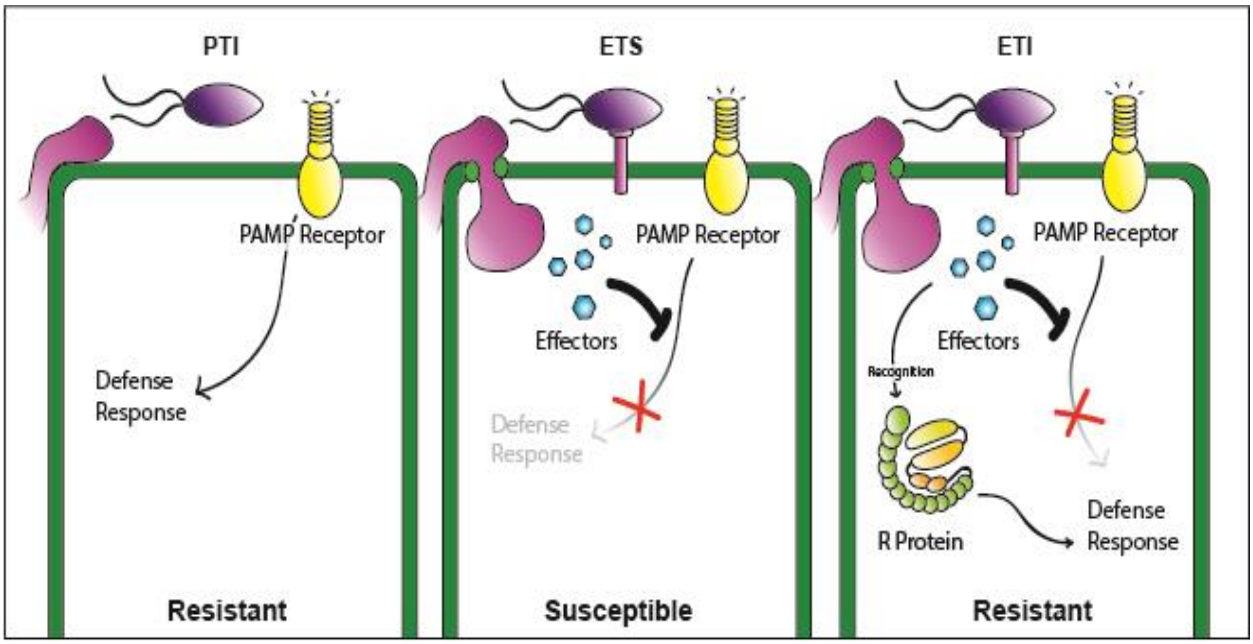

Figure Credit: Kasia Dinkaloo, Department of Plant Pathology, Physiology, and Weed Science, Virginia Tech

Figure 1.1: Overview of PAMP-triggered immunity (PTI), effector-triggered susceptibility (ETS), and effector-triggered immunity (ETI).

During PTI, conserved pathogen PAMPs are recognized by host PAMP receptors leading to a defense response and providing resistance against non-host-adapted pathogens. ETS results when host-adapted pathogens secrete effectors that interrupt signaling leading to defense response resulting in susceptible host plants. Plants can defend themselves from host-adapted pathogens with *R*-genes that directly or indirectly recognize pathogen effectors on a gene-for-gene basis and leads to a robust host defense response and pathogen resistance.

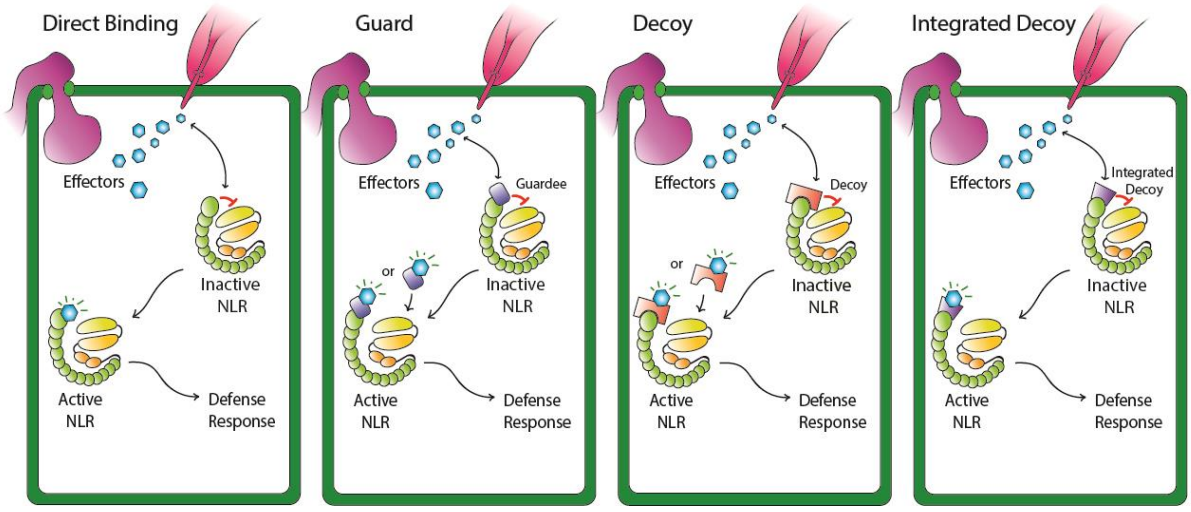

Figure Credit: Kasia Dinkaloo, Department of Plant Pathology, Physiology, and Weed Science, Virginia Tech

Figure 1.2: An overview of the mechanisms of effector recognition by NLR proteins.

Secreted pathogen effectors can be recognized by direct binding between effector proteins and NLR proteins causing an activation of defense responses as shown in the leftmost panel. NLR proteins can also 'guard' key elements of the host immune system that are likely targets for pathogen effectors and activate defense in response to manipulation of their guardees. This is shown in the second panel from the left. Decoy proteins, which mimic host effector targets, are used to bait pathogen effectors into binding and activating downstream defense responses. The advantage of decoys is that they can evolve with the pathogen as they have no role other than effector recognition. This is summarized in the second panel from the right. The final mechanism, which is outlined in the right-most panel, utilizes integrated decoy proteins which work the same way as decoy proteins. However, integrated decoys are encoded on the same or linked genes as the NLR gene which helps prevent auto-immunity in progeny.

Chapter 2: Identification and Mapping of Novel Resistance Genes in *Glycine max* and *Glycine soja* Lines Conferring Durable Resistance to the Oomycete Pathogen, *Phytophthora sojae*.

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Abstract

Phytophthora sojae (P. sojae), the causal agent of soybean root and stem rot disease, is an oomycete pathogen that is among the most devastating pathogens of soybean in the United States and globally. For successful *P. sojae* infection, the pathogen must secrete effector proteins into the host, which function to repress natural defense systems. To date, *P. sojae* has been managed through the inclusion of *P. sojae* resistance (*Rps*) genes and quantitative trait loci into commercial lines. *Rps* genes are able to recognize specific pathogen effectors inside the host and up regulate the defense response in turn. However, the effectiveness of current resistance genes (*R*-genes) is decaying as certain strains of *P. sojae* evolve to overcome the resistance. The reduction of *Rps* gene effectiveness can be attributed to *P. sojae's* loss or manipulation of recognized effectors. This project seeks to identify novel genes conferring durable resistance against *P. sojae*. Resistance gene screening, targeting core *P. sojae* effectors will provide durable resistance, as a loss of these effectors by the pathogen would result in a loss of pathogenicity. An effector-based screening assay has been developed utilizing *Pseudomonas fluorescens* to rapidly screen soybean germplasm for resistance genes. Our strategy is to use the effector-based screening assay, along with a pathogen assay, to develop genetic maps of the novel genes conferring resistance to *P. sojae.*

Introduction

Cultivated soybean (*Glycine max* (L.) Merr.) is a high value crop both in the United States and around the world. Soybeans account for 57% of the world oil-seed production and are utilized for their high oil content and as a protein source in products such as animal feed [1]. The oomycete pathogen *Phytophthora sojae* (*P. sojae)* causes seedling damping off, along with root and stem rot disease in soybean. In 2006, soybean crop losses due to *P. sojae* root and stem rot were estimated at 1.46 million metric tons in the United States and over 2.32 million metric tons worldwide. In the United States each year, *P. sojae* causes approximately $400 million dollars of damage to soybean crops [65]. *P. sojae* overwinters in dry plant tissue and soil in the form of oospores [66]. No-till farming techniques are gaining popularity in the United States to ease crop management cost, but have the negative side effect of increasing soil-borne diseases such as *P. sojae [67]*.

Current management practices are losing effectiveness as the *P. sojae* develops resistance to fungicides and known resistance genes (*R*-genes). We propose to identify core *P. sojae* effectors and use them, along with pathogen-based screening methods, to screen soybean populations and identify potential novel resistance sources. *Glycine max* (*G. max*), the cultivated soybean species, along with *Glycine soja* (*G. soja*), its wild relative are both potential sources of novel *P. sojae R*-genes (Fedkenheuer M. et al, unpublished) [68]. This proposal will also encompass an effector-directed breeding strategy in which recombinant inbred lines will be developed and phenotyped. Finally, identified resistance genes will then be mapped utilizing genotypic markers and collected phenotypic data. Information gathered in this study will be used to develop elite soybean cultivars.

A multi-layered molecular defense network prevents the majority of potential pathogens from becoming pathogenic by recognizing threats and quickly initiating a defense response. A basal level of immunity protects plants from the majority of would-be pathogens, but this immunity can be subverted by secreted pathogen virulence proteins known as effectors. For successful parasitism, *P. sojae* represses the natural defense mechanisms such as recognition by host pattern recognition receptors that stimulate immunity gene expression. This repression is achieved via the extracellular and intracellular secretion of pathogen effector proteins encoded by virulence genes. *P. sojae* effectors were identified based on the presence of an RxLR-dEER motif which evidence suggests plays a role in effector entry into host cells [69, 70]. The RxLR motif was identified due to its conserved presence in highly evolutionarily divergent oomycete effectors [52]. Over 370 RxLR-dEER effectors have been identified in *P. sojae*.

The majority of known *P. sojae R*-genes recognize pathogen effectors with conserved RxLR amino acid motifs. These immune surveillance genes encode proteins that are able to recognize pathogen effectors and initiate immune responses. Recognition of the pathogen effector by a host *R*-gene is characterized by a localized cell death response known as the hypersensitive response (HR) [19]. Using the HR phenotype as an indicator for the presence of a cognate *R*-gene, plants can be bred for recognition of a particular effector, which is known as effector-directed breeding. Ideally, identified *R*-genes recognize "core" pathogen effectors that are conserved in field isolates, monomorphic, and cannot be lost without a loss of pathogen virulence [71]. *R*-genes recognizing core effectors are expected to be more durable against a diverse and constantly evolving pathogen such as *Phytophthora sojae*.

To combat the suppression of host defenses by pathogen effectors plants have developed *R*-genes which comprise a layer of host defense known as effector-triggered immunity or ETI [17]. Effectors are delivered into host cytosol in various ways including the bacterial type 3 and type 4 secretion systems and the fungal/oomycete haustoria [19]. Most *R*-genes encode for nucleotide-binding domain leucine-rich repeat (NBD-LRR or NLR) surveillance proteins that recognize pathogen effectors and activate ETI [25, 70]. Based on the gene-for-gene model of disease resistance an *R*-gene can only provide ETI in the presence of an effector specific to that *R*-gene [26]. Effectors recognized by *R*-genes are referred to as avirulence (*avr*) genes and their recognition leads to a robust host defense response [22, 27]. However, effectors are often redundant and are structurally variable making many of them dispensable which can lead to an "evolutionary arms race" between plant pathogens effectors and host *R*-genes [23]. Structurally similar NLRs have been found in both plant and animal species, but are hypothesized to have developed independently through convergent evolution, reinforcing the utility of NLRs as a surveillance mechanism against host-adapted pathogens [28].

To date, 24 *Response to Phytophthora sojae* (*Rps*) resistance genes have been identified along with additional resistance quantitative trait loci (QTL) [72]. Seven *Rps* genes have been commercially deployed including *Rps*1a, *Rps*1b, *Rps*1c, *Rps*1k, *Rps*3a, *Rps*6, and *Rps*8 [72]. Elite *G. max* cultivars exist with different combinations of these *R*-genes. The effectiveness of current *R*-genes has diminished as the effectors being recognized by NLR proteins are being lost as the pathogen evolves to avoid surveillance. The average lifespan for an *R*-gene under field conditions has been reported as 8-15 years [73]. Functional redundancy of *P. sojae* effectors

allows effectors that are recognized by the host, and initiate ETI, to be lost without a negative impact to virulence [27].

Previous work in Dr. Brett Tyler's lab at Oregon State University identified a number of *P. sojae* core effectors [71]. Our collaborators at Iowa State University under Dr. Alison Robertson identified *P. sojae* resistant soybean germplasm that will form the basis for our effector-based screening [68]. Former Virginia Tech graduate students Dr. Mike Fedkenheuer and Dr. Kevin Fedkenheuer, under the supervision of Dr. John McDowell transformed the identified core effector genes into a *Pseudomonas* type III secretion system for easy effector delivery into soybean (K. Fedkenheuer et al, unpublished)(M. Fedkenheuer et al, unpublished). This research forms the basis of knowledge for this dissertation.

In order to understand the contribution of *P.sojae* effectors on virulence, a transient silencing system was used and 30 notable RxLR effectors were identified [71]. Twelve effectors significantly reduced pathogen virulence when independently knocked-out. Three of these effectors, *Avh16, Avh180,* and *Avh240* greatly reduced pathogen virulence and were selected as ideal core effector candidates. In addition to a reduction in virulence when silenced, each selected core effector was monomorphic and expressed early in the *P. sojae* infection cycle [71].

Concurrent with the identification of *P. sojae* effectors, over 1,000 *Glycine* accessions were screened with known *P. sojae* isolates and 31 *G. max* and 17 *G. soja* accession were determined to likely have novel resistance genes [68]. A combination of three isolates including Iso1005-2.9 (pathotype 1a, 1b, 1c, 1k, 3b, 7), Iso2004 C2.S1 (pathotype 1a, 1b, 1c, 1d, 1k, 2, 3c, 4, 6, 7), and Race 7-2a (pathotype 1d, 2, 3a, 5, 6, 7) were used, which together overcome all

known *Rps* genes with the exception of *Rps 8 [68]*. Testing of *Rps 8* with this combination is underway.

A *Pseudomonas fluorescens* effector-detector system (EtHAN) was developed through transgenic modification of *Pseudomonas fluorescens* to contain *Pseudomonas syringae* secretion components [74]. We further modified this system to deliver known avirulence effectors efficiently enough to provoke a visible cell death response on resistant *G. max* and *G. soja* cultivars (Fedkenheuer K. et al. unpublished). *Pseudomonas syringae* pathovar *glycinea (Psg)* race 4 was used as a helper strain to repress basal host defenses and allow for a robust HR. Screening of the *P. sojae*-resistant *G. max* and *G. soja* accessions with three core effectors using the EtHAN system, revealed 19 *G. max* accessions responding to at least one effector and three *G. soja* accessions each responding to one effector (Fedkenheuer M. et al, unpublished). Each of the identified resistant lines was crossed with the susceptible *G. max* cultivar "Williams" to develop segregating populations. Populations were selfed to the $F_{2:3}$ generation and then used to refine an effector-based screening approach. Recombinant inbred line (RIL) populations have been developed for additional phenotyping and as the basis for mapping. Segregation ratios of the $F_{2:3}$ generation will determine if resistance occurs within independent, dominant loci, as expected based on the gene-for-gene model. RIL populations will be used for mapping of *R*-genes against *P. sojae*.

Results

Our approach for the continuing development of *P. sojae* resistant soybean cultivars is based on previous research completed in our lab and by our collaborators. We will identify novel *P. sojae* resistance genes in available *Glycine* accessions through an effector-directed breeding approach. Our complete research pipeline can be broken down into three parts. Preliminary research includes effector identification, transformation into EtHAN, and identification of resistant parental germplasm. Proposed work includes phenotyping through effector infiltration, genotyping, and mapping. Future work includes the development of elite soybean cultivars with identified *P. sojae R*-genes (Figure 2.1).

Population Development

G. max Plant Introduction (PI) accessions with potential novel resistance gene(s) PI408097, PI408132, and PI567139B along with *G. soja* line PI407076 were selected for screening. All lines were crossed to *P. sojae* susceptible *G. max* cultivar 'Williams'. Williams was chosen because it did not contain any *Rps* genes and its isogenic line, 'Williams 82', had a sequenced genome [75, 76]. The crosses were validated and all the hybrid F1s were selfed to the $F_{2:3}$ generation. PI408097 and PI408132 populations were advanced in the field by selfing and recombinant inbred line (RIL) populations were developed. A complete list of developed populations that were screened is provided in Table 2.1. For the cross of PI408097 by Williams (gm320) 312 lines were developed, while 142 lines were developed for the cross PI408132 by Williams (gm326) and 175 lines for the cross of PI567139B (gm32) by Williams. Additionally, 135 $F_{2:3}$ families were developed for the *G. soja* population of PI407076 by Williams (gs2292).

Overview of Effector Screen

Our effector-based screening assay identifies lines containing cognate resistance genes to single core effectors based on phenotyping for the presence or absence of an HR. A bacterial delivery system with a type III secretion system (T3SS) is utilized to deliver individual RxLR-effectors into the cytosol of host plant cells as shown in figure 2.2. The presence of an HR indicates that a resistance gene is present that recognizes the delivered effector and initiates a robust immune response. The absence of an HR indicates that the RxLR effector remained undetected by the host and no immune response was initiated. Several crossed resistant and susceptible populations were phenotyped in the $F_{2:3}$ and RIL generations for the presence of an *avr* gene. An overview of our screen is shown in Figure 2.3.

Infiltration Screening of $F_{2:3}$ Families

Three $F_{2:3}$ populations were screened (gm32, gm326, and gs2292), using the EtHAN effector delivery system (Table 2.1). Lines that responded with an HR in response to an effector were classified as resistant. Most known *R*-genes encode single, genetically dominant loci, therefore we hypothesized that HRs in response to effector delivery would segregate with a 1:2:1 phenotypic ratio.

A total of 54 $F_{2:3}$ lines for gm326 were screened with effector *avh180*. A phenotypic ratio not significantly different ($x > 0.05$) from the expected ratio of 1:2:1 was observed. These results are shown in Table 2.2 and a histogram is provided in Figure 2.4. A total of 89 and 83 $F_{2:3}$ lines for gm32 were screened with both *avh16* and *avh240* effectors respectively. A phenotypic ratio not significantly different ($x > 0.05$) from the expected ratio of 1:2:1 was observed in each case. These results are shown in Table 2.2 and a histogram is provided in Figure 2.5. A total of 89 $F_{2:3}$

lines for gs2292 were screened with effector *avh180*. A phenotypic ratio not significantly different ($x > 0.05$) from the expected ratio of 1:2:1 was observed. These results are shown in Table 2.2 and a histogram is provided in Figure 2.6. We concluded that gs2292 and gm326 each likely contain one *R*-gene that recognizes our core effectors. We also concluded that gm32 likely contains two *R*-genes that respond to different core effectors and that segregate independently of each other.

Infiltration Screening of RIL Populations

Two RIL populations were screened (gm320 and gm326), using the EtHAN effector delivery system. Lines that responded with an HR in response to an effector were determined to be resistant. Based on $F_{2:3}$ segregation ratios that suggested single dominant loci for *R*-genes responding to our core effectors; we hypothesized that there would be 1:1 phenotypic ratios for our RIL populations.

A total of 98 RILs for gm326 were screened with effector *avh180*. A phenotypic ratio not significantly different ($x > 0.05$) from the expected ratio of 1:1 was observed. These results are shown in Table 2.3. A total of 109 RIL lines for gm320 were screened with the effector *avh240*. A phenotypic ratio not significantly different ($x > 0.05$) from the expected ratio of 1:1 was observed. These results are shown in Table 2.3.

Development of a Facile Assay for Disease Screening

We developed an assay to phenotype *P. sojae* virulence on detached trifoliate leaves in a high-throughput system. Mycelial plugs from *P. sojae* race 2 were used to validate this system and produced large lesions on trifoliate leaves of known susceptible cultivars. *P. sojae* race 2 did not produce an expanded lesion on the resistant control cultivar *Rps*1k-Harosoy. This was the

expected reaction as *P. sojae* race 2 carries *avr1k* with is recognized by *Rps1k*. Using this technique, we confirmed resistance against *P. sojae* race 2 in *G. max* accessions gm32 and gm326. Results from this validation are shown in Figure 2.7.

Disease Assay Screening in Gm326 RIL Population

PI accession gm326 was screened as resistant to *P. sojae* isolates Iso2004 and Iso1005 independently while 'Williams' was screened as susceptible. An RIL population derived from the cross of gm326 by 'Williams' has been screened using our detached trifoliate leaf disease screening assay. Lines that developed lesions when inoculated with a mycelia plug of *P. sojae* isolates Iso2004 and Iso1005 were determined to be susceptible. Lines that did not develop lesions were determined to be resistant. An additional isolate (Race 7) was also tested, but the results were inconclusive and will not be discussed. We hypothesized that there would be a 1:1 phenotypic ratio for the gm326 RIL population.

A total of 88 RILs for gm326 were screened with Iso2004 and Iso1005. A phenotypic ratio not significantly different ($x > 0.05$) from the expected ratio of 1:1 was observed. These results are shown in Table 2.4.

Mapping of *R*-gene in Gm326 RIL Population

JoinMap 4.0 was used to map resistance based on disease screening data with Iso1005 and Iso2004 [77]. Infiltration data was not included as it was not yet available. Mapping population Gm326 consisted of 88 RILs and data set based on 1934 DNA markers including single nucleotide polymorphism (SNP) and simple sequence repeat (SSR). To determine the original linkage between loci a recombination frequency algorithm was used. For map

construction at min LOD of 8.0 regression mapping algorithm was applied with the Kosambi function on.

Preliminary data indicate that resistance gene maps to soybean chromosome 13. Multiple *P. sojae R*-genes have been mapped to chromosome 13, including *Rps3* and *Rps8* [39, 41]. The precise region is still being determined, but current results suggest the resistance does not map to a location with known *P. sojae* resistance. The resulting genetic map is shown in Figure 2.8.

Discussion

The primary goal of this project was to identify novel sources of durable *P. sojae* resistance in soybean. *G. max* and *G. soja* accessions with potential novel sources of *P. sojae* resistance had been identified [68]. In addition, core *P. sojae* effectors, that are monomorphic, expressed early in infection, and required for virulence on soybean were identified [71]. A bacterial effector delivery system was developed to deliver individual effectors into the cytoplasm of soybean cells and initiate a macroscopic HR in the presence of a cognate *R*-gene (K. Fedkenheuer, unpublished) (C. Davis, unpublished). Utilizing this previous work, we sought to identify *R*-genes that specifically recognize core *P.sojae* effectors using the bacterial effector delivery system. Populations were developed from crosses of key *P. sojae* resistant parents to provide germplasm for screening segregating populations. Infiltrations were undertaken in the $F_{2:3}$ and RIL generations. To complement effector infiltration studies, we have also developed a high-throughput live pathogen assay to correlate effector responses to *P. sojae* resistance. *R*-genes that recognize core *P. sojae* effectors should be more durable as *P. sojae* will not be able to remove or disrupt these genes without a loss of virulence. However, it is doubtful that any single *R*-gene will be durable under long-term field conditions. The genes identified in this study can be used in the development of elite soybean cultivars and stacked with other *P. sojae R*-genes and QTLs to provide durable resistance.

Screening of $F_{2:3}$ populations was done to determine if responses to effectors with an HR segregated as single dominant loci as hypothesized. By screening selfed F_3 populations to determine F_2 segregation ratios, we were able to screen each line in replicate. This is key as scoring for the presence of an HR is complex. More details about our scoring method can be

found in the materials and methods section. Our results with gm326, gm32 and gs2292 all supported the hypothesis that effector response segregates as a dominant trait. None of these populations segregated differently from the expected ratio of 1:2:1. Screening of gm32 in a segregating population also revealed that responses to two effectors (*avh16* and *avh240*) while being dominant, appeared to be independent of each other. It remains to be determined if response to *avh180* segregates independently of either *avh16* or *avh240*.

Interestingly, two *R*-genes in PI567139B, the resistant parent of gm32 population, *Rps*Un1 and *Rps*Un2 have independently been mapped to two different chromosomes using an inoculum-based screening method [78]. Our effector-responses in PI567139B against *Avh*16 and *Avh*240 may correlate to *Rps*Un1 and *Rps*Un2. Mapping of the responses to *avh16* and *avh240* in the RIL population would provide more information.

Screening of RIL populations was done to map the resistance loci responsible for the HR response during effector delivery. RIL populations are superior to $F_{2:3}$ populations for mapping because the low levels of heterozygosity reduce the frequency of intermediate HRs and allow for more confident phenotyping. RIL populations, gm320 and gm326 were screened with *Avh240* and *Avh180* respectively. Neither gm320 nor gm326 segregated differently from the expected ratio of 1:1 which further supports the hypothesis that resistance segregates as a dominant loci. Mapping of the effector response is underway.

Screening with our mycelium-based trifoliate assay on the RIL population gm326 was also completed to map resistance to *P. sojae* isolates, Iso1005 and Iso2005. Our system yields effector data and pathogen data for each line within a population. A combination of disease-based and effector-based assays will determine if effector response HRs correlate to resistance

against diverse *P. sojae* isolates. While a correlation between disease screening resistance and effector screening would provide evidence that the effector being tested is encoded by an *Avr* gene, the lack of a correlation would not be sufficient to determine that the same effector is not encoded by an *Avr* gene. For example, gm320 responds to at least three core effectors and the combinatorial effect of multiple uncharacterized *R*-genes could lead to different responses between effector-based and pathogen-based screening methods. There are approximately 400 effectors in *P.sojae* and we have only screened with a minute fraction of those [69]. It is possible that the populations we are screening would provide *P. sojae* resistance in response to an unknown effector that is not being screened in our effector-based screening method. Overall, we believe that mapping the same population using both *Avr* activity and pathogen screens can be used to build a multidimensional map to aid in the elucidation of host resistance mechanisms.

In the future we will continue to develop populations, gather phenotypic data, and map *P. sojae R*-genes. Collecting effector and disease data for additional lines of gm326 and gm320 will provide more data points in our effort to map resistance based on both scoring methods. Preliminary research identified 30 core RxLR effectors, but we have only screened with three of them in segregating populations. Additional efforts to screen more effectors would ideally provide a plethora of novel *R*-genes that could be stacked into elite soybean cultivars and deployed in the field. Over 20 *G. max* and *G. soja* parental lines that have been crossed to Williams responded to at least one core effector that was not *avh16, avh180,* and *avh240*. Further development of these populations would be required for screening with these effectors. The collection of RIL phenotypic and genotypic data has allowed us for the first time to attempt to map *R*-genes in soybean using effector-directed breeding. Mapping is underway and continued

efforts in this area have the potential to yield significant results. I believe we are just scratching the surface of a wealth of natural genetic resistance in soybean with our effector-directed breeding approach and strongly recommend continued efforts to develop, screen, and map our crosses novel *R*-genes.

Materials and Methods

Plant Growth Conditions for Infiltration

Soybean plants were planted in Fafner P2 potting media with Cal-Mag fertilizer. Four inch pots were packed firmly with media. Five seeds were sown per pot and covered with about 2-3 cm of loosely packed potting media. The top soil was treated with Marathon insecticide, put into flats (8 plants per flat), and covered with clear plastic lids for two days to maintain humidity during germination. Germination rates were >95% for all *G. max* seeds and approximately 80% for scarified *G. soja* seeds. *G. max* were germinated and maintained under diurnal light (16 h light at 22 ^{0}C; 8 h dark at 20 ^{0}C). Light intensity was maintained at approximately 300 µM for all experiments. The trifoliate leafs of plants for infiltration experiments were pruned approximately 12 days after germination to promote the growth and health of unifoliate leafs.

Genetic Material and Background

P. sojae resistant parents were identified to have potential novel sources of resistance through screening with a combination of three *P. sojae* isolates that break all known resistance with the exception of *Rps8*. The resistant parents include PI408097, PI408132, PI407076, and PI567139B and all were crossed to the *P. sojae* susceptible cultivar 'Williams'. Crosses were completed under field and greenhouse conditions and verified with simple sequence repeat (SSR) markers. A complete list of crosses is provided in Table 2.1. Seeds harvested from a single plant were used to generate the next successive generation. Seeds were collected in bulk in the RIL generations to provide material for screening. A total of 312 lines were developed for cross gm320, 142 lines for gm326, 175 lines for gm32, and 135 for gs2292.

Culture Preparation

All bacterial cultures were prepared from single colonies and inoculated in KB media with antibiotic selection. The cultures were incubated, shaking at 28 °C for 18-22 h. The bacteria was pelleted and re-suspended in 10 mM $MgSO_4$. EtHAN and EtHAN-effector constructs were prepared at an OD = 0.6. For all infiltrations, EtHAN and *Psg* were co-infiltrated and mixed for at ODs = 0.6 and ODs = 0.3, respectively for a final OD = 0.9.

Effector Infiltrations

Plants were infiltrated after 15-17 days (3 days post pruning). Bacterial solutions were prepared 1 hour prior to infiltration. Needleless syringes were used to pressure infiltrate unifoliate leaves with 100-200 uL of bacterial solution in each of four infiltrations per unifoliate. The same process is performed on each adjacent unifoliate. The best negative control is to co-infiltrate EtHAN-effector and *Psg* into *G. max* cultivars which contain no *R*-gene against the cloned effector Figure 2.3.

***P. sojae* Mycelium Plug Infection Assay on Detached Trifoliate Leaves**

P. sojae was maintained on a PARP V8 plate. Three weeks prior to infection, a mycelium plug is transferred from a PARP V8 plate to a V8 plate. After three weeks, the culture should colonize most of the V8 plate. Contaminated plates should be discarded. Fresh mycelium plugs should be taken from the outside edge of freshly colonized V8 plates for the best results.

At 14 days post planting, trifoliate leaves should appear expanded. Per plant, remove each trifoliate and place each leaf in a wetted petri dish with a piece of filter paper and up to two other leaves. Trifoliate leaf age and size should remain as consistent as possible. Experimental design will depend on leaf and culture sample size. On average, each petri dish will contain three

leaves, one each from three different individual plants. This allowed us to test individuals with multiple pathogen isolates during a single replicate or multiple biological replicates within the same petri dish.

To infect, place a 1cm mycelium plug and lightly wound the area directly underneath the plug. Wet filter paper and Parafilm were used to prevent the leaves from drying out for one week while the infection proceeds. Disease symptoms will begin at about 48 hours post infection. The symptoms conclude after seven days. Each culture should be passage every other month to prevent a loss of virulence.

Scoring Mycelium Plug Infection Assay Results

Seven days post-inoculation soybean trifoliate leaves were images and then scored based on the presence or absence of a lesion. The sizes of the lesions on parental trifoliate controls were used to determine the minimum lesion size to be considered a susceptible interaction. A minimum of three replicates was scored for each line and each isolate.

Scoring Effector Infiltrations

Seven days post infiltration; the unifoliate leaves were detached, imaged, and scored. A plant scored resistant if ≥ 6/8 infiltration spots produced a visual HR on both unifoliate leaves. Plants were scored as susceptible if ≤2/8 infiltration spots produced a visible HR. Plants that fell between 2/8 and 6/8 were scored as ambiguous. Any data, scored as ambiguous was not consider for scoring and the experiment was repeated until >15 plants per line were completed for the majority of the lines within each population. To calculate the percent hypersensitive response of each line within a population we used the equation, $\%\, HR = (\#\ of\ responding\ plants) \div (\#\ of\ infiltrated\ plants)$.

If too much pressure is applied during infiltration, the damage may induce a cell death response around edges of infiltration sites. This can make scoring weak HRs more difficult. If the cell death response is weak, the infiltration site will turn brown with very small HRs throughout the site. Cell death can occur on negative control plants if disease or insects are present. A complete summary is provided in Figure 2.3.

Determination of *R*-Gene Inheritance in $F_{2:3}$ Populations

Plants from segregating $F_{2:3}$ families were screened with a single effector at a time. Segregation ratios were predicted to be 1:2:1. Prior to screening, we determined the background cell death each effector produced on Williams and used the background cell death value plus one standard deviation to determine how to score a line. All $F_{2:3}$ families were tested with an approximate sample size of 15 individuals per line in at least three replicates. For example, 3 pots were planted each with 5 plants per pot for a total of at least 15. Additional replicates of 5 plants per pot were used to account for poor germination and to reach at least 15 scored plants per line.

Determining $F_{2:3}$ Phenotypic Ratios

After compiling the results, we analyzed the data as percent hypersensitive response with variation represented as standard deviation (σ). We determined our range for homozygous dominant using the equation, $RR = 100\%\ to\ (100\% - 1\ parental\ \sigma)$. We determined our range for homozygous recessive using the equation, $rr = 0\%\ to\ (Williams\ \%\ HR + 1\ Williams\ \sigma)$. Lines that fell within the two ranges are considered to be heterozygous.

Determining RIL Phenotypic Ratios

As with $F_{2:3}$ ratios, we analyzed the data as percent hypersensitive response with variation represented as standard deviation (σ). We approximated our range for homozygous dominant using the equation, $RR = 100\%\ to\ (100\% - 1\ parental\ \sigma)$. We determined our range for homozygous recessive using the equation, $rr = 0\%\ to\ (Williams\ \%\ HR + 1\ Williams\ \sigma)$. Lines that fell in between those values were considered to be either ambiguous or heterozygous.

Figures and Tables

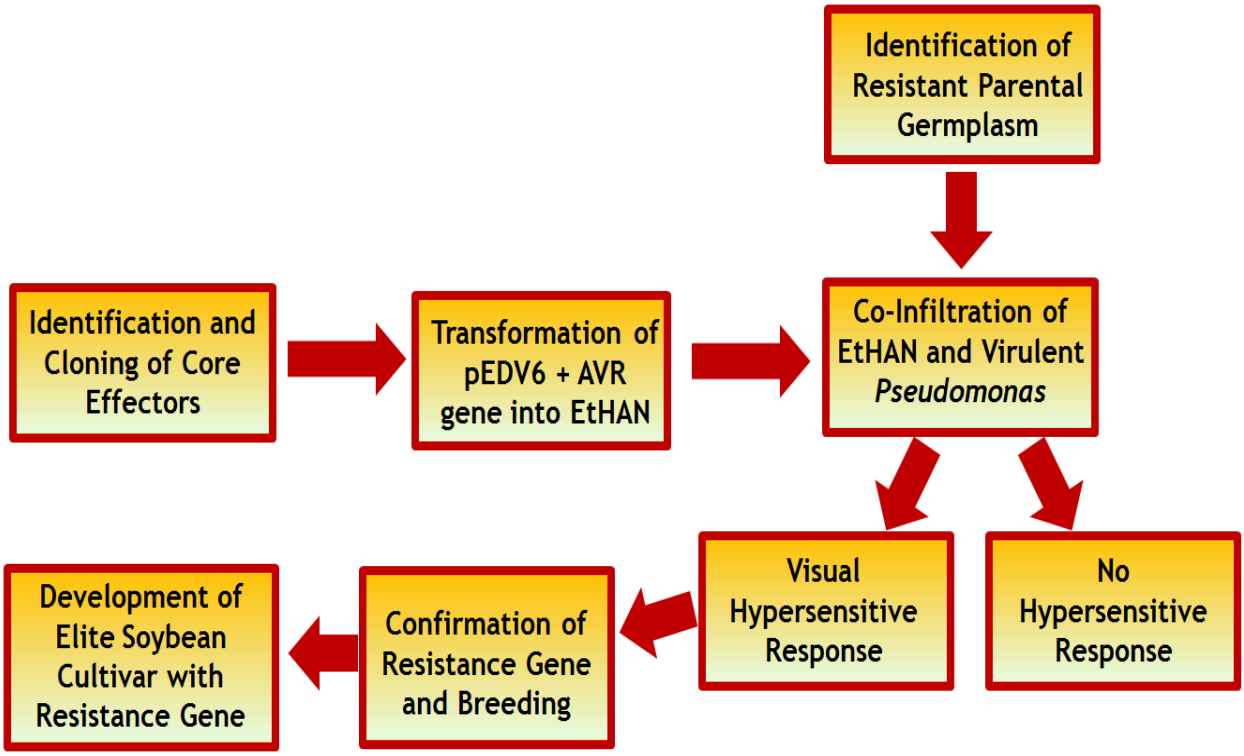

Figure 2.1: Approach to Identify Novel Durable *P. sojae R*-genes

Our approach begins simultaneously at two points with the identification and cloning of core effectors which was completed in the B. Tyler lab at Oregon State University and The identification of *P. sojae* resistant germplasm which took place in the A. Robertson lab at Iowa State University. Approximately 30 core effectors and 48 *G. max* and *G. soja* lines with potential novel resistance were identified. After core effectors were identified and cloned a system was required to deliver individual *P. sojae* effectors into host cells. A *Pseudomonas fluorescens* effector-detector system (EtHAN) system was selected and validated. This work culminated in an effector-directed breeding system in which segregating populations were screened with individual effectors for the presence of an effector based on the hypersensitive response. The phenotyping of effector responses will lead to gene mapping and confirmation of the presence of

a novel *R*-gene. These identified and validated genes could be bred into elite *G. max* cultivars for deployment in the field.

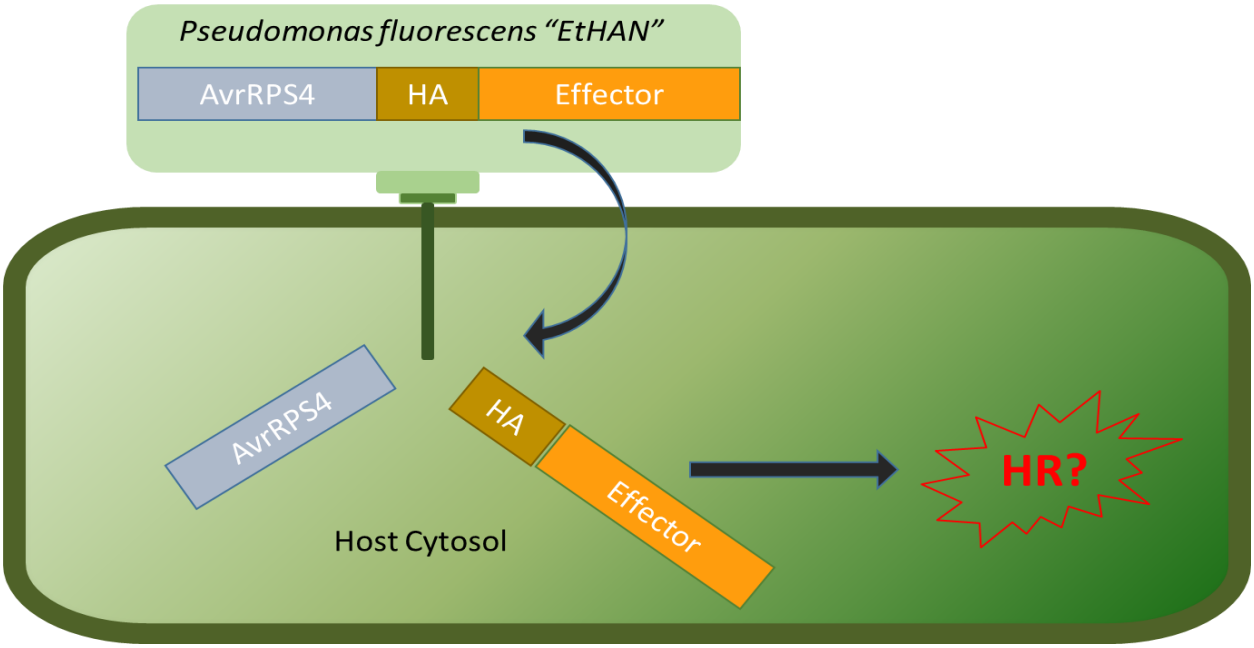

Figure Credit: Ryan Anderson, Department of Plant Pathology, Physiology, and Weed Science, Virginia Tech

Figure 2.2: EtHAN pEDV6 Screening Method Model

Pseudomonas fluorescens "EtHAN" with a type-3 secretion system (T3SS) specifically delivers effectors inserted into the pEDV6 effector detector vector. The AvrRPS4 promoter and leader sequence guides the construct through the T3SS into the host cytosol of soybean cells. The AvrRPS4 promoter and leader sequence are then cleaved and if the delivered effector is recognized by a cognate *R*-gene, a hypersensitive response will result.

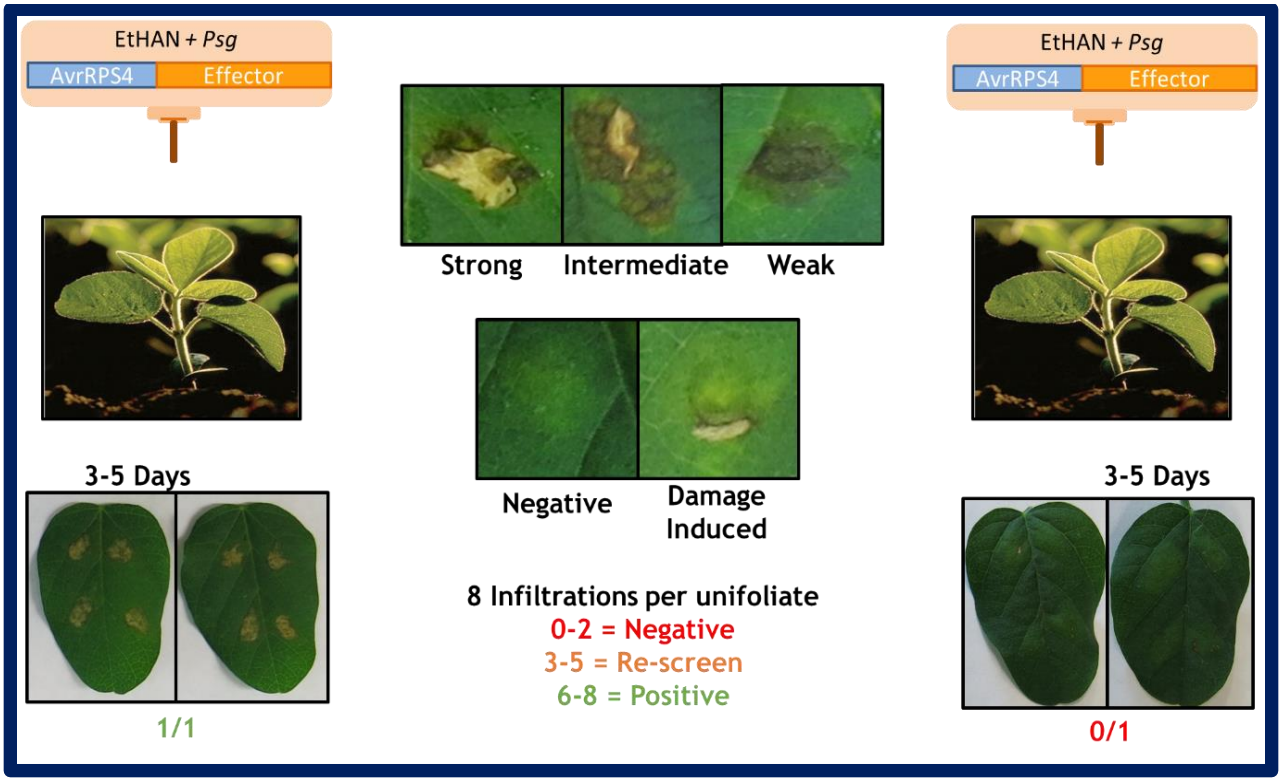

Figure Credit: Mike Fedkenheuer, Department of Plant Pathology, Physiology, and Weed Science, Virginia Tech

Figure 2.3: Overview of Infiltration Scoring

EtHAN carrying a single cloned RxLR effector is co-infiltrated with *Pseudomonas syringae pv. glycinea race 4* into the unifoliate leaves of approximately 2 week old soybean plants. Each unifoliate is infiltrated four times and scored based on the presence or absence of a hypersensitive response three to five days post infiltration. A basal level of background HRs exist even in the susceptible cultivar 'Williams' and must be accounted for with an HR frequency scale. Of the eight infiltrations per plant, if <=2 respond with an HR the individual is scored as susceptible. If >=6 respond with an HR the individual is scored as resistant and if the result is between two and six the result is considered ambiguous and will be re-tested. As shown in this figure, the strength of responses varied from weak to strong from line to line. Damage-

induced HRs from the infiltration protocol must be removed for accurate scoring of effector responses.

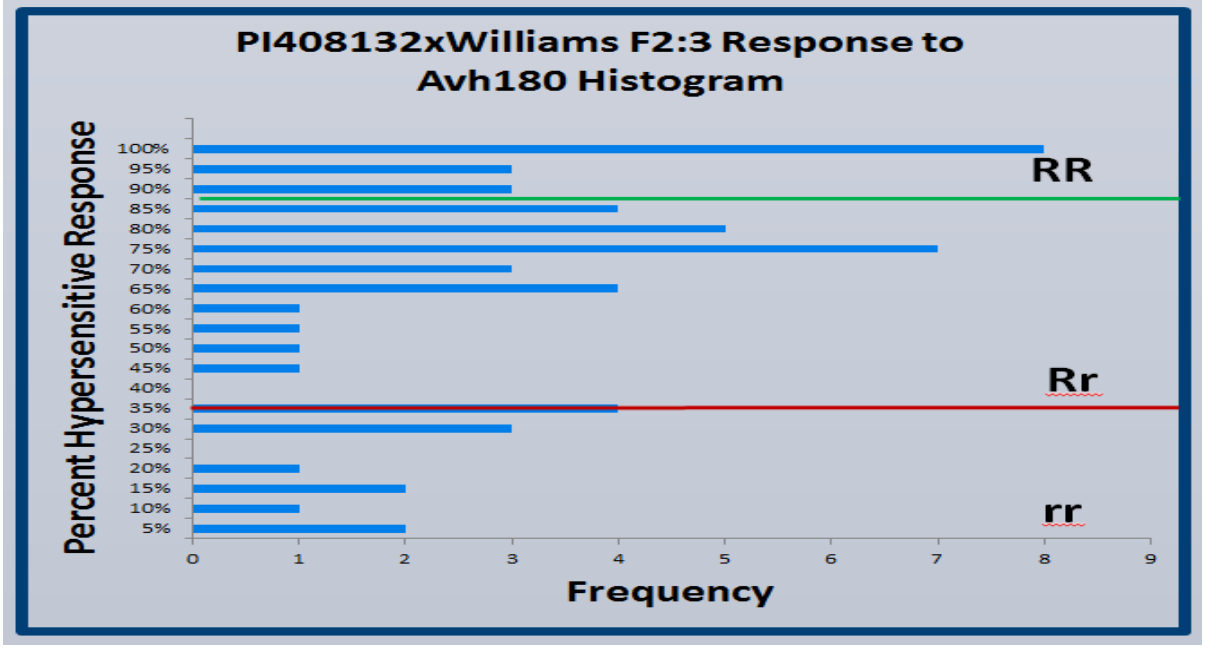

Figure 2.4: Gm326 $F_{2:3}$ Response to *Avh180* Histogram

The y-axis corresponds to the aggregate percent HR for each of the lines that were screened with *Avh180* in the $F_{2:3}$ population gm326. The x-axis corresponds to the frequency at which a line within this population responded to *Avh180* with an HR. The red line represents one standard deviation above the response rate of the susceptible parent Williams while the green bar represents one standard deviation below the response rate of the resistant parent PI408132. Lines that scored at or above the green line were considered homozygous resistant (RR). Lines that scored below the red line were considered homozygous susceptible (rr) and lines in between the red and green lines were considered to be segregating (Rr). We predicted a sharp bell-shaped curve for the $F_{2:3}$ generation peaking around 50% HR. Our data forms a rough bell-shape that skews toward the homozygous resistant score.

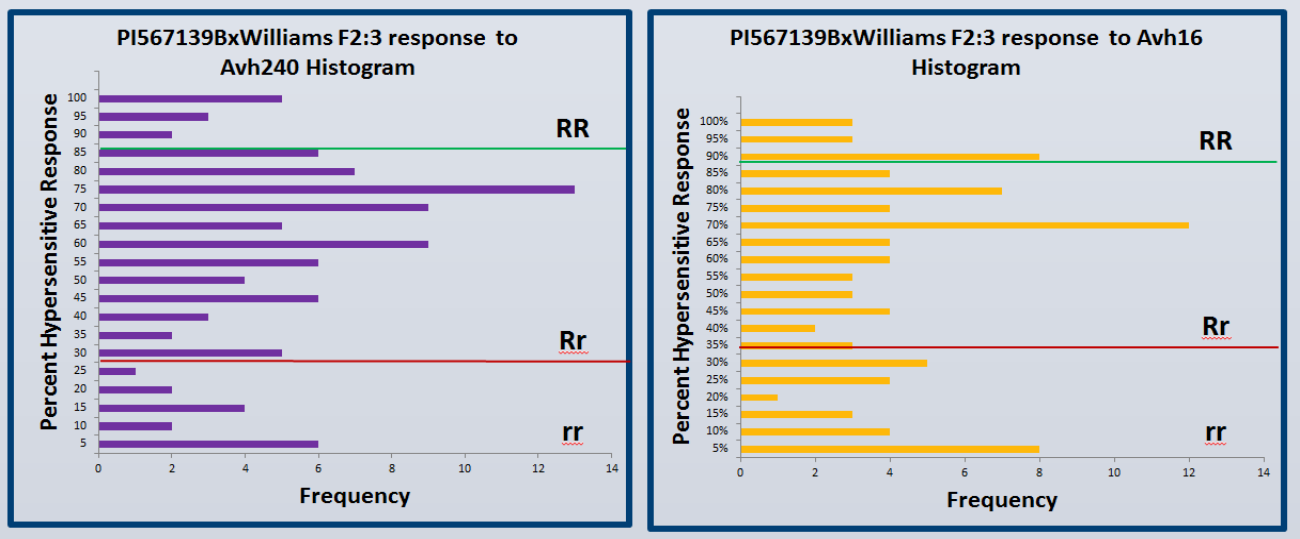

Figure 2.5: Gm320 Response to *Avh16* and *Avh240* Histograms

The y-axis for each histogram corresponds to the aggregate percent HR for each of the lines that were screened with *Avh240* and *Avh16* in the $F_{2:3}$ population gm32. The x-axis corresponds to the frequency at which a line within this population responded to *Avh240* and *Avh16* with an HR. The red line represents one standard deviation above the response rate of the susceptible parent Williams while the green bar represents one standard deviation below the response rate of the resistant parent PI567139B. Lines that scored at or above the green line were considered homozygous resistant (RR). Lines that scored below the red line were considered homozygous susceptible (rr) and lines in between the red and green lines were considered to be segregating (Rr). We predicted a bell-shaped curve for the $F_{2:3}$ generation peaking around 50% HR. The data for each effector tested forms a bell-shaped as predicted.

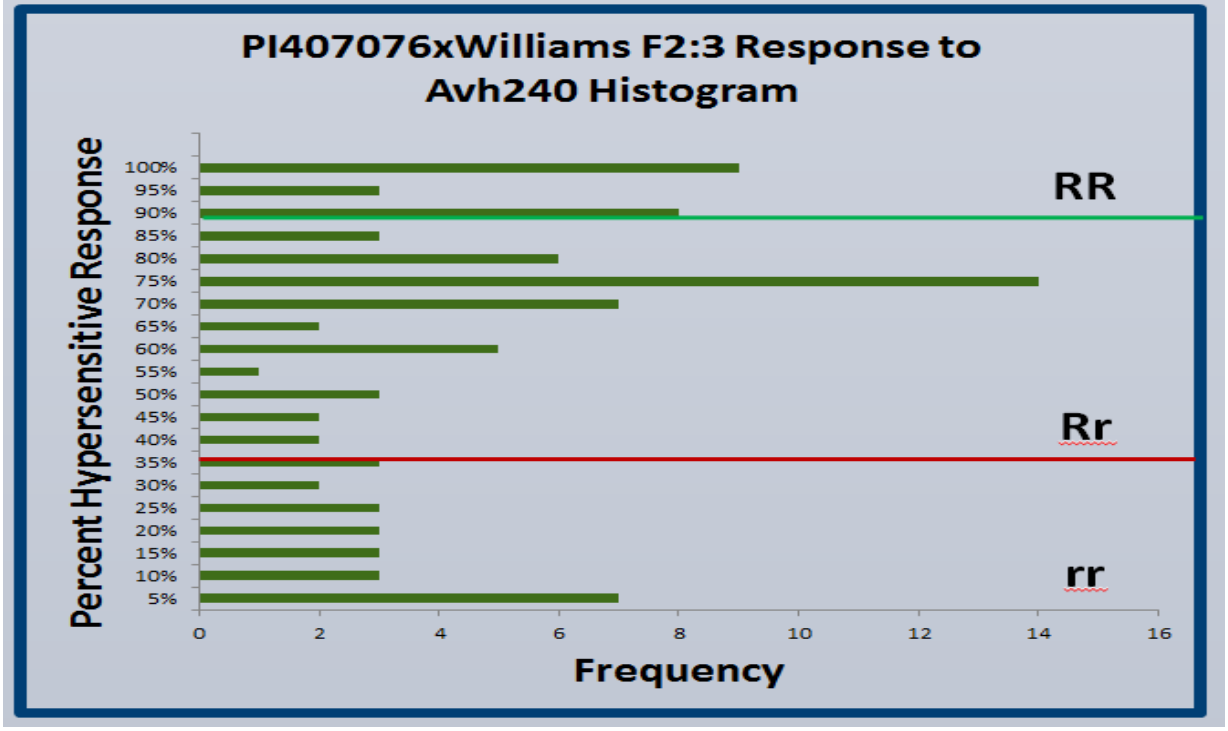

Figure 2.6: Gs2292 Response to *Avh240* Histogram

The y-axis corresponds to the aggregate percent HR for each of the lines that were screened with *Avh340* in the $F_{2:3}$ population gs2292. The x-axis corresponds to the frequency at which a line within this population responded to *Avh240* with an HR. The red line represents one standard deviation above the response rate of the susceptible parent Williams while the green bar represents one standard deviation below the response rate of the resistant parent PI407076. Lines that scored at or above the green line were considered homozygous resistant (RR). Lines that scored below the red line were considered homozygous susceptible (rr) and lines in between the red and green lines were considered to be segregating (Rr). We predicted a bell-shaped curve for the $F_{2:3}$ generation peaking around 50% HR. Our data forms a rough bell-shape that slightly skews toward the homozygous resistant score and towards the positive and negative extremes.

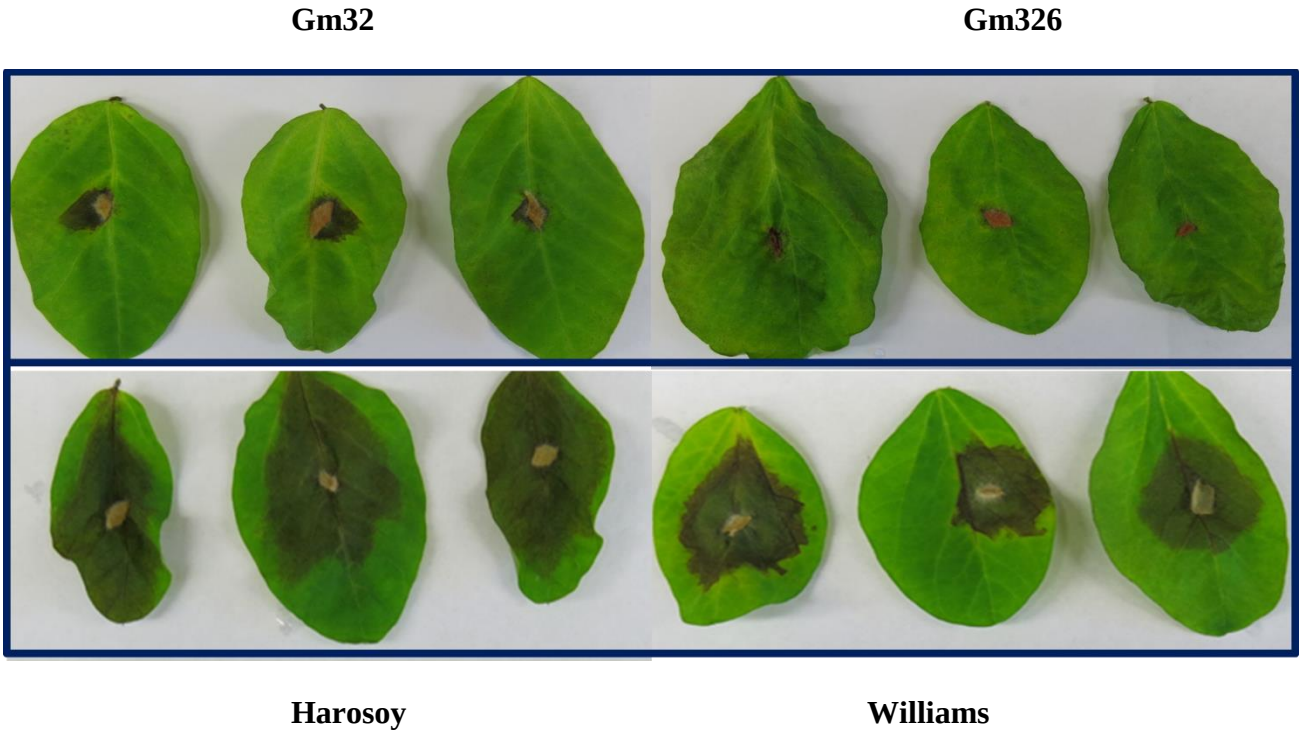

Figure 2.7: Mycelium-based Screening Assay Validation

Two *P .sojae* resistant (gm32 and gm326) and two susceptible (Williams and Harosoy) lines were screened using *P. sojae* race 2 mycelium plugs. Both gm32 and gm326 were shown to be resistant using a hypocotyl inoculation assay as described in Matthiesen et al, 2016 using isolates Iso1005 and Iso2004. Neither Harosoy nor Williams have any reported *Rps* genes. The lesion size clearly indicates that Williams and Harosoy are susceptible in our assay while gm32 and gm326 are resistant. A weak lesion did develop on gm32 but did not grow after the first three days, while the Harosoy and Williams lesion grew until they enveloped the entire trifoliate leaf.

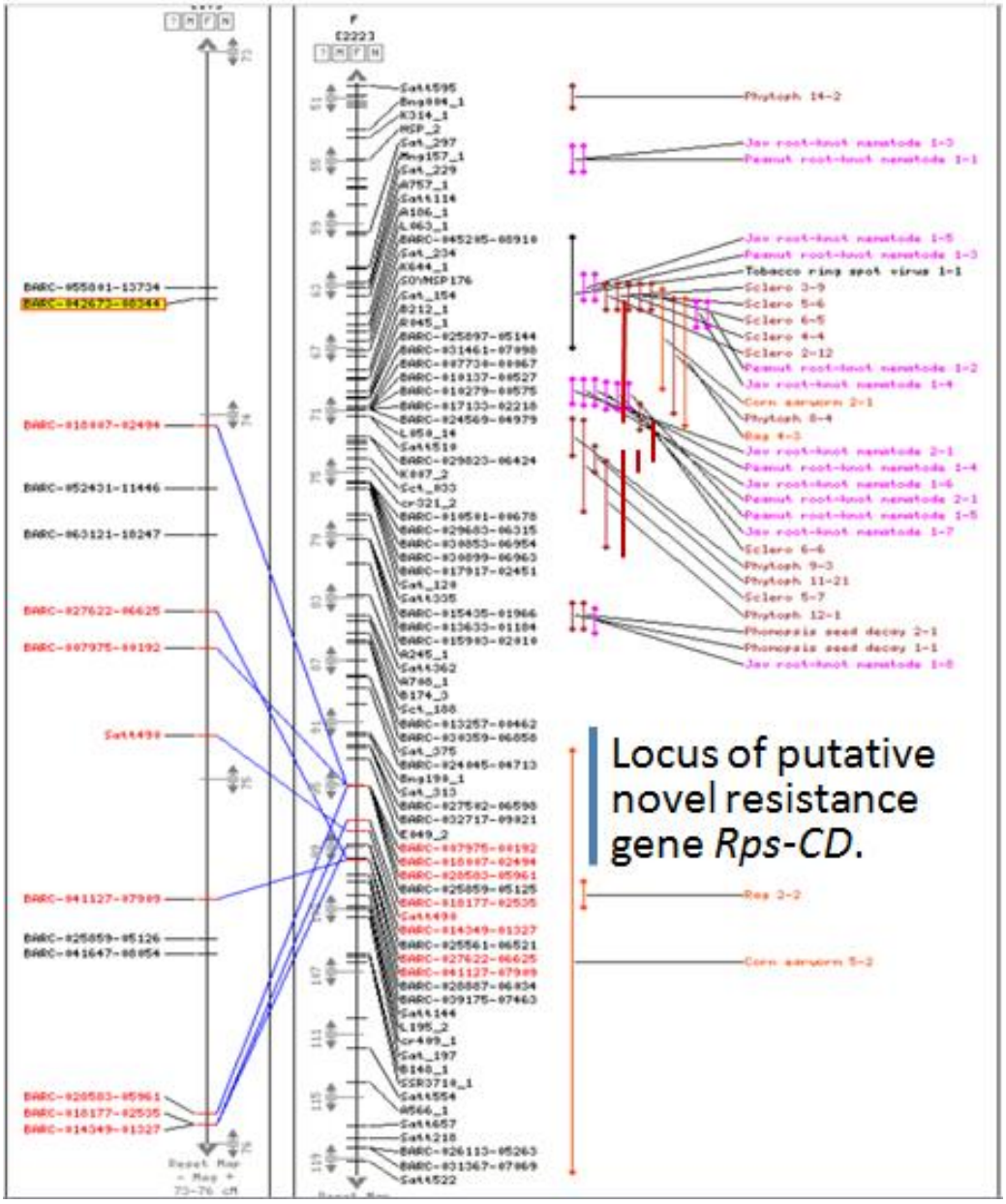

Figure 2.8: Genetic Map of Resistance based on Disease Assay in gm326

Preliminary phenotypic data gathered with our mycelium-based disease assay was combined with approximately 2000 SNP and SSR genetic markers from gm326 to the putative resistance gene Rps-CD. Our phenotypic data clearly maps to chromosome 13 of the soybean genome. However, the precise locus is still being determined. In the above figure, Rps-CD maps

to an approximately 300kb region of chromosome 13 which is denoted by the thick blue line. Thin blue lines correspond to SNP and SSR markers that segregate with the resistance phenotype. The right-hand side of the figure shows the loci of resistance genes against a variety of soybean pathogens. While chromosome 13 does have *Rps* genes, none are physically close to the mapped region. The locations of known *Rps* genes, within this region, are denoted by thick red lines. The results of this experiment are preliminary and need to go through validation, which is underway.

Resistant Parent	Susceptible Parent	Cross Identifier	Avh16 Response	Avh180 Response	Avh240 Response
PI408097	Williams	Gm320	Yes	Yes	Yes
PI408132	Williams	Gm326	No	Yes	No
PI407076	Williams	Gs2292	No	Yes	No
PI567139B	Williams	Gm32	Yes	No	Yes

Table 2.1: Summary of Soybean Parent Lines, Crosses, and Core Effector Responses

This figure summarizes the parental lines that were crossed to produce the populations that are screened in the above experiments. Additionally, it provides data about the initial responses of the parents to the three core effectors that are screened on segregating and RIL populations.

Effector	Cross	Homozygous Resistant	Segregating	Homozygous Susceptible	Expected Ratio	χ^2 Value	P-Value
Avh180	Gm326	14	27	13	1:2:1	0.07	0.97
Avh16	Gm32	21	45	23	1:2:1	0.10	0.95
Avh240	Gm32	19	47	17	1:2:1	1.56	0.46
Avh180	Gs2292	21	48	20	1:2:1	0.23	0.89

Table 2.2: $F_{2:3}$ Population Infiltration Screening Results

This table summarizes the effector-response phenotypic ratios in $F_{2:3}$ populations. We screened segregating $F_{2:3}$ lines for the phenotype of response to an effector with an HR. We observed an approximate 1:2:1 phenotypic ratio in all crosses. Four independent biological replicates were performed (n = 5 plants / 10 leaves per replicate). Statistical significance of our observed ratio was determined using a chi-squared test against a 1:2:1 expected ratio ($x > 0.05$). The observed ratios are not statistically significant suggesting that the response to the effector is provided by a single, dominant locus.

Effector	Cross	Homozygous Resistant	Homozygous Susceptible	Expected Ratio	χ^2 Value	P-Value
Avh240	Gm320	48	61	1:1	1.32	0.25
Avh180	Gm326	40	58	1:1	2.92	0.08

Table 2.3: RIL Population Infiltration Screening Results

This table summarizes the effector-response phenotypic ratios in RIL populations. We screened RIL lines for the phenotype of response to an effector with an HR. We observed an approximate 1:1 phenotypic ratio in all crosses. At least four independent biological replicates were performed (n = 5 plants / 10 leaves per replicate). Statistical significance of our observed ratio was determined using a chi-squared test against a 1:1 expected ratio ($x > 0.05$). The observed ratios are not statistically significant suggesting that the response to the effector is provided by a single, dominant locus. These results are currently being used for gene mapping.

Isolates	Cross	Resistant	Susceptible	Expected Ratio	$\chi 2$ Value	P-Value
Iso1005 and Iso2004	Gm326	40	48	1:1	0.56	0.46

Table 2.4: RIL Population Disease Assay Screening Results

This table summarizes the disease assay response phenotypic ratios in RIL populations. We screened RIL for the phenotype of resistance to *P.sojae* mycelium in trifoliate leafs. We observed an approximate 1:1 phenotypic ratio for both Gm320 and Gm326. At least four independent biological replicates were performed (n = 3 plants / 3 leaves per replicate) up to a total greater than or equal to 10 leaves per line. Statistical significance of our observed ratio was determined using a chi-squared test against a 1:1 expected ratio ($x > 0.05$). The observed ratios are not statistically significant suggesting that the response to the effector is provided by a single, dominant locus. These results have been used for disease mapping and are currently being validated.

Chapter 3: Validation of the Soybean *Rsv3* Gene Conferring Resistance to *Soybean Mosaic Virus*

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Abstract

Soybean Mosaic Virus (SMV) is ubiquitous in soybean fields in the United States and worldwide, and reduces both the quality and value of the produced seed. Soybean has three SMV resistance genes including *Rsv1*, *Rsv3*, and *Rsv4*. *Rsv3* confers resistance to SMV strains G5 through G7. The *Rsv3* locus has been mapped to a region containing five Nucleotide-Binding Domain Leucine-Rich Repeat (NBD-LRR) genes. Sequence comparison between these NBD-LRR genes from different resistant and susceptible cultivars has shown only one gene with nucleotide differences that are consistently associated with resistance. Differences between the alleles of this gene include non-synonymous SNPs (Single Nucleotide Polymorphisms), deletions, and insertions, showing that resistant and susceptible cultivars have different allelic forms of *Rsv3* mediating different defense responses. RNA sequencing studies suggest that the candidate gene (Glyma14g38533) is up-regulated following inoculation with SMV strain G7. Homology modeling of the candidate gene has revealed secondary structure differences in the LRR motif of susceptible cultivars in comparison to resistant cultivars. A transient expression assay using the *Rsv3* candidate gene and the recognized viral effector domain was performed to functionally assess if this candidate gene encodes *Rsv3*. Identification of *Rsv3* will allow for SMV resistant elite soybean cultivars to more easily be developed and utilized, improving soybean seed yield and quality.

Introduction

An ever-present threat to seed quality and, to some degree, quantity is the *Potyvirus*, *Soybean Mosaic Virus* (SMV). Seven strains of SMV, which spread from plant to plant by aphids, are known, and they are ranked based on virulence, G1 to G7, where G1 is the least virulent and G7 is the most [79]. SMV symptoms are specific to host and SMV strain group, but can include stunted plants, cringing and mottled leaves, mottled seed coats, and seed discoloration [80]. Seed coat mottling and discoloration affect the quality and value of the produced seed while stunting of the plant may lower yield.

Utilization of SMV resistant soybean cultivars is considered the most effective control for this disease. Three SMV resistance (*R*) loci have been identified in soybean and include *Rsv1* which is found on chromosome 13, *Rsv3* on chromosome 14, and *Rsv4* on chromosome two [81-85]. The *Rsv3* resistance gene has been stacked with *Rsv1* and *Rsv4* into an SMV resistant line known as RRR [86]. The *Rsv1* locus confers resistance to SMV strains G1 through G5, and has been extensively studied in the past [87] [88]. The *Rsv3* locus confers resistance to the most virulent strains of SMV G5 through G7, and *Rsv4* locus confers resistance to all seven strains of SMV [89] [90] [91] [92]. The *Rsv3* locus has been mapped to a 154kb region on chromosome 14 of soybean using simple sequence repeat (SSR) microsatellite markers [93] [94]. Within the *Rsv3* locus are ten genes including, 'Glyma14g38500', 'Glyma14g38516', 'Glyma14g38533', 'Glyma14g38561', and 'Glyma14g38586' genes that encode for coiled-coil nucleotide-binding domain leucine-rich repeat (CC-NBD-LRR) proteins [93] [94]. CC-NBD-LRR proteins can be broken down into three functional domains, the coiled-coil (CC) domain, the nucleotide-binding domain (NBD) and the leucine-rich repeat (LRR) domain [94] [95]. The LRR domain of NBD-

LRR genes is associated with pathogen recognition [96]. As many *R*-genes encode NBD-LRR proteins all five genes are candidates for *Rsv3* [94]. However, sequence analysis of the NBD-LRR genes points to Glyma14g38533 (G533) as the most likely candidate gene for *Rsv3*, and therefore will be our focus is this project [97].

Gene sequence comparisons of the five CC-NBD-LRR genes between resistant and susceptible soybean lines have identified G533 as the best candidate for *Rsv3* [97]. The mRNA sequencing and quantitative real-time PCR data indicates an up regulation of G533 in response to SMV infection [97]. One hundred and thirty one non-synonymous SNPs, two insertions, and four deletions have been identified when comparing G533 gene sequence of resistant and susceptible cultivars. G533 was the only CC-NBD-LRR candidate gene to show a large degree of sequence variability, with a conserved in-frame deletion and 95% SNP conservation in resistant cultivars. [97]. Notably, 91% of conserved SNP differences between susceptible and resistant lines were found within the protein LRR domain.

NBD-LRR proteins are known to be associated with effector-triggered immunity (ETI). ETI is the second line of defense for host plants against possible pathogens and provides the molecular basis for gene-for-gene resistance [27]. In gene-for-gene resistance, an *R*-gene recognizes a cognate avirulence gene (Avr-gene), which leads to an up-regulation of host defense-related genes. The cylindrical inclusion (CI) protein of SMV, which is involved in RNA replication, is the effector recognized by *Rsv3*. Soybeans lines with *Rsv3* have shown extreme resistance (ER) resistance phenotypes when tested with SMV strains G5-G7 [98] [99]. The ER phenotype results in no visible symptoms in host plant. The *Rsv3* resistance gene will be able to

produce *R*-proteins that can recognize the CI from SMV G5-G7 and up regulate host immunity gene expression [27].

Our previous work has identified G533 as the most promising candidate gene. We intend to functionally validate the *Rsv3* candidate G533 gene using our 3-part research pipeline. Preliminary research to identify a gene of interest based on conserved difference between resistant and susceptible cultivars at a given location [97]. Completed work includes homology modeling, cloning, and transient expression. Future work for this project will involve mRNA sequencing, candidate gene silencing, and the dissemination of information for use by breeders to improve soybean seed quality. Our approach can be summarized in Figure 3.1.

Results

Overview of Transient Expression Assay

Our approach to functionally validate G533 as the gene encoding *Rsv3*-type resistance is based on the presence of an HR resulting from transient expression of G533 and CI in *Nicotiana benthamiana*. DNA was extracted and sequenced from one SMV resistant (L29) and one SMV susceptible (Williams 82) *Glycine max* line. Due to high sequence similarity between G533 and other NBD-LRR genes in the Rsv3 region, G533 could not be specifically amplified using polymerase chain reaction (PCR). G533 cDNA was used to synthesize gene transcripts from L29 (G533-L) and Williams 82 (G533-W) using the sequence obtained from transcriptomic-read assembly and the 'SoyBase' sequence database, respectively (GenScript Inc. USA). Restriction sites were added to the ends of each synthesis product to facilitate entry into an expression vector. Gene synthesis products were transferred from pUC19 entry vectors to a dexamethasone-inducible expression vector pTA7001 [100]. pTA7001 vectors including G533 from L29 and Williams 82 were inserted into *Agrobacterium tumefaciens* strain GV3101 via electroporation. *A. tumefaciens* including pTA7001-G533-L and pTA7001-G533-W were infiltrated together into *N. benthamiana* and observed for the presence of an HR 72 hours after induction of gene expression by dexamethasone. The presence of an HR would indicate that the G533 protein is able recognize the CI protein and initiate a defense response. An overview of our approach is provided in Figure 3.2

Transient Expression of Glyma14g38533 and Viral Cytosolic Inclusion in *N. benthamiana*

To determine if G533 encodes *Rsv3* we transiently co-expressed G533-L and G533-W in *N. benthamiana* with CI. *N. benthamiana* was selected over *G. max* for our *in planta* experiments

because *A. tumefaciens* cannot efficiently transform soybean. Our screens utilized MMA buffer inoculations and un-inoculated tissue as negative controls. The known *Avr/R* pair Rpg-1b and AvrB was acquired from Dr. Aardra Kachroo of the University of Kentucky and used as the positive control [101]. Rpg-1b and AvrB were under the control of a constitutive promoter instead of a dexamethasone-inducible promoter. Rpg-1b and AvrB were infiltrated 24hrs after G533-L and G533-W were infiltrated with CI to account for differences in promoters and normalize the induction of gene expression. Negative controls and G533-W co-expressed with CI did not respond with a visual HR. G533-L co-expressed with CI did produce a visual HR but this response was weaker than the positive control and inconsistent among replicates. Rpg-1b/AvrB produced a strong, consistent, visual HR. An additional experiment was designed to include *A. tumefaciens* expressing EDS1 from Soybean (GmEDS1). EDS1 is required for plant immunity, and EDS1 from *N. benthamiana* may be insufficient for the interaction between G533-L and CI [102]. Visual HRs after the co-expression of G533-L, CI, and GmEDS1 were still weak and inconsistent. An ion leakage assay was utilized to determine if there was cell death that was not visible to the naked eye. Ion leakage experiments revealed significant increases in cell death between G533-L (30% cell death) co-expressed with CI when compared to G533-W (24% cell death) co-expressed with CI. Rpg-1b/AvrB produced the most cell death (44%) and was visible to the naked eye. The results of this screen are shown in Figure 3.3.

Homology Modeling of Conserved Insertion and Deletion regions of Glyma14g38533

To test our initial hypothesis that protein conformational changes exist between NBD-LRR proteins from susceptible and resistant lines, we first sought to identify conserved sequence differences in these lines. Clustal Omega multiple sequence alignment tools were used to first

compare nine resistant lines and three susceptible lines together and identify which regions are conserved between lines expressing the same resistance phenotype [103]. A 30 nucleotide deletion in all resistant lines was identified along with a 21 nucleotide insertion in all resistant lines [97]. The sequence alignment identifying the 30 nucleotide deletion is shown in Figure 3.4. Pairwise sequence alignments were also completed, as they are required to build a homology model. A sequence alignment was done between Williams 82 and a template protein of known structure (PDB ID: 3QFL). The same template was also used in an alignment with L29. The UCSF Chimera-integrated Modeller program was used to produce homology models based on the template protein of known structure and the sequence alignments previously discussed [104]. Initial attempts at modeling the entire LRR domain at the same time produced poor homology models as much of the LRR domain are structurally variable loops lacking secondary structure. Strengths and weaknesses of generated models were determined using model analysis tools available through SwissModel [105]. In order to create more accurate models we decided to only model the section most likely to show conformational change based on the multiple sequence alignments. The conserved ten amino acid deletion and seven amino acid insertion mutations mentioned in the multiple sequence alignment section were selected as regions of possible conformational change. Models were generated for both mutations in resistant L29 and susceptible Williams 82. Generated models for each conserved mutation were then overlayed to show structural differences. The results are summarized in Figure 3.5.

Homology Modeling of Glyma14g38533 LRR from Susceptible Cultivar 'Williams'

The recent publication of a homology model for the complete LRR domain of the L29 G533 allele allowed for modeling of the complete Williams 82 G533 allele LRR domain by

providing a template protein structure [97]. The UCSF Chimera-integrated Modeller program was used to produce and analyze the homology models [104]. Four LRRs out of 23 within the LRR domain showed structural differences between the published L29 LRR domain and the generated Williams 82 LRR domain. First, clear differences in the length and shape of the α-helix of LRR 6 could be seen between the two models. Second, differences in LRR 12 resulted in the early termination of an α-helix in the resistant allele. Third, the complete disruption of an α-helix was seen in the resistant allele of LRR 14. Finally, complete disruption of a β-sheet was seen in the susceptible allele of LRR 19. The results can be seen in Figure 3.6 and Figure 3.7.

Discussion

The primary goal of this project is to validate a candidate NBD-LRR gene as the *Rsv3* gene encoding resistance to SMV. The *Rsv3* genetic locus is known to provide resistance against the three most virulent strains of SMV [89] [90], but the gene encoding *Rsv3* has not been identified. Our work validating G533 as *Rsv3* included transient co-expression in *N. benthamiana* of G533 from Williams 82 and L29 with the viral CI which *Rsv3* is reported to recognize [98]. Homology modeling of the G533 LRR regions from sequenced L29 and Williams 82 cultivars was also used to predict protein structure differences between resistant and susceptible soybean lines. Information gathered in this study will be used in the development of elite soybean cultivars with resistance to SMV to prevent a reduction of seed quality and quantity.

Transient co-expression of G533-L and CI did not yield a consistent visual HR in *N. benthamiana*. The additional co-expression of GmEDS1 was also not sufficient to produce and consistent visual HR. However, ion leakage data suggests that G533-L co-expression with CI results in significantly higher rates of cell death than G533-W co-expressed with CI. This indicates that G533 from the resistant parent has an increase defense response to SMV than the susceptible parent. Neither G533 reached levels of cell death seen in the Rpg-1b/AvrB interaction which indicates that any apparent gene-for-gene recognition of G533 and CI is inefficient relative to Rpg-1b/AvrB. The presence of *G. max* proteins in a *N. benthamiana* system may not have the same effect as they would when expressed in Soybean. Little is known about other proteins required for an *Rsv3*-mediated defense response in Soybean. Many NBD-LRR genes interact with their cognate effectors through intermediary proteins such as guardees

or decoys [24]. It is possible that a protein required for CI recognition by *Rsv3* is not present in *N. benthamiana* or is too structurally different for G533 to recognize it.

It is not known if *Rsv3*-encoded protein structural differences exist between resistant and susceptible soybean lines. We utilized homology modeling to predict whether such differences exist. Though the identification of structural differences does not necessarily mean that these differences are responsible for phenotype, it would provide preliminary data upon which additional protein structure research could be based. The elucidation of the *Rsv3*-type resistance phenotype in soybean could be used as a model to identify and understand NBD-LRR-encoding resistance genes in other plant species. A better understanding of resistance gene function would allow for the creation of elite soybean cultivars that are unaffected by SMV and other pathogens resulting in higher crop yields.

The modeling of the complete Williams 82 LRR domain is a continuation of the modeling work published in Redekar et al and further helps us to understand which polymorphic sites could play a role in SMV resistance. In Redekar et al, the L29 LRR domain was modelled to indicate the location of polymorphic sites, but a comparative model using a SMV susceptible allele was not done [97]. The model of G533-W was generated using the published homology model of the LRR region of L29 as a template which had high sequence identity (>80%). This resulted in a highly confident model that was very similar in overall structure to the template. However, the resulting model revealed four clear structural differences between the L29 allele and the Williams 82 allele. The differences in the length and shape of the α-helix of LRR 6 and LRR 12 could be responsible for phenotypic differences. The complete disruption of an α-helix in the resistant allele of LRR 14 and of a β-sheet in the susceptible allele of LRR 19 are more

significant differences and should be studied in more detail. These observed differences further support the hypothesis that G533 encodes *Rsv3*, by providing evidence that the DNA sequence differences we see translate into actual differences in protein shape. Protein shape dictates protein function, and he we can show that a change in protein shape is predicted and this could lead to a change in protein function. Overall, this work will need to be confirmed with functional experiments, but it does give us insights into which parts of the protein may be responsible for SMV resistance.

Our work supports the hypothesis that G533 encodes *Rsv3*. There is more work to be done to conclusively validate the gene that encodes *Rsv3*. Silencing of the Rsv3 gene in SMV G7 resistant soybean cultivars is underway. If silencing of *Rsv3* from a resistant cultivar results in susceptibility, that would provide additional functional validate of the candidate gene. RNA sequencing of G533 transcript levels has been previously published, but RNA sequencing of defense genes downstream of the NBD-LRR would further validate G533. Elucidation of components in immune response of *Rsv3*-mediated resistance would allow for a thorough transient expression assay. Further expansion of the transient expression assay to include additional CI domains from strains of SMV that are not recognized by SMV and screening of the four other NBD-LRR genes in the *Rsv3* region would provide a complete picture and allow for validation of *Rsv3*.

Materials and Methods

Cloning and Vector Preparation:

High sequence similarity between Rsv3-candidate genes was inferred using gene sequence comparisons. G533 transcript sequence assembled from 'L29' cultivar [97] and the transcript sequence from 'Williams 82' obtained from Soybase (www.soybase.org) was artificially synthesized into pUC19 plasmid vector with N-terminal *XhoI* and C-terminal *SpeI* restriction sites (GenScript Inc. USA). We refer these Glyma14g38533 gene transcripts synthesized from L29 and Williams 82 as 'Gm533-L' and 'Gm533-W', respectively. These vectors were transformed into *DH5α E. coli* via electroporation and selected on LB-agar media with 50 ug/mL ampicillin [106]. The *Rsv3* effector gene encoding cytoplasmic inclusion (CI) was amplified from SMV-G7 virus, using primers CI-Xho14bF: 5'ATCGCT CGAGAT GAGTCT TGATGA GATTCA G3', and CI-Spe14bR: 5'GTATAC TAGTTT ACTGTA ATTGGA CTGCAT TCAAAA TGCCT3' to contain *XhoI* and *SpeI* restriction sites at N and C-terminal, respectively. This amplification product will be referred to as 'SMV-G7-CI'. The Gm533-L and Gm533-W inserts excised from pUC19 vector, and SMV-G7-CI were separately cloned into a dexamethasone-inducible kanamycin-resistant expression vector pTA7001, obtained from Dr. Nam Chua of Rockefeller University [100]. These expression vectors were electroporated into DH5α *E. coli* and transformed colonies were selected on LB agar media with 50 ug/mL kanamycin. The vectors were extracted from DH5α *E. coli* clones, transformed into *A. tumefaciens* strain GV3101 via electroporation and the transformed colonies were selected onto LB-agar media containing 100 ug/mL rifampicin, 50 ug/mL gentamicin, and 50 ug/mL kanamycin (LB-R-G-K) and cultured at 28°C for 48hrs. After 48 hours, individual colonies were

picked and re-plated on identical LB-R-G-K plates and incubated at 28°C for an additional 48 hours. Colonies were grown in LB-R-G-K liquid culture and plasmids were extracted to confirm the vector and insert presence in each colony via colony PCR and Sanger sequencing (Quintara Biosciences Inc.). Successful agro-transformed colonies of Gm533-L, Gm533-W and SMV-G7-CI were selected.

Culture Preparation

Gm533-L, Gm533-W, and SMV-G7-CI were inoculated into LB-R-G-K liquid cultures and grown overnight in a 28C shaking incubator. After 18-24 hrs, the bacterial growth of the cultures were pelleted and re-suspended in MMA (1x MS salt, 10mM MES, 200uM acetosyringone, 2% sucrose) buffer. After 2-5 hrs of room-temperature incubation, concentration of each bacterial solution was brought to OD_{600} of 1.0 SMV-G7-CI culture containing the viral component was mixed with either Gm533-L or Gm533-W, carrying the resistant and susceptible forms respectively, bringing the final concentration to OD_{600} 2.0. The positive control for this experiment was GV3101-INF1. *A. tumefaciens* strain GV3101 containing a constitutively expressed *P. infestans* elicitin (INF1), was similarly prepared to OD_{600} 1.0.

N. benthamiana Growth

N. benthamiana seeds were planted in 4-inch pots on a bed of firmly packed Sunshine Mix #1 potting soil with a half-inch top layer of loosely packed soil. Before planting, the soil was hydrated at a rate of 2L of water + 1 tsp Miracle Gro per 2.5kg dry soil. Pots were placed in plastic flats and kept covered with clear plastic lids for one week post planting to maintain humidity for germination. After one week, the lids were removed and the plants were grown for

an additional week at which point each pot was thinned down to one plant. Plants were then grown for an additional two weeks until ready for bacterial infiltration.

Infiltration

Four-week old *N. benthamiana* plants were infiltrated with transformed *A. tumefaciens* strain GV3101 via pressure-inoculation with a needleless syringe. Younger leaves, positioned near the top of the plant, ranging in length from 3-6 cm, were selected for infiltration. Each leaf received 4 total infiltration spots with the resistant interaction culture in the top two spots closest to the leaf petiole. Two additional leaf spots were infiltrated close to the leaf tip with the gene from the susceptible cultivar. The same process was repeated for multiple leaves on multiple plants for each experiment. Positive control (GV3101-INF1) and negative controls were used to validate the system for each experiment.

Dexamethasone Spray

The chemical dexamethasone (Dex) is required for the induction of the vector promoter and transcription of our genes of interest. 48 hours after infiltration, Dex was sprayed to allow for protein production. Dex was diluted to a concentration of 50uM in water and the surfactant Silwet was added at 0.03%. This mixture was transferred to a spray bottle which was used to spray both sides of each leaf with the pTA7001 vector infiltrated into it. Leaves with the positive control were not sprayed as its expression is not under the control of a Dex-inducible vector. Each sprayed plant was placed inside a plastic bag and sealed to prevent evaporation of the Dex before it could penetrate the leaf.

Scoring Infiltrated *N.benthamiana*

Plants were scored for the presence or absence of a hypersensitive response (HR) 24 hours after Dex spray and 72 hours post-inoculation. The HR is a localized cell-death defense mechanism indicated by wrinkling and a brown discoloration. For each leaf, the infiltration spots containing Gm533-L were compared with those containing Gm533-W. A differential response in which Gm533-L responded and Gm533-W did not was considered a positive result. Differential responses seen between Gm533-L and Gm533-W were compared to responses of the positive control INF-1 and various negative controls.

Ion Leakage Assay

Soybean leaves were inoculated using the above infiltration method and Dexamethasone spray. After 72 hours, six circular leaf punches (1 cm in diameter) were taken from inoculated areas being careful to avoid regions that were damaged by the infiltration process. Leaf discs were then floated in 45 mL sterile ddH_20 to reduce background ion leakage from cutting. After one hour, leaf discs were transferred to new glass tubes with 5 mL of water. After three hours of incubation, the water was removed and transferred to a 10 mL glass beaker. A handheld conductivity meter was used to measure the ion concentration. After conductivity readings were taken, the water was added back to the sample tubes. Samples were then boiled for 8 minutes to release all of the ions from the leaf discs. New conductivity readings were then taken which represent the total ion concentration of the leaf material. To determine percent ion leakage, the initial conductivity reading was divided by the total ion leakage. Three biological replicates were performed (n = 3 plants / 6 leaf punches per replicate). This analysis method compensates for minute differences in the total volume of the leaf discs used.

Homology Modeling

Homology modeling was completed using the UCSF Chimera-integrated Modeller program. Initial modeling attempts were limited in scope due to low sequence identity between template models that were available from the Protein Data Bank (PDB) and G533. The publication of L29 G533 LRR domain allowed for modeling of the complete W82 G533 LRR domain using the published model as a template. Modeller performed the homology modeling using default settings while UCSF Chimera allowed for viewing and analysis of the results. Template structures were downloaded from the PDB or supplied by collaborators and loaded into Modeller. Clustal Omega was used to align the template and target DNA sequences for each homology model. Produced models were color-coded, overlayed, and imaged to highlight the structural differences between models.

Figures and Tables

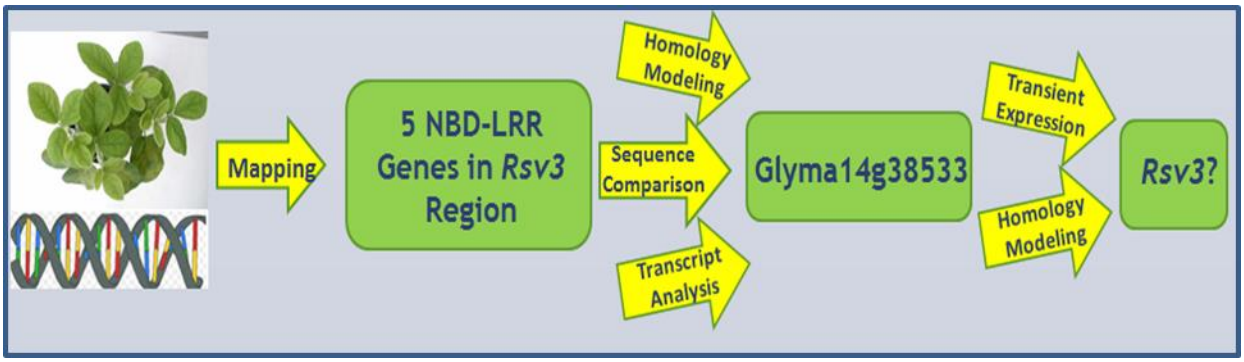

Figure 3.1: An overview of our approach to validation of the *Rsv3* gene.

Previous *Rsv3* mapping experiments identified a region containing five NBD-LRR genes. Published work including transcript analysis, sequence comparison, and homology modeling suggest that Glyma14g38533 encodes *Rsv3*. Our approach utilizes additional homology modeling along with a functional transient expression assay to validate Glyma14g38533 as *Rsv3*.

Figure 3.2: Design of expression vector for transient expression assay.

The dexamethasone-inducible vector pTA7001 was digested with *Xho1* and *Spe1* and synthesized DNA fragments were inserted into the multiple cloning site (MCS). The viral CI and Glyma14g38533 from W82 and L29 were each inserted independently. Engineered vectors were transformed into A. tumefaciens for transient expression in *N. benthamiana*. A hypersensitive response will result from the interaction of an R-gene and its cognate Avr-gene.

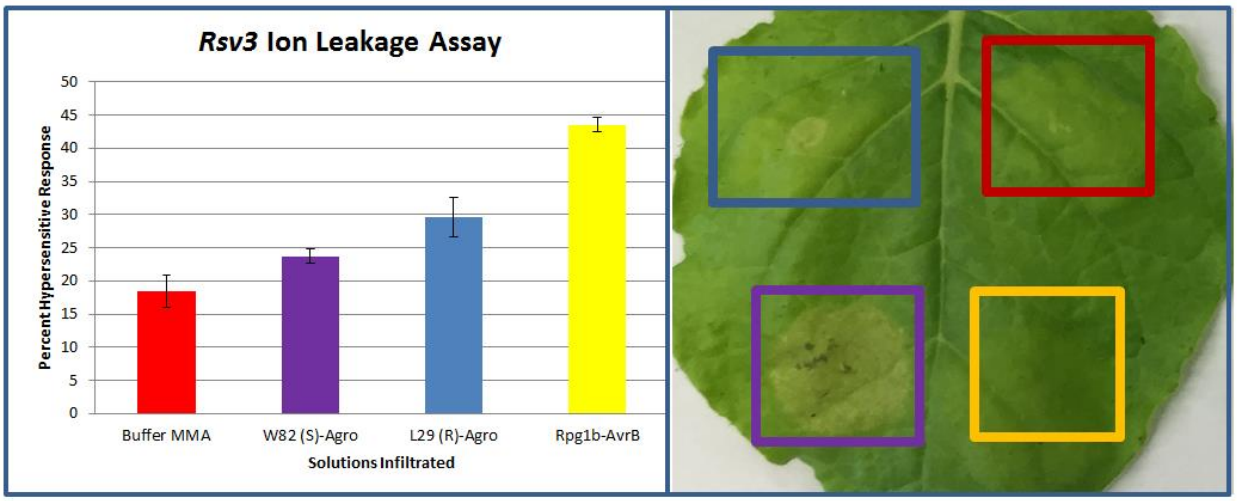

Figure 3.3: Transient Expression Assay Ion Leakage Results.

A. tumefaciens culture with vector containing G533 candidate gene from L29 mixed with culture containing viral CI gene is pressure-infiltrated with needless syringe in location denoted by the blue square and responded with 29.5% cell death. *A. tumefaciens* culture with vector containing G533 candidate gene from W82 mixed with culture containing viral CI gene is pressure-infiltrated with needless syringe in location denoted by the purple square and responded with 23.7% cell death. Positive control Rpg-1b is mixed with AvrB and infiltrated in location denoted by the yellow square and responded with 43.5% cell death. Negative MMA buffer control is infiltrated by similar manner in location denoted by the red square and responded with 18.4% cell death. Error bars determined based on standard error of the mean for 3 replicates.

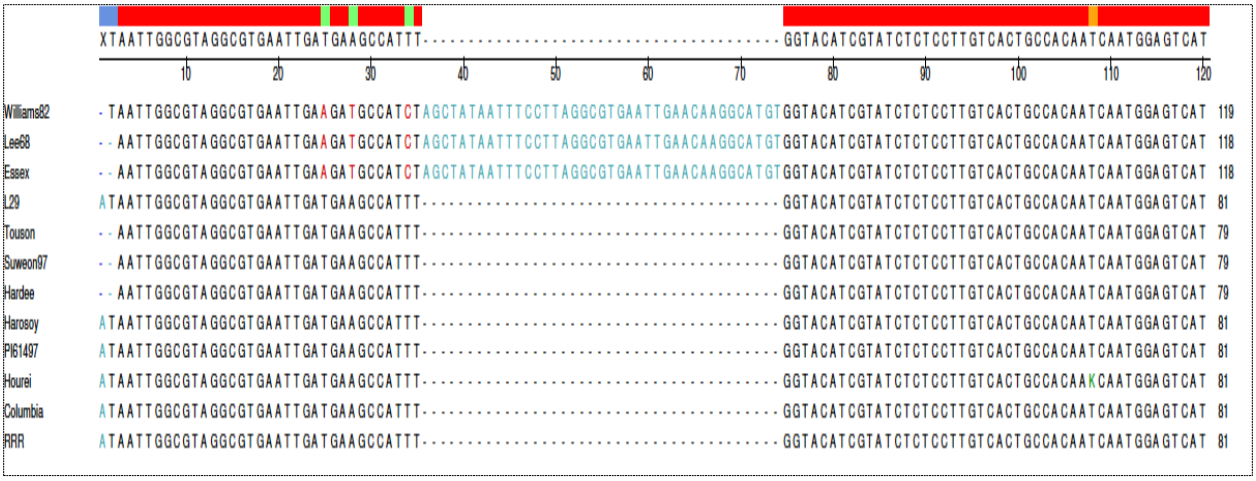

Figure 3.4: Multiple sequence alignment of 12 soybean lines.

The bottom nine lines exhibit *Rsv3*-type resistance while the top three exhibit susceptibility. Sequence alignment shows a 30 base pair deletion in all resistant lines. Deleted nucleotides are shown in blue while point mutations are shown in red. A conserved 21 base pair insertion in resistant lines was also aligned but is not shown. Homology modeling of this region will provide a better understanding if this conserved deletion in resistant lines results in structural protein changes.

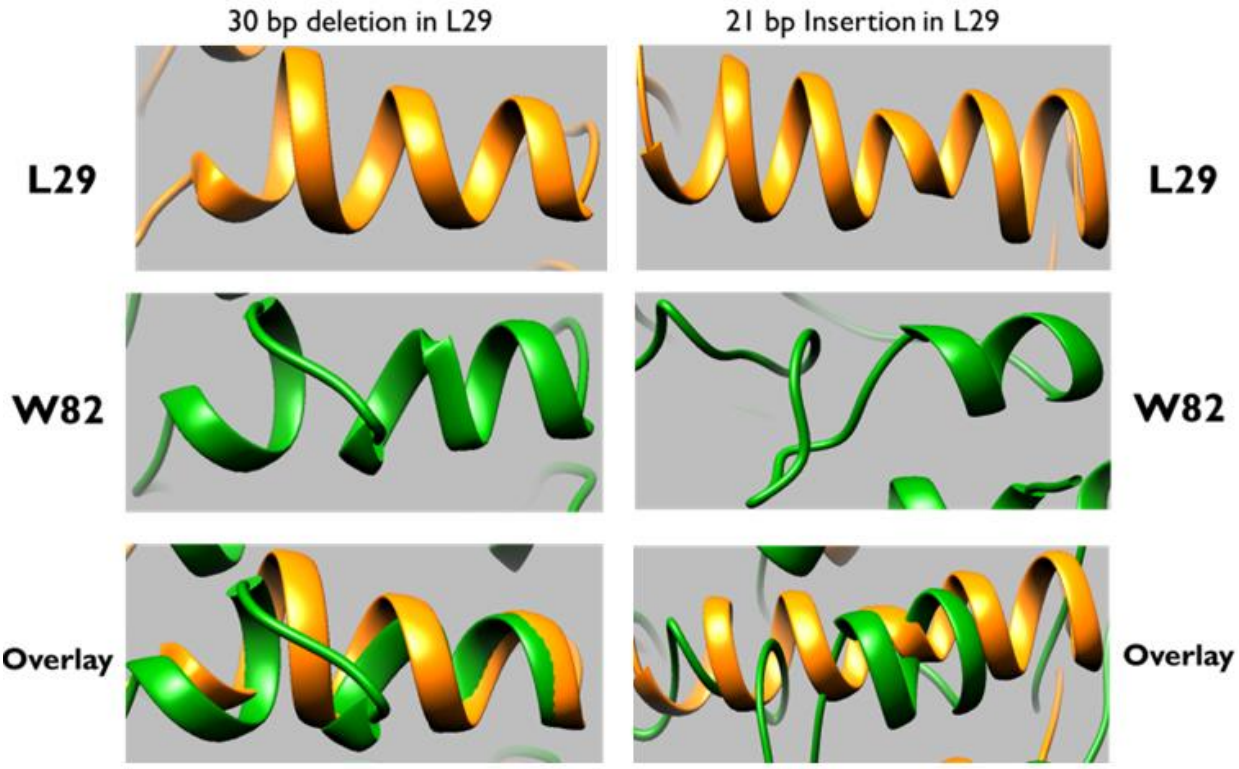

Figure 3.5: Homology modeling results of 21 base pair insertion in L29 line.

The top images show the α-helix structure of the region resulting from conserved insertions/deletions in the resistant cultivar L29. The middle images show the α-helix structure of the region resulting from conserved insertions/deletions in the susceptible cultivar W82. The bottom images show the overlay of the W82 and L29 insertion/deletion sites. A clear secondary structure difference can been seen and may account for phenotypic differences between the cultivars.

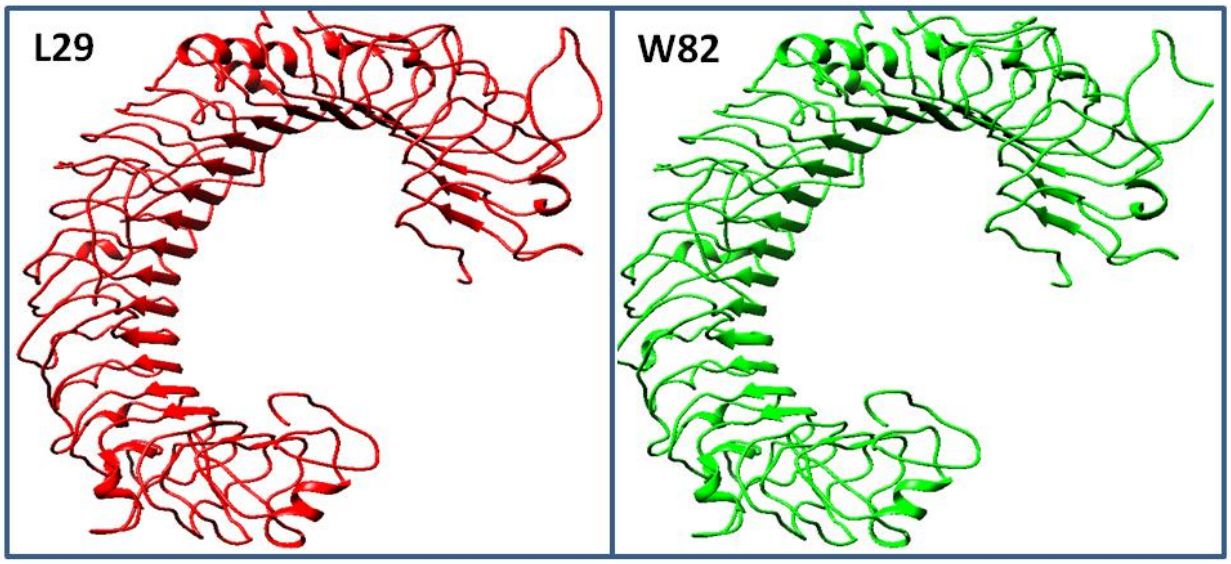

Figure 3.6: Side-by-side comparison of template L29 LRR and generated W82 LRR homology models.

The left model (in red) was published in Redekar et al and represents the entire Glyma14g38533 LRR from the cultivar exhibiting *Rsv3*-type resistance, L29. This model was used as a template for homology modeling of the model on the right (in green). The Glyma14g38533 LRR model from the susceptible cultivar W82 is highly similar in overall structure to L29. Structural differences were present and are highlighted in Figure 3.7.

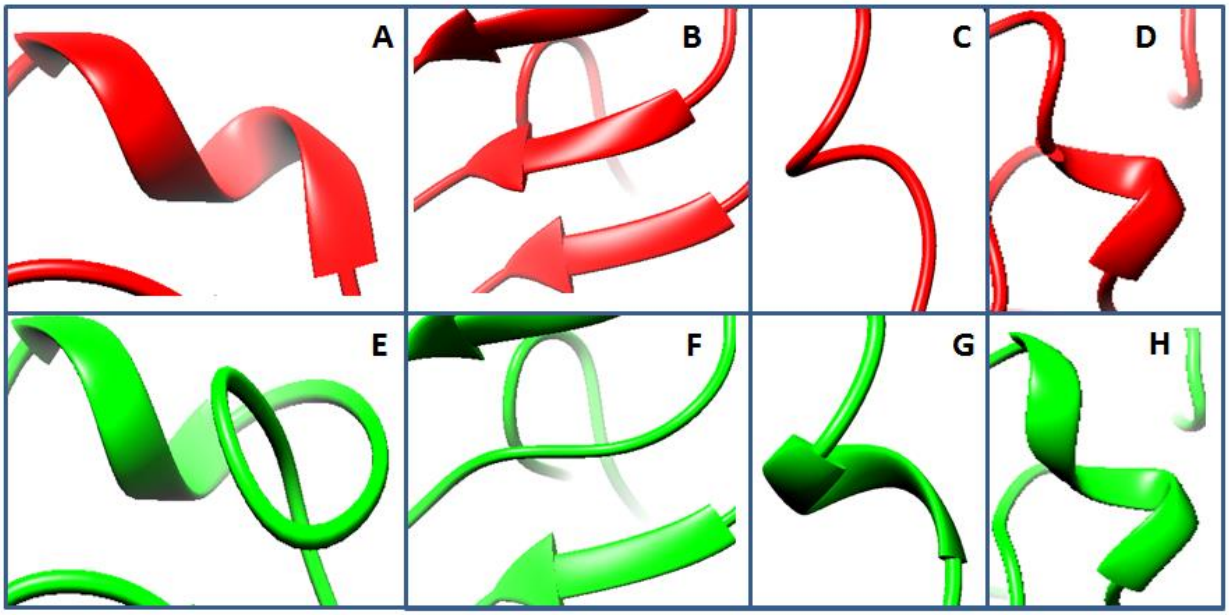

Figure 3.7: Comparison of four LRRs of Glyma14g38533 between L29 and W82.

Panels A-D are models of L29 while models E-H are images of W82. Model A and model E show differences in the length and shape of LRR 6 between L29 and W82. Model B and model F show the complete disruption of a β-sheet of LRR 19 in the W82. Model C and model G show the complete disruption of an L29 α-helix in LRR 14. Model D and model H show an early termination of an α-helix in LRR 12 for the resistant L29 allele.

Chapter 4: Effector-Directed Screens for New Disease Resistance Genes against the Soybean Root and Stem Rot Pathogen Phytophthora sojae

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Summary

Phytophthora root and stem rot disease suppresses global soybean production. We developed a system to screen soybean germplasm for *Resistance to Phytophthora sojae* (*Rps*) genes that recognize core *Phytophthora sojae* RXLR effectors. New *Rps* genes that target core effectors will likely be effective and durable against diverse *P. sojae* isolates. We developed a system using the bacterium *Pseudomonas* to deliver individual *P. sojae* effectors into soybean by Type III secretion. If the delivered effector is recognized by a resistance (*R*) gene, a visual hypersensitive response (HR) is produced. We used *P. sojae* avirulence (*Avr*) effectors to validate that this approach can recapitulate known *Avr/R* interactions. We delivered core *P. sojae* effectors *Avh*16, *Avh*180, and *Avh*240 into disease-resistant *Glycine max* germplasm. *G. max* accessions that produced a HR in response to core effector(s) were selected for crosses with *P. sojae*-susceptible cultivar Williams. We assayed genetic segregation of the response to three effectors in $F_{2:3}$ progeny derived from crosses of resistant X susceptible soybean. In all cases, we observed a simple, genetically dominant inheritance pattern that is consistent with gene-for-gene resistance. We will characterize and breed these potential *Rps* loci for commercial deployment this destructive disease.

Key Words: Breeding, Effector, Germplasm, Oomycete, Pathogen, *Phytophthora sojae*, Resistance, Soybean

Introduction

Phytophthora sojae, an oomycete pathogen, is the causal agent of *Phytophthora* root and stem rot disease on cultivated soybean, *Glycine max* [107, 108]. The United States of America (USA) is the world leader in soybean production, devoting 31% of its cropping area to soybean in 2014 and in 2015 [109]. Soybeans are crushed to extract valuable oil. The crushed remainder is processed into soybean meal [67]. Soybean meal is marketed globally as a food source for animals and fish [67]. In 2006, soybean crop losses due to *Phytophthora* root and stem rot were estimated at 1.46 million metric tons in the USA and over 2.32 million metric tons worldwide [65]. This was a loss of 1.75% of all soybean produced during that year in the USA [65]. Robust management options are needed to mitigate these losses.

P. sojae overwinters in dried plant tissue and soil, typically as oospore [43] ([108]. Germinating oospores can infect seedlings directly, or can produce sporangia, containing asexual zoospores [108, 110]. Once released, zoospores will swim towards root exudates, encyst, and invade root tissue with filamentous hyphae [111]. *P. sojae* hyphae grow intercellularly and produce feeding structures, called haustoria, which penetrate the cell wall and interface with the plant cell membrane [112]. *P. sojae* secretes effector proteins into soybean cells at this interface to suppress plant immunity and to promote virulence [108, 110, 112]. A compatible infection is propagated asexually during the soybean growing season [108, 110].

Pests and pathogens secrete effectors to subvert plant immunity [113]. The genome sequence of *P. sojae* revealed hundreds of genes encoding candidate effectors [114-117]. One large effector family, RXLR effectors, can enter plant cells [113]. These effectors contain an N-terminal signal peptide, RXLR-dEER motif, and an effector domain [118]. The *P. sojae* genome

contains nearly 400 genes encoding candidate RXLR effectors [119]. Many effector genes have variable sequence and expression in diverse *P. sojae* isolates [114, 119]. If a *P. sojae* effector is recognized by a soybean *Rps* gene (effector-triggered immunity or ETI), the plant produces localized cell death (i.e., a Hypersensitive Response or HR) [110]. These gene-for-gene interactions trigger a robust immune response which impedes pathogen growth [113].

Gene-for-gene resistance has been effective at controlling *Phytophthora* root and stem rot under field conditions [108, 110, 120]. A total of 24 genes have been identified. Of these, seven (*Rps*1a, *Rps*1b, *Rps*1c, *Rps*1k, *Rps*3a, *Rps*6, and *Rps*8) have been commercially deployed to manage this disease [108, 120]. Because *R* gene function is dependent on effector(s) recognition, *Rps* genes can be defeated when *P. sojae* genetic diversity includes isolates for which *Avr* effectors are mutated or silenced [108, 120]. *P. sojae* isolates that defeat all commercial *Rps* genes have been described [108]. The average effectiveness of a soybean *R* gene in the field was estimated at 8-15 years, underscoring the need to identify new sources of genetic resistance against *P. sojae* [121].

Effectors have been identified that interact with all of these *Rps* genes except for *Rps*8 [108]. In every case (*Avr*1a, *Avr*1b, *Avr*1c, *Avr*1k, *Avr*3a/5, *Avr*3b, *Avr*3c, and *Avr*4/6), all recognized effectors are from the RXLR family [110]. With the exception of *Avr*3b, these effectors make minor contributions to *P. sojae* virulence on soybean [110]. Non-essential *P. sojae* effector genes can be silenced, deleted, or mutated in response to *Rps*-mediated selective pressure with little or no fitness cost to the pathogen. These mechanisms for evading host immunity can subvert conventional *R* genes, rendering the *R* gene useless for disease control.

Effector-directed breeding strategies (termed “effectoromics”) can accelerate disease resistance breeding against plant pathogens [122]. Effectoromics has been successfully applied to improve potato resistance against potato late blight [123]. Vleeshouwers et al assayed 54 effectors from *Phytophthora infestans* for *Avr* activity on wild *Solanum* [123]. The RXLR family lpiO induced a HR on three species which revealed the *Rpi*-blb1 *R* gene. They identified the cognate effector *Avr*-blb1 by transiently expressing IpiO and *Rpi*-blb1 in a heterologous *Nicotiana benthamiana* system [123].

We chose to search for novel *Rps* genes which recognize core *P. sojae* effectors. We define core effectors as virulence genes that are highly expressed in early infection, conserved amongst diverse isolates, and necessary for virulence based on reverse genetic criteria. *Rps* genes against core *P. sojae* effectors will likely be more durable and more effective against genetically diverse isolates when compared to traditional *Rps* genes because *P. sojae* cannot easily discard a core effector without a major fitness penalty. In this study, we designed a system for delivering oomycete effectors from *Pseudomonas* into soybean. This system was used to screen soybean germplasm for potential new *Rps* genes against numerous core *P. sojae* effectors. *G. max* accessions with potential new *Rps* genes were crossed with susceptible cultivar Williams. Inheritance of three candidate *Rps* genes in $F_{2:3}$ populations suggested single, dominant loci, indicating that the effectors are recognized by R proteins that can be easily bred into elite soybean cultivars.

Results

Overview of the Screen

Our approach tests *G. max* for a localized cell death (hypersensitive response or HR) against specific pathogen effectors, when the effector is delivered to the inside of plant cells by a bacterial surrogate. A visual HR suggests the presence of a *R* gene in a tested population. To identify novel and durable *R* genes, we assayed *P. sojae* resistant soybean germplasm for HRs against core *P. sojae* effectors. *G. max* accessions which contained predicted *R* genes were crossed with susceptible Williams. *Avr* activity was assessed in $F_{2:3}$ populations to determine gene inheritance. An overview of the strategy is provided in Figure 4.1.

Identification of Resistant Soybean Germplasm

In a recently published study, Matthiesen et al. screened 1,019 Glycine accessions with a mixture of three *P. sojae* isolates which defeat 13 commercial *Rps* genes [68]. They predicted that 17% of the *G. max* accessions contained novel *Rps* loci [68]. We used these accessions in our screen with core effectors. We listed the *G. max* accession IDs and its corresponding plant introduction (PI) number for tested *G. max* germplasm in Table 4.1.

Identification of Core Effectors from P. sojae

This work will be described in a forthcoming manuscript [71]. In this study, Wise et al. identified 30 RXLR effectors (from ~400 predicted RXLR genes [124]) which are highly expressed at early time points in the infection and which are genetically monomorphic amongst four *P. sojae* reference [71]. These isolates collectively encompass much of the *P. sojae* genetic diversity in the USA [119]. RNA silencing of effector genes was used to test whether 17 effectors are important for virulence [71]. The results indicated that three effectors have major

effects on virulence, four have no effect, and the remaining ten have intermediate effects [71]. The three essential effectors (*Avh*16, *Avh*180 and *Avh*240) were subsequently silenced in an additional three reference isolates and were shown to be essential in all three isolates [71]. *Avh*16, *Avh*180 and *Avh*240 were shown to be highly expressed in all four *P. sojae* reference isolates [71]. These results suggest that *Avh*16, *Avh*180 and *Avh*240 are broadly important across the species, and we therefore selected them as the first effectors for our screen [71]. Germplasm was tested with nine additional effectors: *Avh*53, *Avh*137, *Avh*261, *Avh*488, *Snel*1, *Avh*94, *Avh*241, *Avh*23, or *Avh*110 [71]. This second set of effectors was chosen for screening based on gene expression and sequence conservation [71]. RNA interference experiments indicate that *Avh94* and *Avh241* make major contributions to virulence and that *Avh110* and *Avh23* only make moderate contributions to virulence. The remaining genes have not yet been tested with RNAi.

Development of a Bacterial System for Transient Effector Delivery

Our primary objective was to develop a system for delivering *P. sojae* effectors, one at a time, to the interior of plant cells so that recognition of the effector by an *R* protein would trigger a visible cell death response. The screens with *P. infestans* effectors were accomplished using *Agrobacterium*-mediated transient transformation [123]. This approach works well in tomato and related species, but soybean is recalcitrant to *Agrobacterium*-mediated transient transformation. Therefore, we decided to focus on developing a system through which the effectors could be delivered by the *Pseudomonas* Type III secretion system. Effector delivery was accomplished using the effector-detector vector (pEDV6) that provides a Type III secretion system (T3SS) signal [125]. Cloning into pEDV6 produces a *AvrRps*4-RXLR fusion product [125]. The bacterial *AvrRps*4 leader sequence enables secretion of the construct through the T3SS into plant

cells [125]. This approach has proven useful when screening for virulence or avirulence activity of *Hyaloperonospora arabidopsidis* RXLR effectors in *Arabidopsis* [125].

We chose several *Pseudomonas* strains to evaluate for the ability to deliver *P. sojae* effectors into soybean. *Pseudomonas syringae pathovar tomato DC3000* is a widely used reference strain. D28E is a mutant version of the reference strain *Pst DC3000* in which 28 Type III effectors have been removed by targeted deletions [126]. D28E (*Avr*PtoB) is D28E with the *Pst DC3000* effector *Avr*PtoB, which can potentially suppress PTI [126]. *Pseudomonas syringae* pathovar *glycinea* (*Psg*) race 4 is a virulent pathogen on soybean [127]. *Pseudomonas fluorescens (Pf0*) is a soil bacterium that is non-pathogenic and does not contain Type III effectors nor a T3SS [74]. *Pf0* strain EtHAN contains a transgenic, stably integrated operon of genes encoding the *Pst DC3000* Type III secretion components [74].

First, we tested whether these strains provoke a HR in soybean. *Pst DC3000* produced a visual HR on all tested soybean cultivars. No visual HR was observed on leaves in response to infiltration with *Pst DC3000* D28E, *Pst DC3000* D28E (*Avr*PtoB), *Psg* race 4, or *Pf0* EtHAN. Next, we tested whether these strains could deliver *P. sojae* effectors into soybean. We cloned *P. sojae Avr* effectors *Avr*1k, *Avr*1b, and *Avr*4/6 into pEDV6 and transformed the five *Pseudomonas* strains with these constructs. For each strain, we challenged cultivars containing *Rps*1k, *Rps*1b, and *Rps*4 with their cognate effectors. Each *Pseudomonas* strain was evaluated for its ability to produce a visual HR on *Rps* cultivars in known *Avr/R* interactions. Pf0 EtHAN was the only strain to produce a visual HR on soybean leaves in known *Avr/R* interactions.

Optimization of the Growth of EtHAN in planta

To improve HR consistency and intensity, we explored options to enhance effector delivery in EtHAN. We hypothesized that increasing the *in planta* growth of EtHAN might improve visual signal in response to *P. sojae* effectors. This hypothesis was based on prior work by Keen et al. that demonstrated that the *in planta* growth of *P. fluorescens* could be improved by co-infiltration with a virulent strain of *Psg* [128].

We confirmed these results by assaying the growth of EtHAN in soybean leaves with and without *Psg* race 4. The addition of *Psg* race 4 by co-infiltration improved the *in planta* growth of EtHAN two-fold in a compatible interaction (Figure 4.2). In an incompatible interaction, the addition of *Psg* race 4 augments the growth of EtHAN tenfold (Figure 4.2). This was seen in incompatible interactions of EtHAN delivering *Avr*1k, *Avr*1b, or *Avr*4/6 on soybean cultivars *Rps*1k, *Rps*1b, and *Rps*4 respectively (Figure 4.2). Based on these results, we chose to use *Psg* race 4 to augment the growth of EtHAN for screening soybean germplasm. There are no known *R* genes against *Psg* race 4 [127]. We confirmed that our starting germplasm was susceptible to this race by pre-screening accessions with this *Psg* race 4.

Optimization of Plant Growth

We optimized several aspects of plant growth to achieve consistent results with our system. We found that a light intensity of 250-300 µM to be key for the production of consistent macroscopic HRs during screening. Approximately 12-14 days post planting, *G. max* cultivars will begin producing immature trifoliate leaves. This is described as the V2 growth stage for soybean [129]. Because results are scored seven days post infiltration, senescence of the unifoliate leaves can interfere with data collection. We discovered that pruning the first set of

trifoliate leaves causes a reprogramming of the unifoliate leaves (Figure 4.5): After two days the unifoliate leaves become thicker, darker, and resilient (Figure 4.5). By removing trifoliate leaves at the stalk apex, unifoliate leaves are exposed to more direct light as they are unshaded. In *Avr/R* interactions, effector-responses were stronger and more consistent on unifoliate leaves from pruned plants compared to unifoliate leaves from unpruned plants.

A Facile Assay for P. sojae Virulence

We also developed an assay to determine *P. sojae* virulence on detached trifoliate leaves. Mycelial plugs from *P. sojae* race 2 produced large lesions on trifoliate leaves from susceptible cultivars which were wounded at the plug site: Williams and Harosoy (Figure 4.3). This isolate did not produce an expanded lesion on the resistant control cultivar *Rps*1k-Harosoy (Figure 4.3). Using this technique, we confirmed *P. sojae* resistance in *G. max* accessions, accession 32 (gm32) and gm326 (Figure 4.3).

Validation of the Pseudomonas/EDV Delivery System

After optimizing plant growth, the next step was to test whether *Avr*1k, *Avr*1b, and *Avr*4/6 can elicit a strong and consistent HR when delivered by EtHAN to soybean cultivars *Rps*1k, *Rps*1b, and *Rps*4 respectively (Figure 4.4ab). We quantified the consistency of the macroscopic HR as a percentage of infiltration sites (Figure 4.4b). *Avr1k* had the highest percent HR on *Rps*1k and *Avr4/6* had the lowest percent HR on *Rps*4 (Figure 4.4b). We produced similar results using *Rps* cultivars in the Williams background (Table 4.2). EtHAN alone, or with *P. s. glycinea race 4*, did not produce a HR on any tested soybean cultivar (Table 4.3).

To quantify cell death, we measured ion leakage from the soybean leaves into water using a conductivity meter. Infiltration of strains carrying *Avr*1k, *Avr*1b, and *Avr*4/6 into

cultivars containing a cognate *R* gene produced higher levels of ion leakage (20-30% higher) when compared with the Harosoy control (Figure 4.6). Co-infiltration of EtHAN and *Psg* race 4 produced some background cell death (Figure 4.6). These results indicate that the *Pseudomonas* delivery system is suitable for screening *G. max* germplasm to find novel gene-for-gene interactions.

Screening of Resistant G. max Germplasm with P. sojae Core Effectors

To identify potential new *R* genes, we screened 32 *G. max* accessions which are predicted to contain novel *Rps* loci [68]. Our first set of screens utilized EtHAN strains carrying the three most promising core effectors, *Avh16*, *Avh180*, and *Avh240*. From the 32 *P. sojae*-resistant *G. max* accessions, six accessions responded to *Avh*16 with a visible HR, six accessions responded to *Avh*180, and six accessions responded to *Avh*240 (Table 4.4). *Avh*7a, a homolog of *Avh*16, produced a HR on the same accessions that responded to *Avh*16 (Table 4.5). Three accessions responded to two effectors, and one accession responded to all three effectors (Table 4.4). Seventeen accessions did not respond to these three effectors. Additionally, six *G. max* accessions with partial resistance against *P. sojae* were included as blind negative controls and did not respond to effector treatment.

Screening Resistant G. max Germplasm with Additional Effectors

The positive results from screens with *Avh*16, *Avh*180, and *Avh*240, encouraged us to screen the same 32 *G. max* accessions with a second set of core *P. sojae* effectors, namely *Avh*53, *Avh*137, *Avh*261, *Avh*488, *Snel*1, *Avh*94, *Avh*241, *Avh*23, and *Avh*110 (Table 4.6). These effectors were selected by the same criteria as *Avh*16, *Avh*180 and *Avh*240, except that their function in P7076, P7074 and P7063 was not tested by silencing. We found that many *G. max*

accessions respond to one or more of these effectors (Table 4.6). *Avh*23 and *Avh*110 did not provoke a HR on any tested accessions.

Genetic Segregation of Effector Response in $F_{2:3}$ Populations

Seven effector-responsive accessions were selected for crossing with susceptible Williams (Table 4.7). We chose to breed resistant accessions with Williams because it has a sequenced genome [75] and no *Rps* genes [76]. A sequenced genome enables us to design single nucleotide polymorphism (SNP) chips which are essential for soybean locus mapping. A SNP is a nucleotide variation at a genomic position within a population and these chips can assay multiple SNPs concurrently [130]. Williams was chosen for breeding based on maturity group as well. The maturity group refers to the cultivar development period which is characterized by growth stages [129]. Soybean producers use maturity group information to accurately time chemical treatments. Williams' 3.9 maturity group is ideal for growth in the Midwest.

We analyzed $F_{2:3}$ seed for two crosses, accession 32 (gm32) x Williams and gm326 x Williams. Because most known *R* genes segregate as single, genetically dominant loci, we predicted that the HR response to the effectors would segregate similarly, producing a 1:2:1 phenotypic ratio in segregating $F_{2:3}$ populations.

We tested segregating $F_{2:3}$ families for *Avr* activity using the effector delivery system. For gm32, we observed a phenotypic ratio not significantly different ($x > 0.05$) than 1:2:1 in response to *Avh*16 and in response to *Avh*240 (Table 4.8). For gm326, we observed a phenotypic ratio not significantly different ($x > 0.05$) than 1:2:1 in response to *Avh*180 (Table 4.8). We conclude that gm32 likely contains two dominant *R* genes and gm326 likely contains one dominant *R* gene.

Discussion:

Phytophthora root and stem rot disease on soybean was first described in 1954 by soybean farmers in southwestern Ontario and in the Midwest [131]. *P. sojae* owes its "success" as a crop pathogen to a plastic genome which supports rapid evolution. *P. sojae* isolates have been described which defeat all commercial *Rps* genes. To mitigate this disease in the future, we need new and robust disease management options. Genetic resistance is the most economic tool, but most *R* genes against *Phytophthora* pathogens are not durable because they recognize effectors that do not play essential roles in virulence.

Our strategy is based on screening for novel *R* genes against core *P. sojae* effectors and to breed these *R* genes for commercial deployment. To probe soybean germplasm recognition for core *P. sojae* effectors, we first needed a flexible system for transient expression in soybean. We exploited the *Pseudomonas* T3SS for effector delivery into soybean, drawing from early work by Staskawicz et al., who produced visual cell death symptoms by transforming *Psg* race 1, race 4, and race 5 with molecular components from *Psg* race 6 [132]. *Avr*B1 was shown to produce an HR on *Rpg*1 [127]. Keen and Buzzell transformed *Psg* race 4 with *Avr*D from *Pst* and observed visual incompatibility [133]. They suggested that *Rpg*4 may be responsible for *G. max* resistance against *Pseudomonas* carrying *Avr*D [133]. These experiments demonstrate that *Psg* is capable of delivering exogenous, plasmid borne effectors to soybean leaves in sufficient quantity to produce *Avr* activity in *Avr/R* interactions. Using this information, we constructed a *Pseudomonas* system for transient expression in soybean.

Our system uses Type III secretion by *Pf0* strain EtHAN to deliver *P. sojae* effectors. A critical component of effector delivery to *G. max* by EtHAN is the addition of *Psg* race 4 to the

inoculum. We hypothesize that *Psg* improves the growth of EtHAN *in planta* by suppressing plant immunity. To validate this system, we exploited known *Avr/R* interactions between *P. sojae Avr* effectors and *G. max R* proteins. When *Avr*1k, *Avr*1b, and *Avr*4/6 were delivered to *G. max* cultivars which contained a cognate *R* gene, we observed visual HRs. We confirmed cell death symptoms using an ion leakage assay. This new system gave us the unique ability to study oomycete effectors in soybean. Importantly, the bacterial system recapitulated the same gene-for-gene specificity as is seen in experiments with *P. sojae* containing the same *Avr* genes. In fact, the previously documented strength of resistance was recapitulated in our system: The response to *Avr*1K was strongest and the response to *Avr*4/6 was weakest, mirroring the strength of the responses in either *P. sojae*-based assays or when the effectors are delivered by biolistics [134, 135]. Thus, this system was validated for studying oomycete effectors in soybean. The broad applicability of EtHAN as an effector-screening system is further underscored by recent reports of its successful use in monocot wheat leaves [136, 137].

We tested 12 core effectors, and observed that ten produced a visual HR on at least one of the 32 tested *G. max* accessions. Six accessions responded to one effector, twelve accessions responded to two effectors, seven accessions responded to three effectors and one accession responded to four effectors. By selecting for *P. sojae* resistant germplasm and by then selecting for accessions with no known resistance, we likely enriched our germplasm with accessions containing new *Rps* genes.

We selected seven accessions which produced a HR in response to a core effector(s) for breeding with susceptible Williams. We observed *Avr* activity in $F_{2:3}$ progeny from crosses, gm32 x Williams and gm326 x Williams. In $F_{2:3}$ populations, a 1:2:1 phenotypic ratio was

expected and observed suggesting dominant inheritance. We concluded that gm32 likely contains *Rps* genes which recognize core *P. sojae* effectors *Avh*16 and *Avh*240. Gm326 likely contains one dominant *Rps* gene which targets *Avh*180.

In addition to resistance loci *Rps*1 to *Rps*6 for which *Avr* effectors have been identified, twelve additional *Rps* loci have been described [120]. These uncharacterized resistance loci may explain some of our *Avr* activity. In 2013, Lin et al. independently mapped two *R* genes in PI567139B, *Rps*Un1 and *Rps*Un2 [78]. We independently identified effector-responses in PI567139B against *Avh*16 and *Avh*240. This effector-response may correlate to *Rps*Un1 and *Rps*Un2. It is unlikely that effector-responses were due to effector-toxicity in soybean leaves because effector-responses were accession specific and were similar to effector-responses by *Rps* cultivars in known *Avr/R* interactions.

Effector-directed breeding provides many advantages over classical pathogen-based breeding. Effector-directed breeding allows for the identification of potential *R* genes which recognize core pathogen effectors. This approach can be applied to find potential *R* genes against any pathogen with a sequenced genome and identifiable effectors. Non-host plants can be screened for effector recognition as well. Effector-directed breeding can facilitate stacking *R* genes.

We suggest using both effector-directed breeding and pathogen-based screening to identify novel, durable *R*-genes. This may help to eliminate accidental duplication of *R* loci during resistance breeding. Mapping the same population using both *Avr* activity and pathogen screens will create a multidimensional map to more accurately determine resistance mechanisms. For this reason, we have developed a technique to detect *Avr* activity in simultaneous effector

and pathogen assays. This system will yield effector data and pathogen data for a single individual.

In principle, our effector delivery system could be used to screen any host plant for *R* genes against a pathogen for which effectors are characterized. EtHAN was used to successfully deliver effectors to wheat leaves [136, 137]. This demonstrates that EtHAN may be useful for effector delivery in a broad range of systems. Our immediate goal is to use effector data and pathogen data to map potential *Rps* genes in soybean RI lines. In the future, we hope to introduce new and durable *Rps* genes to soybean growers for protection against *Phytophthora* root and stem rot.

Materials and Methods

Effector Plasmid Clones

Clones encoding RXLR effectors were cloned into pENTR using the pENTR™/D-TOPO® Cloning Kit – ThermoFischer Scientific or they were provided by the Tyler lab at Oregon State University. The coding region of the ORF began with a methionine codon, followed by the first codon following the signal peptide cleavage site as predicted by SignalP. An LR reaction was used to clone the effector genes into the destination vector pEDV6 [74]. Constructs were transformed into10 *E. coli*. Single colonies were selected on KB agar containing Gentamycin (10 µg/µl) and were screened for the desired plasmid by PCR, using a pEDV6 forward (F) primer and a gene specific reverse (R) primer. Positive transformants were maintained at 37 °C under Gentamycin selection.

Transformation of EtHAN by Tri-Parental Mating

Pf0 strain EtHAN was provided by the Chang lab at Oregon State University [74]. EtHAN was transformed using tri-parental mating as follows: Bacterial strains RK600, EtHAN, and positive transformants in top10 *E. coli* were streaked onto KB agar plates with the proper selection. Onto one King's Broth (KB) agar plates, we streaked our positive transformants in top10 then the helper strain RK600 followed by EtHAN. The plates were incubated for three days at 28 °C. Following the incubation, colonies were streaked onto KB agar containing Chloramphenicol (10 µg/µl), Nitrofurantoin (20 µg/µl), and Gentamycin (10 µg/µl). Single colonies were screened by PCR using the pEDV6 F primer and a gene specific R primer. All bacterial strains are described in Table 4.9. A list of primers used for cloning can be found in Table 4.10.

Culture Preparation

Bacterial cultures were prepared from single colonies and inoculated in 5 mL aliquots of KB media containing the appropriate antibiotic selection. The cultures were incubated at 28 °C for 18-22 h. The bacteria was pelleted and re-suspended in 10 mM $MgSO_4$. EtHAN and EtHAN-effector constructs were prepared at an OD = 0.6. For co-infiltration, EtHAN and *Psg* were mixed for at ODs = 0.6 and ODs = 0.3, respectively for a final OD = 0.9.

Plant Growth Conditions

G. max was planted in Fafner P2 potting media. About 1.5 kg Fafard P2 potting media was hydrated with 2 L of water with Cal-Mag fertilizer. 4 inch pots were packed firmly with media. 5 seeds were sown per pot and covered with about 2-3 cm of loosely packed potting media. The top soil was treated with Marathon insecticide, put into flats (8 plants per flat), and covered with clear plastic lids to maintain humidity during germination. After 24-36 h, the lids were removed from the flats. Germination rates were >95% for all *G. max* tested. *G. max* were germinated and maintained under diurnal light (16 h light at 22 ^{0}C; 6 h dark at 20 ^{0}C). Light intensity was maintained between 250-300 µM for all experiments.

Effector Infiltrations

Plants were infiltrated after 15-17 days (3 days post pruning). Bacterial solutions were prepared 1 hour prior to infiltration. *G. max* unifoliates from pruned plants infiltrate best 1 h after the lights turn on. Needless syringes were used to pressure infiltrate unifoliate leaves. Each unifoliate received 4 infiltrations of 100-200 uL of bacterial solution. The identical process is performed on the adjacent unifoliate. Co-infiltration of EtHAN and *Psg* did not produce a visible HR on any tested *G. max* cultivar. The best negative control is to co-infiltrate EtHAN-effector

and *Psg* into *G. max* cultivars which contain no *R* gene against the cloned effector. A list of soybean cultivars which were used for these experiments is detailed in Table 4.11.

Scoring

Seven days post infiltration (DPI), the unifoliate leaves were detached, imaged, and scored. A plant scored positive if ≥ 5/8 infiltration spots produced a visual HR on both unifoliate leaves (Figure. 4.7b). Plants were scored as negative if ≤2/8 infiltration spots produced a visible HR (Figure 4.7b). Plants that fell between 3-4/8 were scored as ambiguous (Figure 4.7b). Any data, scored as ambiguous, was discarded, and the experiment was repeated until the appropriate sample size was reached. To calculate % HR, we used the equation, $\%\ HR = (\#\ of\ responding\ plants) \div (\#\ of\ infiltrated\ plants)$.

If too much pressure is applied during infiltration, the damage may induce a cell death response around edges of infiltration sites (Figure 4.7a). This can make scoring weak HRs more difficult. If the cell death response is weak, the infiltration site will turn brown with very small HRs throughout the site (Figure 4.7a). Cell death can occur on negative control plants if disease or insects are present (Figure 4.7c). The Compendium of Soybean Diseases was used as a reference to identify disease [129].

Determination of R Gene Inheritance in $F_{2:3}$ Populations

Plants from segregating $F_{2:3}$ families were screened with a single effector at a time. Prior to screening, we determined the background cell death each effector produced on Williams. $F_{2:3}$ families were tested with an average sample size c. 15 individuals per family or line. Seed was limited.

Determining $F_{2:3}$ Phenotypic Ratios

After compiling the results, we analyzed the data as % HR with variation represented as standard deviation (σ). We determined our range for homozygous dominant using the equation, $RR = 100\%\ to\ (100\% - 1\ parental\ \sigma)$. We determined our range for homozygous recessive using the equation, $rr = 0\%\ to\ (Williams\ \%\ HR + 1\ Williams\ \sigma)$. Families that fell within the two ranges are considered to be segregating.

In planta Bacterial Growth Curves

All bacterial strains were grown at 28 °C on KB agar containing the appropriate antibiotic selection. Single colonies were inoculated in 5 mL liquid KB media containing antibiotic selection. The cultures were incubated at 28 °C for 18-22 h. Bacterial cultures were then pelleted by centrifugation at 2,000 x g for 15 minutes and resuspended in 3-5 mL 10 mM $MgSO_4$. Optical densities (OD) were calculated by measuring absorbance at 600nm (OD_{600}). Bacterial solutions were then diluted to appropriate ODs with 10 mM $MgSO_4$. In bacterial growth assays containing only one strain of bacteria, solutions were pressure infiltrated into soybean unifoliates using a needless syringe at an OD_{600} = 0.01. In the case of co-infiltration experiments, the target strain (EtHAN) for the assay was infiltrated at an OD =0.01 while the non-target strain (*Psg*) was infiltrated at an OD = 0.3. Leaf disks were collected at 0 days post inoculation (0 DPI) and 5 days post inoculation (5 DPI). Leaf disks were homogenized in 200 µL 10 mM $MgSO_4$ using 1 mm glass beads in a bead beater. Samples were transferred to a sterile 96 well plate and diluted 1:10 serially. The dilution series was plated on KB agar containing the appropriate antibiotic selection. Plates were placed at 28 °C for 2 days at which point colony forming units (CFU) were counted (n = 5 plants / 10 leaves per replicate). Three biological replicates were performed.

Ion Leakage Assay

Soybean leaves were inoculated according to the methods for the visual hypersensitive response assay. After seven days, six circular leaf punches (1 cm in diameter) were taken from inoculated areas. Leaf disks were then floated in 45 mL sterile ddH_20 to reduce background ion leakage from cutting. After one hour, leaf disks were transferred to new tubes with 5 mL of water. After a three hour incubation, the water was removed and transferred to a 10 mL beaker. A handheld conductivity meter was used to measure the ions in solution. After conductivity readings were taken, the water was added back to the sample tubes. Samples were then boiled for 8 minutes to release all of the ions from the leaf disks. New conductivity readings were then taken to measure total ion leakage. To determine percent ion leakage, the initial conductivity reading was divided by the total ion leakage. Three biological replicates were performed (n = 3 plants / 6 leaf punches per replicate).

In vitro and in planta Plasmid Ejection Assay

Plasmid ejection of pEDV6 containing *P. sojae* avirulence genes *Avr*1K, *Avr*1B, or *Avr*4/6 was measured in all *Pseudomonas* strains used in this study. Bacterial colonies were grown in a 5 mL liquid KB cultures containing the appropriate antibiotic selection. Bacteria were pelleted and resuspended in 10 mM $MgSO_4$. For the *in vitro* assay, the bacterial solution was left to incubate at 28 °C and 10 µL aliquots were plated every 15 minutes onto KB agar containing an antibiotic to select for the bacteria and Gentamycin to select for the pEDV6-effector plasmid. Three independent biological replicates were performed (n = 3 cultures per replicate). The plates were then incubated for two days at 28 °C and CFU were counted. The *in planta* bacterial growth curve described above was used to observe plasmid ejection after

pressure infiltration of the culture into soybean leaves. For the *in planta* assay, time points were at 0 DPI and 3 DPI.

P. sojae Mycelium Plug Infection Assay on Detached Trifoliate Leaves

P. sojae was maintained on a PARP V8 plate. Three weeks prior to infection, a mycelium plug is transferred from a PARP V8 plate to a V8 plate. After three weeks, the culture should colonize most of the V8 plate. Contaminated plates should be discarded. Fresh mycelium plugs should be taken from freshly colonized V8 plates for the best results.

At 14 days post planting, trifoliate leaves should appear expanded. Per plant, remove each trifoliate and place each leaf in a wetted petri dish with a piece of filter paper. Experimental design will depend on leaf and culture sample size. On average, each petri dish will contain three leaves from three different individual plants. This allows for testing individuals with multiple pathogen isolates during a single replicate.

When ready to infect, scar each leaf in the center and place a 1cm mycelium plug on top of the scarred area. Parafilm the plates to maintain high humidity. Disease symptoms will begin at about 48 hours post infection. The symptoms conclude after seven days. Each culture should be passage every 1.5 months to maintain virulence.

Figures and Tables

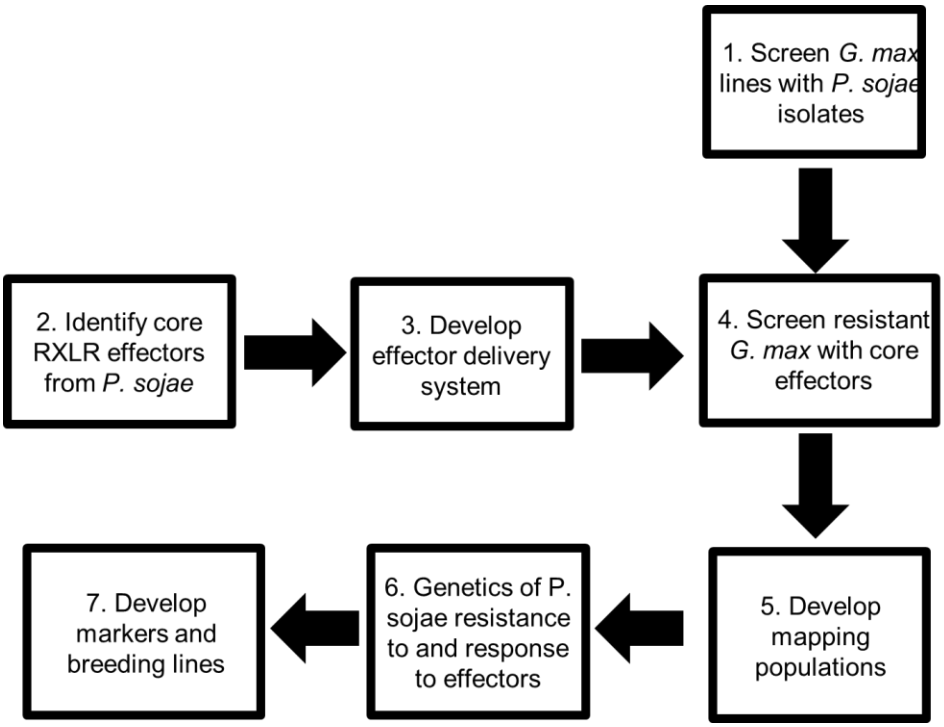

Figure 4.1: An overview of effector-directed breeding in soybean. This summarizes the development of soybean lines with R genes that recognize core P. sojae effectors.

1. *Glycine max* germplasm is screened with a collection of isolates that collectively break all commercially available *Rps* genes. **2.** Core effectors are identified with functional genomics tools. **3.** A bacterial system to individually deliver those effectors to plants is developed. **4.** The *P. sojae*-resistant germplasm from Step 1 is screened with core effectors. **5.** Lines with putative *R* genes against the effectors are crossed to a susceptible line, and the progeny from the cross are used to develop mapping populations. **6.** Genetic analysis is used to validate the phenotypes and identify lines in which effector recognition and pathogen resistance are conferred by the same locus. **7.** Such lines will be used to develop breeding lines.

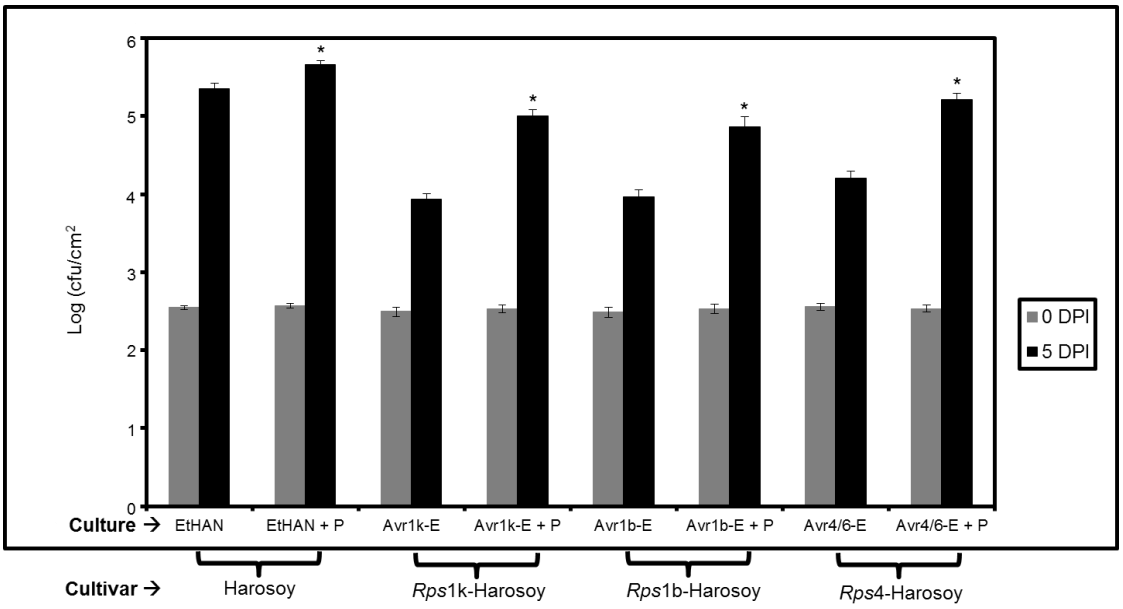

Figure 4.2: Co-infiltration with virulent Psg race 4 improves the growth of EtHAN in planta.

EtHAN or EtHAN with the indicated *Avr* genes was infiltrated into the indicated plant cultivars that carry *R* genes against the cognate *Avr* effector proteins. The addition of *Psg* race 4 to a culture is indicated by a "+ P". These data were produced from one biological replicate (n = 5 plants / 10 leaves per culture). Standard deviation represents leaf-to-leaf variation. Statistical significance of *Avr-Psg* race 4 CFU was determined by a pairwise t-test against the cognate *Avr-P. f.* EtHAN CFU ($p < 0.05$) and is denoted by an *. Two additional biological replicates produced similar results.

Figure 4.3: A simple P. sojae mycelial plug assay for testing pathogen virulence on detached soybean leaves.

We detach trifoliate leaves after the V2 growth stage. These trifoliate leaves are inoculated with P. sojae plug. The infection is scored at 7 DPI. *P. sojae* produced large brown/black legions on susceptible cultivars Williams and Harosoy in all cases. *Rps*1k-Harosoy is included as a positive control for resistance against *P. sojae* race 2 and showed no expanded disease legion. *P. sojae* race 2 did not produce expanded disease legions on gm32 or gm326 suggesting that they are resistant to this isolate. This figure was produced from one biological replicate (n = 3 plants / 3 leaves per replicate). Two additional biological replicates produced similar results.

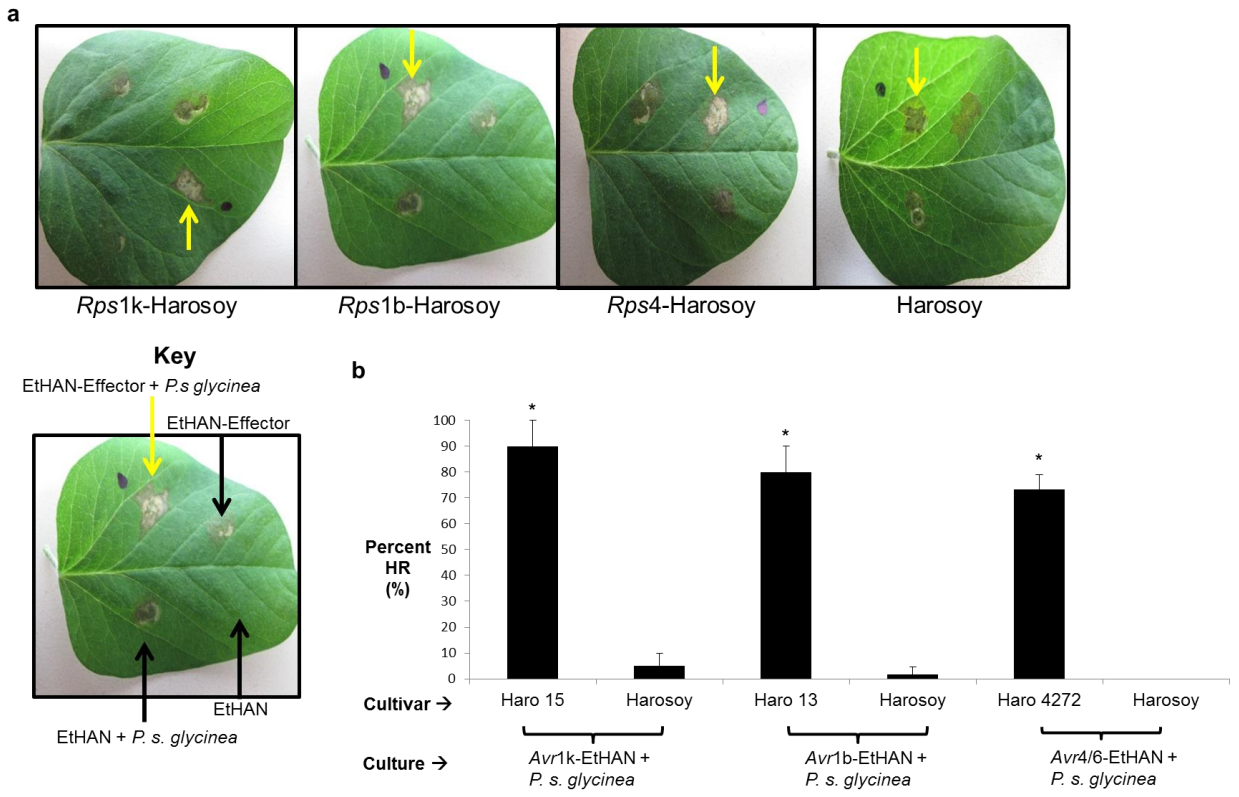

Figure 4.4: Validating the Pseudomonas effector delivery system using known Avr/R interactions.

(a) P. sojae avirulence proteins trigger gene-for-gene resistance when delivered from P. fluorescens by Type III secretion. The key depicts the infiltration strategy. The yellow arrow indicates the sites at which EtHAN expressing the avirulence effector was co-infiltrated with *Psg* race 4. The adjacent infiltration is EtHAN expressing the avirulence effector without *P. s. glycinea* race 4. The negative controls for each treatment were infiltrated on the opposite side of the leaf. (**b)** The graph quantifies the amount of times a visual HR was produced on a cultivar, represented as % HR. Harosoy was used as a negative control. The error bars depict standard deviation between the three biological replicates (n = 10 plants/ 20 leaves per replicate).

Statistical significance was determined by a pairwise t-test against Harosoy ($p < 0.05$) and is represented by an asterisk.

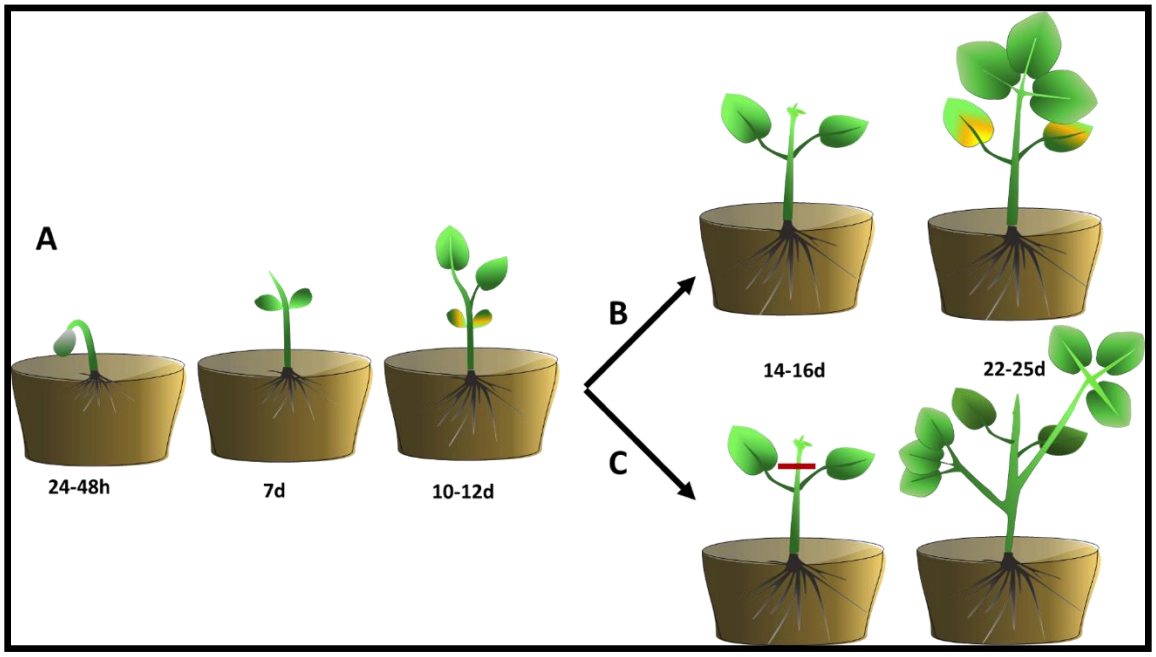

Figure 4.5: Soybean growth and pruning techniques for optimizing effector-response.

G. max plants germinate, first producing cotyledons. a) Unifoliate leaves emerge after 10-12 days at which point the cotyledons senesce. b) The plant will then begin producing trifoliate leaves which will mature, leading to senescence of unifoliate leaves. c) If the immature trifoliate leaves are pruned between days 14-16 (Red Hash Mark) the unifoliate leaves will become thicker and darker. New inflorescence will be generated below the point of pruning.

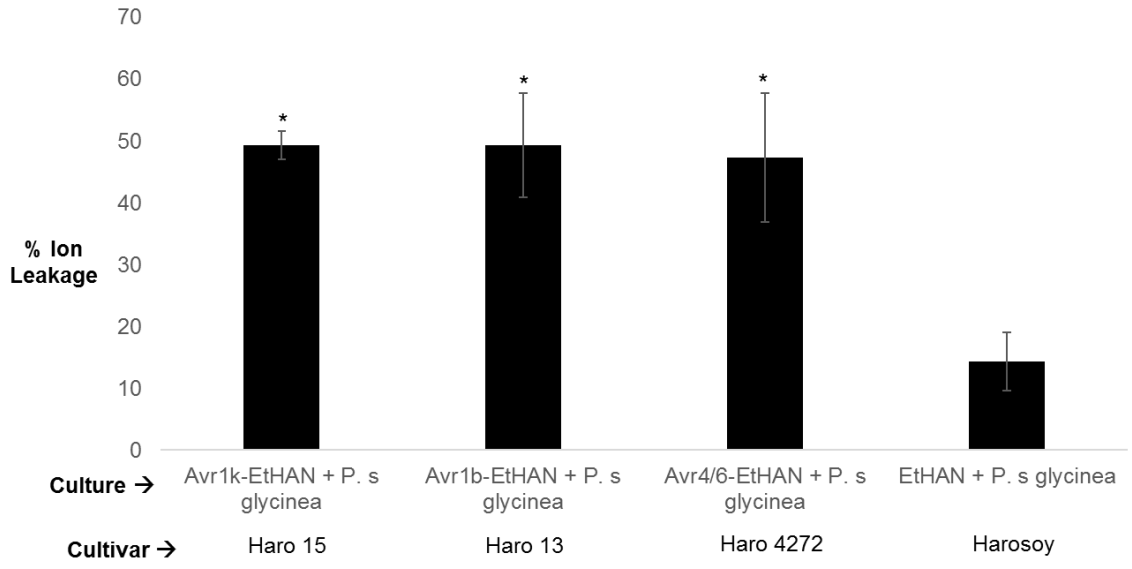

Figure 4.6: Ion leakage assay to quantify cell death symptoms in visual assays.

*Avr*1k, *Avr*1b, and *Avr*4/6 were inoculated onto cultivars which contained a cognate *R* gene. EtHAN + *Psg* race 4 on Harosoy was used to measure background cell death. Three independent biological replicates were performed (n = 3 plants / 6 leaf punches per replicate). Standard deviation was calculated between the three replicates.

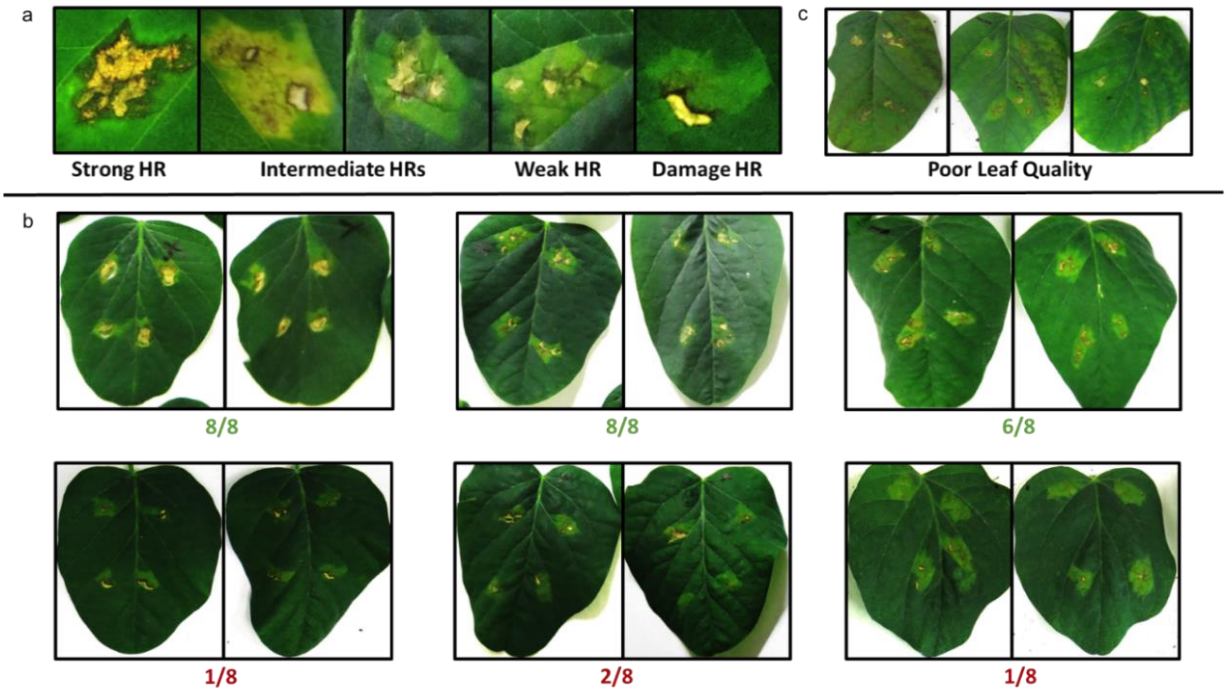

Figure 4.7: Effector-response was scored by presence or absence of a HR.

a) The strength of the effector response (aka Strong/Medium/Weak) was not recorded for individual infiltration site. b) During all screens, each plant received 8 effector inoculations, 4 per unifoliate leaf. Each of the 8 infiltration spots were scored as either 1 or 0 for the presence or absence of a HR. If ≥5 individual sites produced a HR, the plant was considered a positive responder. If ≤2 spots produced a HR the plants were negative. If between 3-4 infiltration spots responded, the data was considered too ambiguous to score.

Table 4.1: The PI numbers for all referenced accessions are listed.

G. max **Parental Accession**	**PI Number**
gm13	PI407985
gm20	PI408319C
gm31	PI398440
gm32	PI567139B
gm41	PI200553
gm48	PI274508
gm78	PI398666
gm92	PI398775
gm94	PI398791
gm96	PI398946
gm98	PI398996
gm99	PI399004
gm106	PI399036

gm121	PI408287
gm123	PI423741
gm144	PI424477
gm291	PI399079
gm314	PI408015
gm315	PI408020A
gm317	PI408029
gm320	PI408097
gm321	PI408111
gm326	PI408132

Table 4.2: Validating effector delivery using *Avr*1k, *Avr*1b, and *Avr*4/6 on *Rps* cultivars in the Williams background.

These effectors produce about 30% background cell death on cultivar Williams. Three independent biological replicates were performed (n = 10 plants / 20 leaves per replicate). Standard deviation was calculated between the three replicates.

G. max Cultivar	**% HR *Avr*1k**	**% HR *Avr*1b**	**% HR *Avr*4/6**
Williams82	84 ± 11	28 ± 14	30 ±6
L77-1863	46 ± 7	72 ± 16	28 ± 11
L85-2352	22 ± 8	27 ± 6	68 ± 18
Williams	34 ± 11	25 ± 6	32 ± 6

Table 4.3: A EtHAN and Psg race 4 mixture of bacteria do not produce a HR on tested cultivars.

Three independent biological replicates were performed (n = 10 plants / 20 leaves per replicate). Standard deviation was calculated between the three replicates.

Isoline	**Resistance Gene**	**EtHAN + *Psg* race 4**
Williams 82	*Rps*1k	8 ± 2
Haro 15	*Rps*1k	4 ± 1
L77-1863	*Rps*1b	12 ± 5
Haro 13	*Rps*1b	2 ± 1
L85-2352	*Rps*4	13 ± 3
Haro 4272	*Rps*4	0

Table 4.4: Soybean germplasm respond to core P. sojae effectors, Avh16, Avh180, and Avh240.

Responses of 30 *G. max* accessions to core effectors *Avh*16, *Avh*180, and *Avh*240. Data are presented as percentage of infiltration sites responding with a macroscopic HR, with standard deviation between the three biological replicates (n = 10 plants / 20 leaves per replicate).. Statistical significance was determined by pairwise t-test against Harosoy ($p < 0.05$). No background cell death was recorded for the *P. sojae* resistant *G. max* germplasm.

G. max **Parental Accession**	***Avh*16** **% HR**	***Avh*180** **% HR**	***Avh*240** **% HR**	***Avh*16** **T-Test (p)**	***Avh*180** **T-Test (p)**	***Avh*240** **T-Test (p)**
99	54 ± 7	0	0	P<0.001	0	0
321	51 ± 28	0	0	P<0.04	0	0
48	67 ± 31	0	0	P<0.03	0	0
144	70 ± 24	0	0	P<0.02	0	0
31	0	86 ± 4	0	0	P<0.0003	0
326	0	86 ± 16	0	0	P<0.005	0

314	0	70 ± 10	0	0	P<0.003	0
20	0	0	60 ± 10	0	0	P<0.004
13	0	0	73 ± 21	0	0	P<0.01
32	88 ± 11	0	93 ± 7	P<0.001	0	P<0.0006
291	0	73 ± 15	77 ± 15	0	P<0.005	P<0.006
78	0	90 ± 10	91 ± 9	0	P<0.002	P<0.001
320	68 ± 16	63 ± 6	97 ± 6	P<0.008	P<0.001	P<0.0005
Harosoy	3	0	0	0	0	0

Table 4.5: Homologous effectors, Avh16 and Avh7a, provoke similar response patterns in resistant germplasm.

We identified *Avh*7a as a homolog of *Avh*16. Resistant *G. max* accessions which responded to *Avh16* with a HR, were tested with *Avh7a* as well. We obtained similar results in responding cultivars. This data was produced from three biological replicates (n = 10 plants / 20 leaves per replicate). Standard deviation was calculated between the three replicates.

***G. max* Parental Accession**	***Avh*16 % HR**	***Avh*7a %HR**
99	54 ± 7	87 ± 6
321	51 ± 28	100 ± 0
48	67 ± 31	67 ± 6
144	70 ± 24	63 ± 12
32	88 ± 11	93 ± 6
320	68 ± 16	100 ± 0

Table 4.6: Response of *G. max* germplasm to twelve core *P. sojae* effectors.

Blank cells indicate that the accession did not respond to the corresponding effector with an HR % >30. The delivery of *Avh*23 and *Avh*110 to the resistant germplasm did not produce a HR on any accession. Three independent biological replicates were performed (n = 5 plants / 10 leaves per replicate). Standard deviation was calculated between the three replicates.

***G. max* Parental Accession**	**Response to Core *P. sojae* Effectors**									
	***Avh*16**	***Avh*180**	***Avh*240**	***Avh*53**	***Avh*137**	***Avh*261**	***Avh*488**	**Snel1**	***Avh*94**	***Avh*241**
13			73 ± 21						55 ± 16	84 ± 10
20			60 ± 10							
31		86 ± 4		82 ± 8					71 ± 16	
32	88 ± 11		93 ± 7							
40				83 ± 10			85 ± 8			
41				85 ± 8	81 ± 14					
48	67 ± 31								86 ± 4	
78		90 ± 10	91 ± 9							
92					77 ± 7					

94				55 ± 6	62 ± 10	81 ± 6				
96					81 ± 6	84 ± 15				
98									72 ± 10	
99	54 ± 7									
106				86 ± 8	60 ± 24					
121				78 ± 6		62 ± 14				80 ± 10
123				54 ± 26		76 ± 14				
144	70 ± 24									
291		73 ± 15	77 ± 15		56 ± 10		66 ± 14			
314		70 ± 10			83 ± 6					
315									77 ± 12	
317								84 ± 8		60 ± 24
320	68 ± 16	63 ± 6	97 ± 6							
321	51 ± 28				62 ±14					
326		86 ± 16					66 ± 20			

Table 4.7: *G. max* accession which responded to core effectors were bred with susceptible Williams.

We tested multiple individuals from segregating $F_{2:3}$ populations for a response to the effector with a HR. The tested $F_{2:3}$ populations have a * next the cross name.

Cross	ID	*Avh*16	*Avh*180	*Avh*240	Maturity Group
32(R) x Williams(S)*	cr32				V
326(R) x Williams(S)*	cr326				IV
144(R) x Williams(S)	cr144				IV
284(R) x Williams(S)	cr284				IV
291(R) x Williams(S)	c291				IV
13(R) x Williams (S)	cr13				IV
320(R) x Williams(S)	cr320				IV

Table 4.8: Effector-response phenotypic ratios in F2:3 populations.

We screened segregating $F_{2:3}$ families for the phenotype of response to an effector with a HR. We observed a 1:2:1 phenotypic ratio in all crosses. Four independent biological replicates were performed (n = 5 plants / 10 leaves per replicate). Statistical significance of our observed ratio was determined using a chi-squared test against a 1:2:1 expected ratio ($x > 0.05$). The observed ratios are not statistically significant suggesting that the response to the effector is provided by a single, dominant locus.

		Number of F3 Families			Expected	x^2 value
Effector	**Cross**	**HmR**	**Seg**	**HS**	**Ratio**	**(2 df)**
*Avh*180	*G. max* 326(R) x Williams(S)	14	27	13	1:2:1	0.071
*Avh*16	*G. max* 32(R) x Williams(S)	21	45	23	1:2:1	0.101
*Avh*240	*G. max* 32(R) x Williams(S)	19	47	17	1:2:1	1.56

Table 4.9: Bacterial strains and their corresponding selectable markers are listed.

All *Pseudomonas* strains were grown at 28°C and all *E. coli* strains were grown at 37°C.

Bacterial Strain	**Selectable Marker**	**Temperature (°C)**
Top 10 *E. coli*	None	37
P. fluorescens, EtHAN	Chloramphenicol	28
E. coli, RK600	Chloramphenicol	37
P. s pv glycinea race 4	Riffampicin	28
P. syringae DC3000	Tetracyclin	28
P. s DC3000, D28E	Riffampicin / Spectinomicin	28
P. s DC3000, D28E + *Avr*PtoB	Riffampicin / Spectinomicin	28

Table 4.10: The diagnostic primers used screen for positive transformants into pEDV6 are listed.

In all cases, the pEDV6 F primer was used with a gene specific R primer to confirm the insert and proper directionality.

Primer	**Sequence (5'-3')**
pEDV6 F	GGCACCCCAGGCTTTACACTTTATG
*Avr*1K R	TCAGATAATCATGATGCTGT
*Avr*1B R	CCGGTGAAAGGTGTATCCGTTGTAG
*Avr*4/6 R	CGTTAGGTGGTGTAGTCCGACGGAC
*Avh*16 R	CCATCTTCTTTGCTTCCTTAGC
*Avh*7a R	CTACAAGCCGTCCGTGTTCATGCCCA
*Avh*180 R	CTAAGCGATGTTCGTCTG
*Avh*240 R	CTAGTTTGCGGGTTGG
*Avh*23 R	TCATGCATTGTCGGAAAGTTTGAAGT
*Avh*110 R	TTATCCTACGCGGACACTCGCTACGC
*Avh*53 R	GTTATTTTCGTTAGCCCCATA
*Avh*137 R	CGGGTAAATGTATCCCTCAAG

*Avh*261 R	AATGTGATTTTGCGGGTTGTC
*Avh*488 R	CGGCGACTGGCCCATGCGAGC
Snel1 R	CGATCGCCGGTGCTGACGACT
*Avh*94 R	CTAAGCGGTGTCCTCCTCCTCC
*Avh*241 R	GTTCAGCTCGTGCCACTTGAA
*Avh*7a R	CTACAAGCCGTCCGTGTTCATGCCCA

Table 4.11: Williams and Harosoy isolines are listed.

Resistance Gene	Cultivar	Isoline
Rps1k	Williams	Williams 82
Rps1k	Harosoy	Haro 15
Rps1b	Williams	L77-1863
Rps1b	Harosoy	Haro 13
Rps4	Williams	L85-2352
Rps4	Harosoy	Haro 4272

Chapter 5: Development of an Effector-Based Screening System to Identify Novel *R* Genes in a Wild Relative of Cultivated Soybean

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Abstract

*T*he oomycete *Phytophthora sojae* causes a destructive root and stem rot disease of cultivated soybean (*Glycine max*). Over time, commercially available resistance (*R*) genes are losing efficacy against *P. sojae*. We hypothesized that *Glycine soja*, a sexually compatible wild relative of *Glycine max*, could be exploited as a useful source of new *R* genes against soybean pathogens. We developed a system to screen *G. soja* with core RXLR effectors from *P. sojae* to identify novel *R* genes. We focused our screening on conserved RXLR effectors that are essential for virulence. *R* genes that recognize core effectors are expected to be durable, because mutations in the effector genes will result in a fitness penalty for the pathogen. We screened 24 *P. sojae*-resistant *G. soja* accessions with individual RXLR effectors delivered by *Pseudomonas fluorescens* Type III secretion. We identified several *G. soja* accessions for which delivery of one or more core effectors triggered a localized cell death, suggestive of effector-triggered immunity. We developed a segregating population from a cross of one *G. soja* line to a susceptible *G. max* line, to track inheritance of the *R* gene. Response to the effector *Avh*240 segregated in a 1:2:1 phenotypic ratio in the $F_{2:3}$ generation suggesting that this line contains one dominant *R* gene against the core *P. sojae* effector *Avh*240. These results indicate that an effector-based screening approach can be utilized to identify useful new *R* genes from a wild relative of soybean.

Introduction

Soybean is an important crop which accounts for 60% of world oilseed production [138]. The United States is a world leader in the production of various soybean products such as oil, crush, and meal [139]. Soybean production and trade continues to increase globally and in the US [139]. *Phytophthora* root and stem rot is one of the most destructive disease affecting soybean yield [140]. In 2006, losses associated with *Phytophthora* root and stem rot in the United States were 1,464 metric tons, equivalent to nearly $500 million USD [140].

Phytophthora sojae (*P. sojae*), the causal agent of this disease, is responsible for pre- and post-emergence damping off of susceptible seedlings [110]. On mature soybean plants, *P. sojae* causes root and stem browning and leaf chlorosis [129]. The disease symptoms generally appear 1 to 2 weeks after heavy rainfall [110]. *P. sojae* produces sporangia and oospores during infection. Sporangia release motile zoospores that swim to root exudates through wet soil. Zoospores then encyst and penetrate roots, producing hyphae that grow in the spaces between cells [72]. The filamentous hyphae create specialized feeding structures, known as haustoria, that draw nutrients out of the cell [112]. Additionally, these structures function as an interfaces where virulence factors, called effectors, are secreted to subvert immunity and reprogram the cell for feeding [112]. Oospores are thick-walled sexual structures produced in plant tissue that can survive for many years dominant in the soil and endure winter frosts. Oospores germinate in the spring and infect emerging seedlings. Heavy rains will induce oospore germination making this disease very volatile in seasons with extended periods of flooding, which typically initiates disease epidemics [72].

P. sojae secretes several different types of effector proteins to subvert plant immunity and alter host cell structure and function [56, 141]. The best-studied effector protein superfamily, called RXLR effectors, are defined by an N-terminal secretion signal, followed by the consensus amino acid motif RXLR [142]. The RXLR motif is often, but not always, followed a short motif enriched for acidic amino acids ("DEER") and by so-called "W" and "Y" domains that form helical "WY folds" which are unique to RXLR effectors in the *Phytophthora* and downy mildew families [141, 143]. These domains can be arranged to form complex folds, which make the WY fold a highly evolvable platform that supports structured surface polymorphisms upon rearrangement [56]. Virulence targets of RXLR proteins have been validated as key regulators of immunity, and it is becoming increasingly clear that RXLR proteins play a major role in promoting virulence of oomycetes in the *Phytophthora* genus [144].

Despite their importance for virulence, RXLR proteins can also be detrimental to the pathogen if recognition of an RXLR effector by a plant *R* protein leads to gene-for-gene resistance (more recently termed "effector triggered immunity" or ETI [145]). Indeed, every case of ETI against *Phytophthora* species, characterized at the molecular level, involves recognition of an RXLR protein by a nucleotide-binding, leucine-rich repeat immune receptor protein [56]. ETI is often characterized by localized cell death (termed the hypersensitive response or HR) which impedes pathogen growth [145]. This type of resistance is generally regarded as one of the most cost-effective tools to manage diseases caused by *Phytophthora* pathogens.

In soybean, there are 24 known Resistance to *Phytophthora sojae* (*Rps*) genes that provide gene-for-gene resistance against *P. sojae* infection. Seven of these genes have been commercially deployed with varying degrees of effectiveness against the most common *P. sojae*

pathotypes [108, 146]. Unfortunately, even the most effective *Rps* genes are now being overcome by pathogen co-evolution. This co-evolution produces diverse *P. sojae* strains ("pathotypes") characterized by different patterns of pathogenicity on *Rps* soybean cultivars. In other words, a soybean cultivar containing a *R* gene against *P. sojae* may be resistant to one pathotype and susceptible to a different pathotype [108]. It would be desirable to identify *R* genes with resistance to most or all of the *P. sojae* pathotypes encountered in field situations.

Genome analyses of oomycete phytopathogens is revealing the mechanisms behind the rapid evolution of pathotype diversity. *Phytophthora* genomes contain large, variable collections of RXLR-encoding genes which in turn has given rise to multiple *P. sojae* pathotypes [146]. Pathogen genomes are often organized by a bipartite distribution of fast and slow evolving genetic elements. It is hypothesized that pathogens create a two 'speed-speed genome' to modulate the evolutionary speeds of genes [147]. Effector genes are located in a part of the genome where rates of polymorphisms, copy-number variation, and recombination are extremely high [148]. Effector genes can be deleted or altered to avoid *R* gene detection. Due to genomic instability, expression of effectors is often variable. Transcriptional changes can cause effectors expression to fall below detection thresholds [56]. Polymorphisms that affect the coding sequence can cause effectors to have variability or off target catalytic activity. By this mechanism effector function can be changed or modulated to achieve new virulence mechanisms [56]. The proximity of RXLR effectors to transposable elements suggest a potential epigenetic adaptation to globally silence a group of RXLR effectors under environmentally favorable conditions [149]. These silencing events could potentially be reversed if the environmental pressures for the silencing are reduced, thereby providing the pathogen with a powerful

mechanism for rapid co-evolution. Altogether, these mechanisms illustrate why host resistance to *Phytophthora* species is short-lived, and underscore the challenges of breeding soybean with durable resistance to *P. sojae* field isolates with diverse pathotypes, as are frequently encountered in the field [150].

Wild relatives of domesticated crop species often contain useful agronomic traits such as tolerance to abiotic stresses, yield enhancement, and disease resistance [151]. These traits can be bred into commercial cultivars by conventional breeding if the relative is sexually compatible with the crop or if somatic cell hybrids and bridge species can be used to circumvent compatibility issues. Working with sexually compatible species is advantageous because it simplifies the breeding process. There have been successful reports of breeding resistance from wild relatives into commercial varieties. One of the first examples of introgression of resistance from a wild species was in potato [152]. Since then, disease resistance traits have been introgressed from wild species in most major crop plants.

Soybean was domesticated 6,000-9,000 years ago in Eastern Asia [153]. Although many aspects of domestication remain unclear, *Glycine soja* (*G. soja*) is accepted as the closest known wild relative to *G. max* [153]. *G. max* seed contains about 17% more oil than *G. soja* seed. *G. max* germinates consistently to produce uniform plants with large seed and thin seed coats. *G. soja* produces small, vine-like plants with small seed and thick seed coats. Despite these phenotypic differences, *G. max* and *G. soja* are sexually compatible [129], such that useful traits from *G. soja* can, in principle, be bred into soybean cultivars. Many studies of soybean genetic diversity suggest that most landraces of *G. max* are monophyletic, and *G. soja* germplasm is reported to contain more genetic diversity than *G. max* germplasm. Wen et al. and Joshi et al.

both hypothesized six *G. soja* genetic subgroups [154, 155]. The subgroups are separated geographically and correspond to Japan, Korea, Russia, northeast China, southern China, and the Huanghuai Valleys in China. Long-term seed dispersal is rare and does not appear to impact population structure of native populations.

Resistance against soybean cyst nematode [156], soybean mosaic virus [157], and *Phytophthora* root and stem rot [68] have been reported in *G. soja*. There are no reports of using directed breeding to intentionally introgress disease resistance traits from *G. soja* to *G. max*. Using molecular diagnostics, *G. soja* can be exploited as a source of resistance genes against *P. sojae*. Our goal is to identify novel and durable *R* genes against *P. sojae* in *G. soja* accessions, using an approach that combines traditional, pathogen-based screens with effector-based screen in which plant germplasm is probed for immunity triggered by delivery of individual effectors. This effector-based approach was pioneered against *Phytophthora infestans* [123]. 54 RXLR effectors from *P. infestans* were assayed for avirulence activity, by delivering effectors one-at-a-time to *Solanum* germplasm via *Agrobacterium*-mediated transient expression. In this system, *Agrobacterium* suspensions were infiltrated into plant leaves, which were then screened for a macroscopic cell death response that could result from hypersensitive cell death triggered by recognition of the effector. Vleeshouwers et al. used wild *Solanum* species to identify the *Rpi*-blb1 *R* gene using this approach. The avirulence response was triggered by the IpiO RXLR family [123]. More recently, Oh et al. bred novel *R* genes *Rpi*-blb1 and *Rpi*-blb2 against potato late blight from a wild *Solanum* species into potato [158]. Efforts are now underway to commercialize these genes in potato. This example illustrates how useful *R* genes were discovered by probing wild *Solanaceae* with RXLR effectors.

Following the *P. infestans* example, our approach is based on screening with RXLR effectors; however we focused our approach on "core effectors" that are highly expressed early in infection, conserved and monomorphic among the four most common *P. sojae* pathotypes, and required for virulence. By focusing on effectors that are conserved and contribute most to virulence, we hope to find resistance genes that target the pathogen's most static or most vulnerable points. This would make it more difficult for the pathogen to evolve quickly by mutating or silencing the gene and activating genes with redundant functions. In addition, *R* genes that recognize conserved effectors are more likely to provide recognition and resistance to a broad range of field isolates.

This study builds on a recent effort in which comparative genomics and transcriptomics were used to identify RXLR effector genes in *P. sojae* which were highly expressed early in infection and conserved among the four most common *P. sojae* pathotypes [159, 160]. Then, transient gene silencing was used to suppress expression of candidate core *P. sojae* effectors. The silencing of core *P. sojae* effectors *Avh*16, *Avh*180, and *Avh*240 greatly reduced pathogen growth on the susceptible cultivars, while the silencing of 7 other effectors *Avh*53, *Avh*137, *Avh*23, *Avh*110, *Avh*488, SNEL1 and *Avh*241showed moderately reduced pathogen growth. These are the effectors that we used in the current study.

Our screening strategy was based on an effector-based screening approach that was developed in *G .max* (Chapter 2). Importantly, this study developed an alternative system for effector delivery (see below). We were able to effectively optimize techniques developed in this study for use in wild soybean. After normalizing the variables of germination and growth stage in wild soybean we found that the effector-based screening was suitable for use with minor

technical modifications. Using effector-based screening we were able to identify putative resistance genes in accessions containing novel resistance to *P. sojae*. Additionally, we were able to confirm simple inheritance of one putative *R* gene in segregating populations. This suggests that this technique can be used to identify *R* genes in *G. soja* and is sensitive enough to map resistance in crosses between *G. soja* and *G. max*, thereby validating its utility for disease resistance breeding.

Results

Overview of the Strategy

Our approach for delivering effectors was based on a screening system described in Fedkenheuer et al. 2016 (Chapter 2). Because *Agrobacterium*-mediated transient transformation is not efficient in soybean, we developed a system for delivering effectors via Type III secretion from *Pseudomonas*. Trials with different *Pseudomonas* species revealed that the best strain for delivery in soybean is the soil bacterium *P. fluorescens*, EtHAN. EtHAN contains an artificially engineered type III secretion system which can be utilized to deliver individual effectors. *P. sojae* effectors were cloned into the Effector Detector Vector (pEDV6) that contains the bacterial *AvrRps*4 promoter and leader sequence which guides proteins through the type III secretion system [74]. We co-infiltrated EtHAN with *P. syringae pathovar glycinea* (*Psg*) for effector-based screens to improve signal intensity. *Psg* has been shown to improve the growth of EtHAN *in planta* presumably by suppressing host immunity (Chapter 2). Consequential to improved growth, more effector protein is likely delivered. If a host *R* gene recognizes the delivered effector, a macroscopic HR is triggered that can be scored visually.

In this study, we validated that the EtHAN-effector system efficiently delivers core *P. sojae* effectors into *G. soja* unifoliate leaves. Recently, Matthiesen et al. screened ~1,000 Glycine accessions with a mixture of three *P. sojae* isolates which defeat 13 commercial *Rps* genes [68]. From these accessions, we chose 24 *G. soja* accessions for effector-based screening. These resistant lines are likely to contain novel *R* gene loci or new alleles at known loci. The effector-based approach described below provides a mechanism through which to sort and prioritize these gene for breeding.

Optimizing the Screening System

The first objective of this study was to optimize growth conditions for *G. soja*, because our experience with this screen in *G. max* demonstrated that robust plant health is of key importance to obtaining reliable results from bacterial inoculations. This required several modifications of the protocols developed for the *G. max* screen .*G. soja* seeds are small and are coated with a thick outer wall. Germination of *G. soja* seed requires removal of the seed coat (scarification). We found that *G. soja* seeds germinated best when scarified in 100% H_2SO_4. We determined that 5 minutes in these conditions was sufficient to remove all seed coats. Immediately following chemical treatment, seeds were removed and rinsed with dH_2O to remove all traces of acid.

Another complication is that vegetative growth and development is more variable in *G. soja* than in cultivated soybean. Unlike *G. max*, *G. soja* must be germinated at 100% humidity to achieve consistent germination. Another important step was to prune of the first set of trifoliates leaves at three days after germination. This allowed us to standardize growth stage prior to effector infiltration. This is critical when screening diverse accessions of *G. soja* because timing

of unifoliate leaf maturity is often variable among accessions. Pruning of trifoliate leaves also improves unifoliate leaf quality. After pruning, unifoliate leaves became thicker and acquired a darker green color. In plants treated in this manner, resistance responses are stronger and the infiltration zone is more clearly visible. When scoring for visual HRs, it is important to judge the spread of cell death outside of the zone where the syringe interfaced with the leaf surface. This is done to avoid false positives caused from damage induced by the infiltration process. The negative control and the effector treatment must be done on different plants because a strong effector response can trigger cell death in the negative control (a priming effect). Individuals were subjected to only one treatment for this reason. When screening individual effectors, the experimental treatment can be treated as a negative control on non-responding accessions. When recognition does not occur, levels of cell death are often below those of the untransformed control (Figure 5.1).

Screening G. soja accessions with 3 P. sojae pathotypes

As mentioned above, our screen focused on *G. soja* accessions identified in a previous study to be resistant to a combination of *P. sojae* pathotypes which collectively overcome all known *Rps* genes. Table 5.1 lists the relevant accession information including PI numbers and geographic origin. To confirm disease resistance responses we performed disease screening on *G. soja* germplasm using individual isolates rather than the combination of three used in the previous study. We believed it was important to look at infections on an individual level because it is impossible to predict the complexity of interactions when using isolates in combination. For example, a strong immune response to one pathotype may override the virulence mechanisms of another pathotype. We optimized infectivity of our cultures at monthly intervals by passaging

isolates through infected soybean (cv. Williams). The process of refreshing *P. sojae* cultures is essential to maintain a healthy strength for infection. A loss in virulence over time was observed in all isolates during stem and leaf inoculation assays.

We developed a disease assay in which trifoliate leaves were inoculated with agar plugs from each of three virulent accessions: *Pt*1005, *Pt*2004, and *Pt*Race 7 (Figure 5.2). This assay is reproducible, convenient, and allows the same plants to be scored simultaneously for pathogen resistance and effector response. Wounding of the leaf prior to inoculation was essential for reproducibility. After 5-7 days, resistance versus susceptibility was very clear, and we scored the plants as resistant or susceptible without taking measurements of disease lesions. The *G. max* accession Williams was used as a control for susceptibility. We confirmed that many of the *G. soja* accessions were resistant to all three isolates; however, several accessions were partially susceptible to one or two pathotypes. No accessions were susceptible to all three isolates. Hypocotyl inoculations were done to confirm instances of susceptibility in foliar assays. In all cases, we observed perfect agreement between hypocotyl and leaf inoculation. The major technical challenge of these assays is providing a consistently moist infection site for pathogen infection following inoculation. This refined pathotype information will be important for prioritizing accessions for further characterization and for accurately mapping pathogen resistance loci in segregating populations. Hypocotyl inoculations were laborious and had limited throughput when compared to the trifoliate assay.

Three G. soja Accessions Contain Novel Resistance to Psg race 4, a Bacterial Pathogen of Soybean

As mentioned above, our effector delivery system is based on co-inoculation with EtHAN (containing the effector transgene) and *Psg* race 4 (to boost growth of the EtHAN strain). Thus, it was necessary to pre-screen *G. soja* accessions for immune responses to *Psg* race 4 that would interfere with effector delivery during the co-inoculation with EtHAN + *Psg*. This was important to eliminate false positives from our screens that could occur from ETI triggered by the native effectors secreted by *Psg* race 4. We found three *G. soja* accessions (gs2763, gs730, and gs727) that produced a HR when pressure infiltrated with *Psg* race 4 (Figure 5.3 and Table 5.3). Visual HRs were apparent one to two days post inoculation (DPI). Gs2763 and gs730 produced strong HRs. Accession gs727 produced a weak and inconsistent visual HR against *Psg*. Thus, we were unable to screen for effector specific HRs in these accessions due to the immune interaction with the helper strain *Psg*. However, these accessions likely contain potentially useful resistance genes against *Psg*.

Effector-based screening

Our first screen of 20 *P. sojae* resistant *G. soja* accessions was conducted with three of the most promising core *P. sojae* effectors: *Avh*16, *Avh*180, and *Avh*240. These effectors are strongly expressed and make major contributions to virulence in all four of the reference *P. sojae* accessions, so *R* genes against any of these effectors could be very effective. Effectors were delivered individually for each of the 20 accessions via co-infiltration with EtHAN and *Psg* race 4, as described in Figure 5.1. We scored responses to these effectors with a HR as a percentage (%HR). We observed 3 accessions (gs2777, gs2514, and gs2292) that produced HRs against

*Avh*16, *Avh*180, and *Avh*240 respectively (Figure 5.4). Effector responses in these accessions were strong and extremely specific (Table 5.4). Gs2292 produced a HR against *Avh*240 in 95% of individuals tested. Accessions gs2777 and gs2514 produced HRs against *Avh*16 and *Avh*180 respectively in 90% of individuals tested. These accessions produce strong and consistent HRs against their corresponding effectors and display no background cell death from our internal control of untransformed EtHAN co-infiltrated with *Psg*.

A second round of screens was performed with core effectors *Avh53*, *Avh*137, *Avh*23, *Avh*110, *Avh*488, SNEL1 and *Avh*241. *Avh*53 and *Avh*137 elicited resistance responses in many accessions from multiple subgroups (Figure 5.6). Additionally, we observed paired responses to effectors *Avh*53 and *Avh*137 only in accessions collected in Primorsky, Russia. This observed geographic distribution suggests that these accessions share common resistance loci. *Avh*23 and *Avh*110 did not produce a visual HR on any tested *P. sojae* resistant *G. soja* accession (Table 5.5 A-G). We compiled effector screening data and accession information in Table 5.2.

Determining the Inheritance of Potential R Genes in a Segregating $F_{2:3}$ Population

P. sojae-resistant *G. soja* accessions which responded with a HR to a core *P. sojae* effector were crossed with the susceptible *G. max* cultivar Williams as part of our breeding program. In order to test the viability of using effector-based screening to map a resistance gene, we looked at the inheritance of a potential *R* gene in a segregating $F_{2:3}$ population (Figure 5.5). We analyzed the cross, gs2292 (R) x Williams (S), for the phenotype of response to core *P. sojae* effector *Avh*240 with a HR. Figure 5.5A describes the screening and scoring individuals within segregating $F_{2:3}$ populations. We collected data from 89 $F_{2:3}$ families with a sample size n>10. Because most *R* genes segregate as major-effect, genetically dominant loci, we expected a 1:2:1

phenotypic ratio. HR strength was variable throughout biological replicates; however the presence or absence of visual HRs was extremely consistent (Table 5.6). As expected, we observed a 1:2:1 phenotypic ratio in the $F_{2:3}$ progeny of a cross between gs2292 (R) x Williams (S) (Figure 5.5B and Figure 5.7). By analyzing this population, we determined that effector based screening is both sensitive and accurate enough to identify resistance responses on an individual plant basis. Additionally, the response to *Avh*240 in this accession appears to segregate as a single locus, easily amenable to introgression into *G. max* cultivars.

Discussion

Soybean is harvested for oil and protein, and ranked second for area planted in the US in 2014 [161]. In 2015, 55% of vegetable oil consumed in the United States of America (USA) came from soybean, underscoring the importance of this crop. *Phytophthora* root and stem rot substantially affects the profitability of soybean by reducing soybean harvests in pathogen suited environments across the world [140]. In the USA, *Phytophthora* root and stem rot is most severe in the Midwest, and resistance to *P. sojae* can be broken by many factors [150]. In the past, soybean *Rps* genes were reported to confer full resistance against most *P. sojae* pathotypes. Unfortunately, *P. sojae* is evolving to evade recognition by soybean *Rps* genes, and the average effectiveness of an *Rps* gene in the field is 8-15 years [121]. Thus, it is important to identify novel *R* genes against *P. sojae* which are durable in the field and which recognize the potentially broad diversity of isolates encountered in field situations.

We began by assessing *G. soja* germplasm for resistance to *P. sojae* to confirm and extend the results from Matthiesen et al. [68]. We used the same three pathotypes, which collectively overcome all known *Rps* resistance genes except Rps8, that were used by [68]. We developed a trifoliate infection assay to complement our new effector based screening system. Agar plugs were used to infect a wounded trifoliate leaf. Our trifoliate agar plug infection assay can be used to measure quantitative resistance by measuring lesion diameter, or it can be used to measure qualitative resistance by assaying for the presence or absence of disease lesions in pronounced examples. We used specialized tools to quickly generate uniform agar chunks, greatly increasing throughput and consistency. Most importantly, this assay can be used in combination with effector-based screening since trifoliates are removed during the pruning

process. Using this disease screening technique, we evaluated *G. soja* germplasm for novel resistance to *P. sojae*. Screening with pathotypes individually versus in combination generated uniquely complimentary data. For example, the individual isolate approach generates a more accurate pathotype profile; while screening in combination allows us to infer the strength of the resistance response. Resistance observed in screening with a combination of pathotypes indicate that resistance responses will not be easily overcome through diversification of effector profiles.

We chose to screen *P. sojae* resistant *G. soja* germplasm with core *P. sojae* effectors to identify new sources of *R* genes against *Phytophthora* root and stem rot disease. A core *P. sojae* effector is highly expressed in early *P. sojae* infection, conserved among the 4 most common *P. sojae* pathotypes, and essential for *P. sojae* virulence. To assess effector impact on virulence, Tyler et al. used transient gene silencing to suppress expression of candidate core *P. sojae* effectors. The silencing of core *P. sojae* effectors *Avh*16, *Avh*180, and *Avh*240 greatly reduced pathogen growth on the susceptible *G. max* cultivar Williams. We selected these 3 effectors as our most promising *P. sojae* core effectors. The silencing of core *P. sojae* effectors *Avh*53, *Avh*137, *Avh*23, *Avh*110, *Avh*488, and SNEL1 moderately decreased pathogen growth. We used these 9 core *P. sojae* effectors to probe for potential *R* genes in *P. sojae* resistant *G. soja* germplasm against core *P. sojae* effectors. Because these *P. sojae* effectors are monomorphic and important for virulence, we hypothesize that *P. sojae* will be unable to easily discard core effectors to evade *R* gene detection. For this reason, we predict that a *R* gene against a core *P. sojae* effector will provide durable resistance against *P. sojae* in the field. We hypothesize that *R* genes against core effector targets will provide broad spectrum resistance because these core effectors contain conserved sequences present among the most common field isolates of *P. sojae*.

Prior to effector-based screening of *G. soja* germplasm with our library of *P. sojae* core effector candidates we established the background of *Psg* in all accessions. This screen identified three *G. soja* accessions: gs2763, gs730, and gs727 which contain resistance to *Psg* race 4. *Psg*, a bacterial pathogen, is the causal agent of bacterial blight on soybean [162]. *Psg* is most virulent in the northern most states and into parts of Canada [162]. By testing *Psg* resistant accessions with other pathotypes (races) of *Psg* we can determine whether this resistance is durable against all isolates of this pathogen. We can use segregating populations available in our germplasm collection to track resistance against both *Psg* and *P. sojae* using the assays developed in this study. It will be interesting to determine if there is a linkage between bacterial and oomycete resistance mechanisms.

Resistance responses against *Psg* in these three accessions excluded these accessions from effector-based screening. We compiled effector based screening data from the remaining 20 *G. soja* accessions with 10 effectors. We found putative *R* gene candidates against 8/10 core effector candidates. Effector responses for 6 core effectors were very specific (only one or two responding accessions per effector). We observed strong and clear resistance responses against our top candidate effectors: *Avh*16, *Avh*180, and *Avh*240. In some cases, we found a correlation between the effector response of a *P. sojae* resistant *G. soja* accession and geographic collection location. *Avh*53 provoked responses in 6 accessions, while *Avh*137 provoked resistance responses in 9 accessions. We confirmed that these HRs were effector specific by extensively screening *Avh*53 and *Avh*137 on non-responding *G. soja* accessions. Four *P. sojae* resistant Russian *G. soja* accessions responded with a HR to both core *P. sojae* effectors *Avh*53 and *Avh*137. From the 2 remaining *P. sojae* resistant Russian *G. soja* accessions, one accession

responded to *Avh*53 and one accessions responded to *Avh*137. No other effector responses were observed in *P. sojae* resistant Russian *G. soja* accessions. This could indicate that *R* genes against *Avh*53 and *Avh*137 are under positive selective pressure in Russia. We found multiple *P. sojae* resistant South Korean *G. soja* accessions which responded to *Avh*137 and 1 South Korean accession which responded to *Avh*53. One Japanese *G. soja* accession responded to *Avh*137 as well. Since, subgroups are genetically isolated from each other we hypothesize that evolution has either converged on these effector targets or that these accessions share *R* genes from ancestral origins.

We used the *Pseudomonas* effector screening system to analyze a segregating $F_{2:3}$ population, gs2292 (R) x Williams (S). Similar to the *G. soja* parental accession, $F_{2:3}$ seed required scarification prior to planting. We found that scarifying *G. soja* seeds and the $F_{2:3}$ seeds in 100% H_2SO_4 optimized seed germination and experimental throughput. Individuals in $F_{2:3}$ families shared morphological characteristics more similar to *G. max* cultivar Williams. We observed a 1:2:1 phenotypic ratio for the phenotype of response to the core *P. sojae* effector *Avh*240 with a HR. This suggests a classical dominance inheritance. All *P. sojae* resistant *G. soja* accessions which produced a HR against a core *P. sojae* effector were crossed with *G. max* cultivar Williams as part of our breeding program. We will use recombinant inbred lines to further evaluate and fine-map potential *R* genes. A limitation of our approach is applying this system in the *G. max* Williams background. Effectors which produce a strong HR will often produce background cell death in Williams. This makes scoring individuals within segregating populations more challenging. We hope to mitigate these limitations by further optimization of our system or by exploring other elite accessions. Since effector-based screening was not paired

with pathogen infection assays in $F_{2:3}$ populations it is impossible to say for certain whether our identified effector response genes are truly responsible for pathogen resistance. The parental accession gs2292 is resistant to three accessions which overcome all known *Rps* resistance. Additionally, this accession responds to *Avh*240, one of our most promising core effectors, in effector-based screens. These data taken together suggest that these techniques will complement each other in more advanced populations. We will analyze individuals within recombinant inbred populations using our trifoliate infection assay in combination with our effector-based screening assay to understand this interplay in future studies.

Effector-based screening is a new but powerful technique. We believe that this technique is an excellent complement to classic pathogen infection assays. These techniques can be used together to map resistance loci more accurately. Additionally populations containing multiple resistance loci can be more clearly separated. Some of the most successful examples of breeding for resistance have come from wild species. For example, the *R* gene *Pto* which confers resistance to *Pseudomonas syringae pathovar tomato* has been used to control speck disease for over 70 years [163]. Until recently, the only resistance against root-knot nematode in tomato was the *R* gene Mi-1[164]. Introgressed from the wild tomato species, this gene from *Lycopersicon peruvianum* is an excellent example of an effective resistance gene derived from a wild species. In potato, the *R* gene *Pi-ber* was derived from *Solanum berthaultii* and confers complete resistance against the *P. infestans* isolate US8 [165]. We built on work in *G. max* to develop an effector-based screening system for *G. sojae*. We were able to utilize bacterial type III secretion to deliver individual effectors to the interior of *G. soja* unifoliates. After growth conditions were optimized, this system was seamlessly transitioned into *G. soja*, underscoring the potential of this

system to be deployed across broad spectrum of crop plants. We used resources (germplasm and cultivars with specific *Rps* genes) available in *G. max* to develop a disease system that would be effective in both *G. max* and *G. soja* for any pathotype.

By targeting our effector screens to identify *R* genes against core *P. sojae* effectors, we can look for durable resistance in a unique way. Additionally, we designed a disease assay which can be performed concurrently with effector response screens on the same individual plants, to facilitate linking effector responses with pathogen resistance. The introduction of these genes into cultivated soybean by traditional breeding allows for product introgression into all soy-based markets. We hope to map these genes to specific loci by examining phenotypes in segregating progeny. Mapping resistance genes using genetic markers is an effective and proven method [166]. We hope to use the unique information acquired from an intelligently designed effector-based screens and disease assays to predict *R* gene specificity and durability in a field setting [167].

Materials and Methods

Culture Preparation and Maintenance

Phytophthora sojae cultures of pathotypes *Pt*1005, *Pt*2004, and *Pt*Race 7 were kindly provided by the Robertson Lab at Iowa State University [68]. Agar plugs were used to inoculate trifoliate leaves of susceptible Williams. Seven days post inoculation, visibly infected areas were cut and washed in 70% EtOH. Sterilized plant material was washed with diH_2O, dried, and plated on to PARP-V8 plates. Cultures were incubated in the dark at room temperature. Agar plugs containing *P. sojae* mycelium were taken from the edges of the growing colonies and used to start new V8 plates. *P. sojae* grows quickly on V8 media and fully colonized plates within two

weeks. Pathogen material prepared in this fashion was used for infection assays. On average, *P. sojae* will colonize a PARP-V8 plate in 6-8 weeks. After 8 weeks, *P. sojae* pathotypes were re-isolated using the process outlined above, to maintain virulence.

Trifoliate Leaf Agar Plug Infection Assay

We used agar plugs from pathotypes *Pt*1005, *Pt*2004, and *Pt*Race 7 to infect mature trifoliate leaves from our *G. soja* germplasm collection. Trifoliate leaves were detached and placed into Petri dishes containing filter paper saturated with water. A scalpel was used to wound trifoliate leaves. Wound size, shape, and depth are not important within reason; however the position and angle of the wound will sometime effect the direction of the disease lesion. Agar plugs were then placed over the wound and a drop of H_2O was added to the agar plug to hydrate the site of infection. We used a specialized tool, specifically a Large/Jumbo Double End Steel Amalgam Carrier ®Miltex Instrument Co. to rapidly generate consistent sized agar plugs. Plates were sealed with parafilm to prevent moisture loss and maintain humidity during infection. Sealed plates were stored under normal growing conditions until imaging. After 7-10 days, disease lesions on susceptible plants covered 80-100% of the leaf surface. While disease lesions are often present on resistant accessions, these lesions do not expand past the wound site.

Hypocotyl Inoculation Assay

We confirmed our results using standard hypocotyl inoculation assays [72]. We applied agar plugs to wounded hypocotyls of soil grown plants. After inoculation, lesions developed in resistant and susceptible accessions. Lesions did not expand outside of the site of inoculation in resistant germplasm. Lesions expanded rapidly in susceptible individuals making differences in

susceptibility clear. We confirmed resistance and susceptibility phenotypes using this technique to validate our trifoliate screen.

Scarification

Seeds were scarified in 100% H_2SO_4, for 5 minutes. Seeds were then rinsed vigorously in H_2SO_4 and directly planted or dried for storage. As a high-throughput modification, accessions were scarified using a 98 well 1.5 mL centrifuge tube holder with holes bored through the wells (Figure 5.8). This allowed seed to be scarified in batch, while keeping accessions separate. Manual scarification using coarse sandpaper is effective; however germination rates and emergence times were improved with acid scarification.

Planting and Maintenance

For all screens, *G. max* was planted in Fafner P2 potting media. Fafner P2 potting media was hydrated with 2 L of water and Cal-Mag Fertilizer®. 2.5 inch pots were packed firmly with media. 5 seeds were sown per pot and covered with about 2-3 cm of loosely packed potting media. The top soil was treated with Marathon insecticide, put into flats and covered with clear plastic lids to maintain humidity during germination. After 24-36 h, lids were removed from the flats. Germination rates were between 80-95% for all *G. soja* accessions tested. *G. soja* were germinated and maintained under diurnal light (16 h light at 22 ^{0}C; 6 h dark at 20 ^{0}C). Light intensity was maintained between 250-300 µM for all experiments. Light intensity is critical for the production of consistent macroscopic HRs during screening. Flats were watered with 1.0 L of H_2O per flat every 36-48 hours or as needed. Before the end of the V2 stage of development, trifoliate leaves were pruned as described in the Results section.

Culture Preparation

Gateway clones of 10 RXLR core effectors (*Avh*16, *Avh*180, *Avh*240, *Avh*53, *Avh*137, *Avh*241, *Avh*488, SNEL1, *Avh*23, and *Avh*110) were generously provided by the Tyler Lab at Oregon State University. The unstructured N terminus and secretion peptides of core RXLR effectors were removed during entry vector cloning in order to complement the design of the pEDV6 system. Bacterial cultures were prepared from single colonies and inoculated in 5 mL aliquots of KB media containing the appropriate antibiotic selection. The bacteria was pelleted as mentioned above and re-suspended in 10 mM $MgSO_4$. Single inoculums were prepared at ODs = 0.3-0.6 and pressure infiltrated into soybean unifoliates. Co-infiltration of EtHAN and *Psg* were done by mixing the two at ODs 0.6 and 0.3 respectively for a final OD = 0.9.

Infiltrations

Plants were infiltrated after 15-17 days (3 days after pruning the trifoliate leaves). Bacterial solutions were prepared 1 hour prior to infiltration. Needless syringes were used to pressure infiltrate attached unifoliate leaves. Each unifoliate received 4 infiltrations of 50-100 µL of bacterial solution. The identical process is performed on the adjacent unifoliate. Negative controls were performed on separate plants to avoid priming induced by effector recognition.

Scoring

Seven days post infiltration (DPI), unifoliate leaves were detached, imaged and, scored. A plant was scored positive if ≥6/8 infiltration spots produced a visual HR on both unifoliate leaves. Plants were scored negative if ≤2/8 infiltration spots produced a visible HR. Plants that fell between 3-5/8 were scored as ambiguous. Any data, scored as ambiguous, was discarded, and the experiment was repeated until the appropriate sample size was reached (Chapter 2). To

calculate % HR, we used the equation, $\% HR = (\#\ of\ responding\ plants) \div (\#\ of\ infiltrated\ plants)$.

Delivery of *P. sojae* Core Effectors to Resistant *G. soja* Germplasm

In experiments with *Avh16*, *Avh180*, and *Avh240*, cultivars were screened with effectors individually in three biological replicates (n = 20 plants per replicate). Standard deviation was calculated between the three replicates. Statistical significance ($p<0.05$) was determined by a pairwise t-test against Harosoy.

Determination of *R*-Gene Inheritance in $F_{2:3}$ Populations

Individuals from segregating $F_{2:3}$ families were screened with *Avh*240 as described above. Before effector screening, we determined the background that *Avh*240 produces in Williams. $F_{2:3}$ families were tested with an average sample size of n≥10 individuals per family. We determined our range for homozygous dominant as ≥85 % HR. We determined our range for homozygous recessive as the background response of *Avh*240 on Williams ≤30 % HR. Families that fell within the two ranges are considered to be segregating. We use the Chi squared (χ^2) value to determine the probability at which the deviation is expected.

Figures and Tables

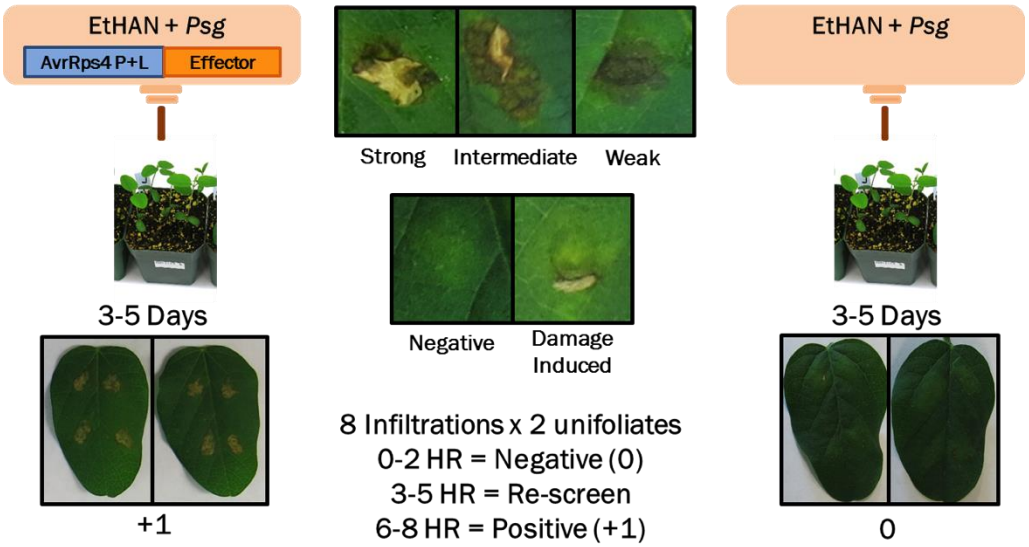


Figure 5.1: Overview of the procedure for screening *G. soja* accessions with core *P. sojae* RXLR effectors.

P. fluorescens EtHAN was used to deliver *P. sojae* effector proteins to the interior of plant cell. The pEDV6 vector contains the Avr*Rps*4 promoter and leader sequence which were used to guide effector proteins through the Type III secretion system. We co-infiltrated EtHAN with *Psg* race 4 in a 2:1 ration to suppress PTI. This suppression creates a favorable environment for *P. fluorescens* to achieve high levels of growth, such that it delivers higher quantities of our desired effector and produces a more robust phenotype. Pressure infiltration was used to inoculate four areas per unifoliate leaf and we infiltrated both unifoliate leaves per plant. Plants were scored 3-5 DPI. If at least 6 out of 8 spots produced a visual HR, the plant was scored +1. If 0 out of 2 spots produced a visual HR, the plant was scored as 0, and if 3-5 spots produced a visual HR, the data point was discarded and the population was retested. An internal control of untransformed *P. fluor*escens co-infiltrated with *Psg* was used during screening.

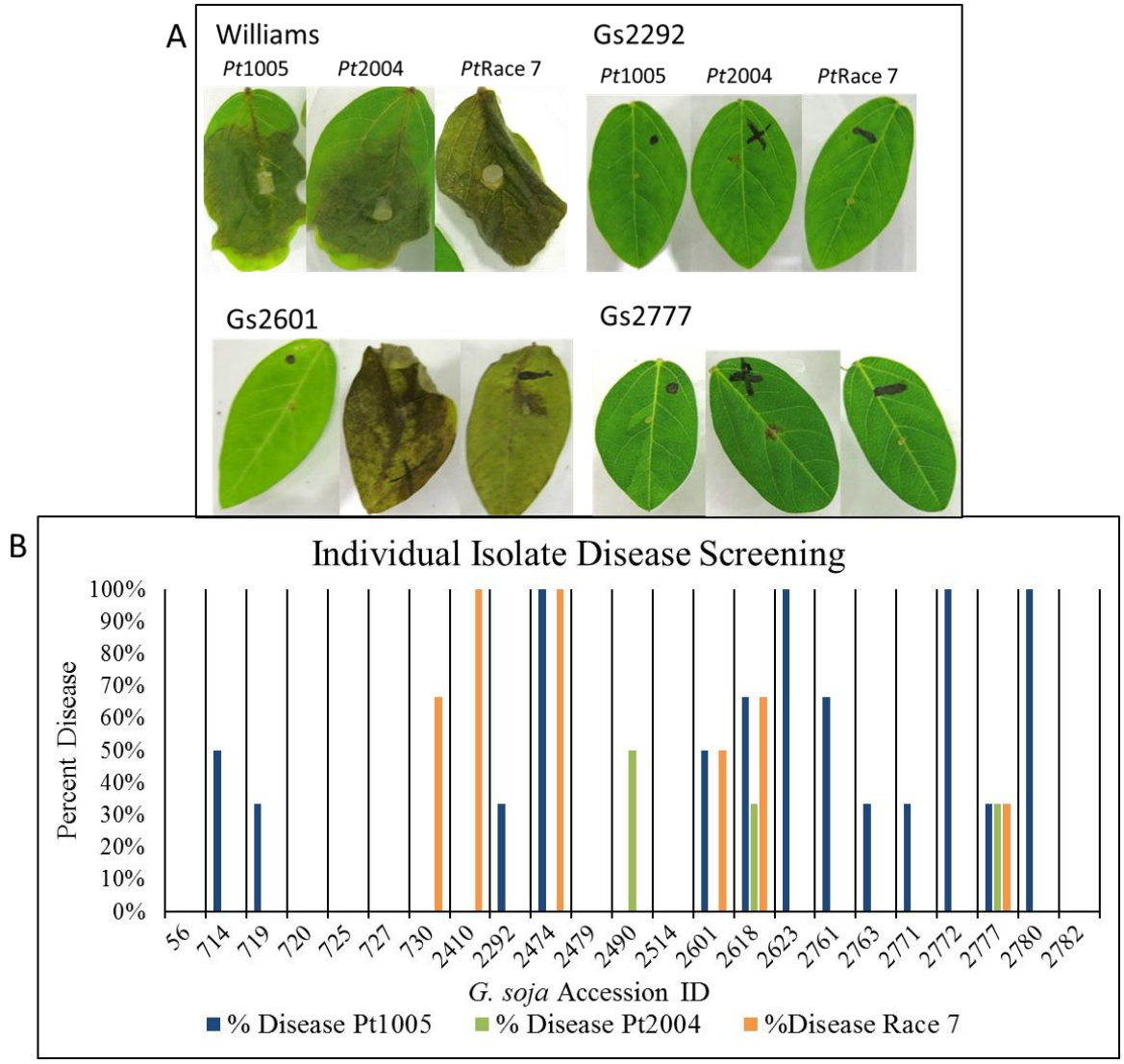


Figure 5.2: **Disease screening of *G. soja* accessions with three common field isolates of *P. sojae*.**

A) Agar plugs containing mycelia were used to infect trifoliate leaves with isolates Pt1005, Pt2004, and PtRace7. We found susceptible interactions among *G. soja* accessions; however most cultivars showed nearly complete resistance. **B)** Percent disease was determine for all *G. soja* germplasm. On the X axis, *G. soja* germplasm is listed and on the Y axis, susceptibility was measured for n = 5 individuals in three biological replicates.

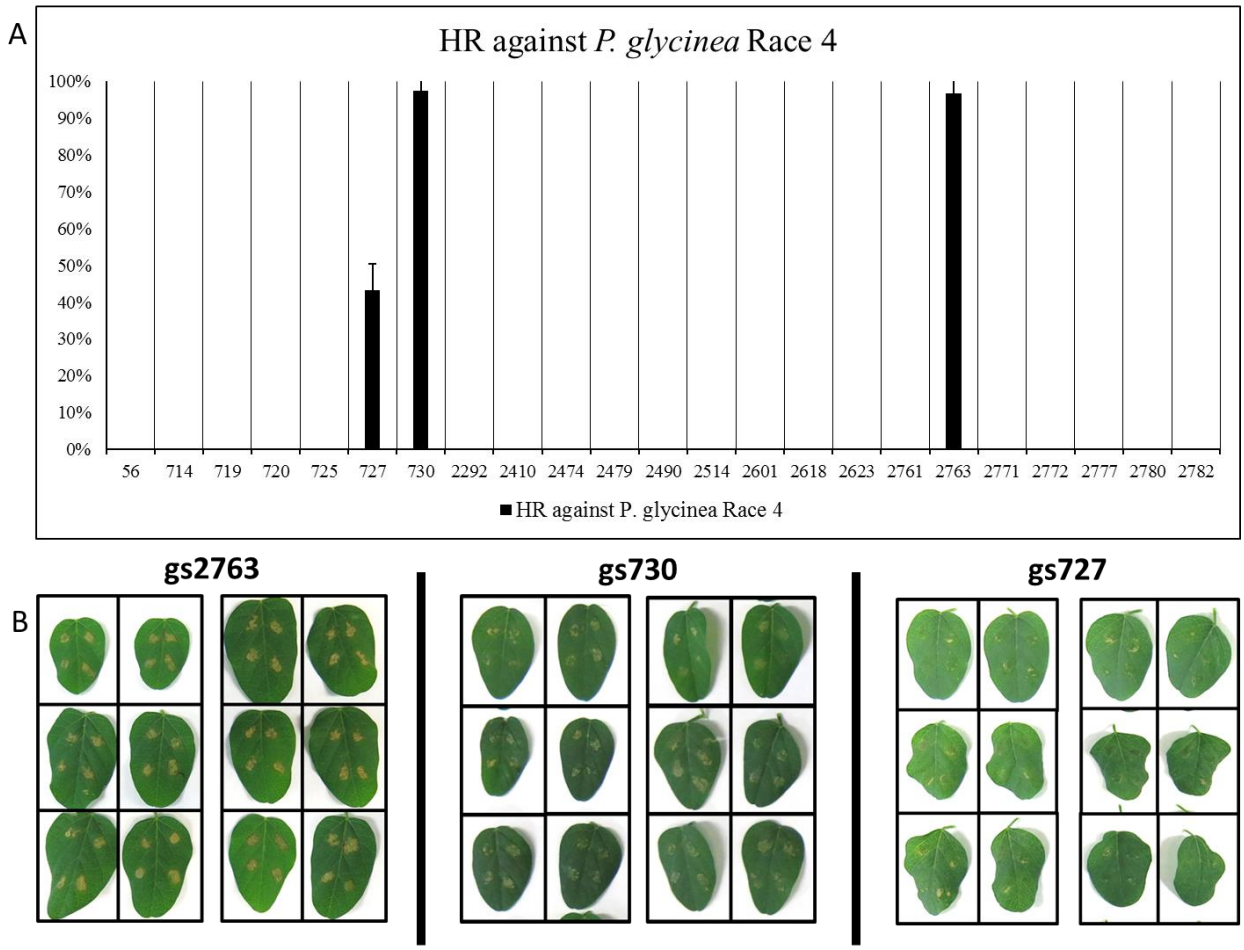


Figure 5.3: **Gs2763, gs730, and gs727 are incompatible with *Psg* race 4**.

These *P. sojae* resistant *G. soja* accessions produced a faster, stronger HR when inoculated with *Psg* as opposed to when inoculated with a core *P. sojae* effector. **A)** The %HR in response to *Psg* was recorded over 3 biological replicates. **B)** The images were taken from 1 biological replicate where these accessions were infiltrated with *Psg*.

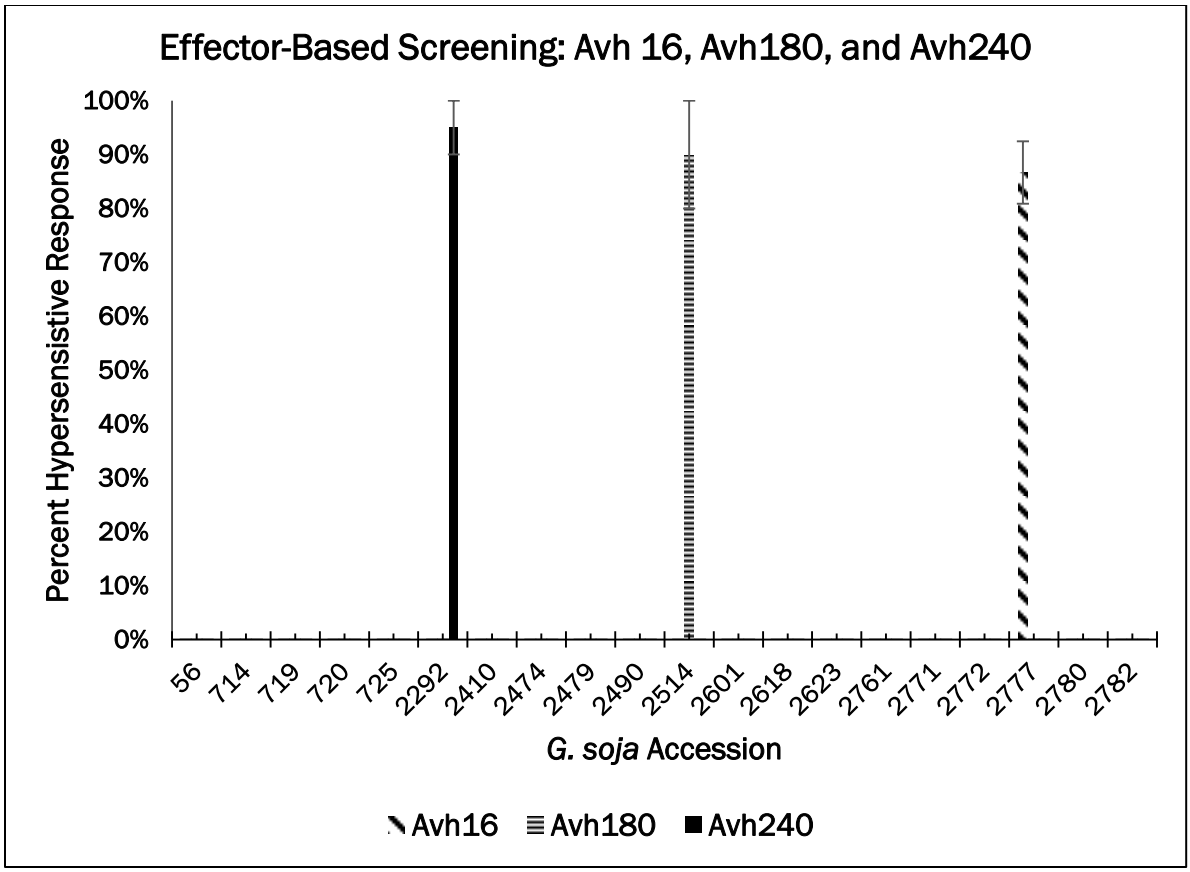
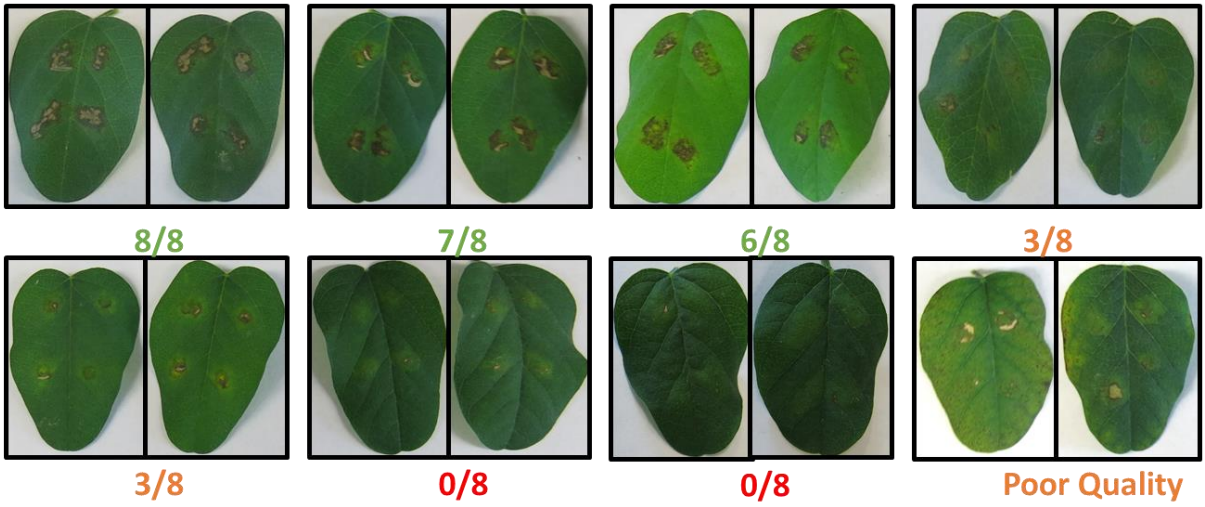


Figure 5.4: Gs2777, gs2514, and gs2292 produced HRs in response to *Avh*16, *Avh*180, and *Avh*240 respectively.

On the X axis, *G. soja* accessions subjected to effector-based screening are listed. On the Y axis, percent hypersensitive response among individuals within accessions recorded. We confirmed the observed effector responses over 3 biological replicates to determine standard deviation (n≥60). The error bars represents the percent standard deviation between 3 biological replicates. Neither *P. fluorescens* nor *Psg* produced HRs individually or in combination in these accessions.

A



B

Cross	# of $F_{2:3}$ Families[1] HgD	SEG	HgR	Expected Ratio	χ^2 value	Probability
gs2292 (R) X Williams (S)	21	48	20	1:2:1	0.230	>98%

Figure 5.5: **Segregating $F_{2:3}$ families were scored for response to *Avh*240**.

A) HR strength was variable in $F_{2:3}$ families. Individuals that produced HRs against *Avh*240 in ≥6/8 infiltrations were scored positive, whereas individuals which produced a HR in ≤2 infiltrations were scored negative. Individuals that produced HRs in 3-5/8 infiltrations were not included in scored data. **B)** This table shows the distribution of families in response to the trait HR against *Avh*240. Families were separated into 3 groups homozygous dominant (HgD), homozygous recessive (HgR), and segregating (SEG).

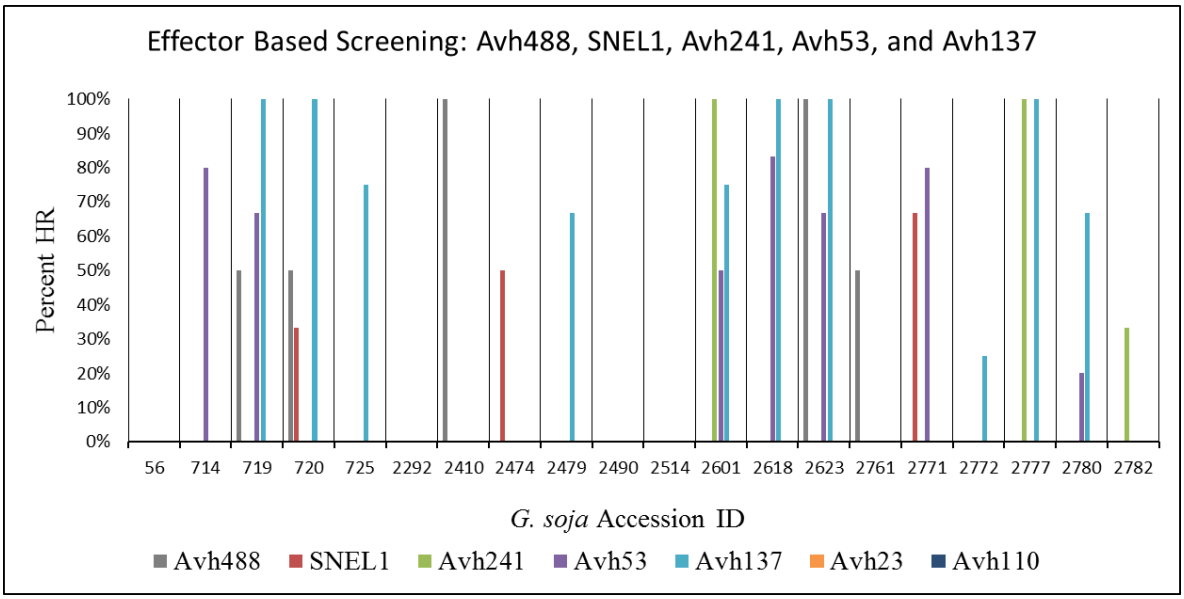


Figure 5.6: **Cumulative effector based screening with *Avh*488, SNEL1, *Avh*241, *Avh*53, and *Avh*137**.

On the Y axis, percent hypersensitive response is reported for effectors screened individually on *G. soja* germplasm.

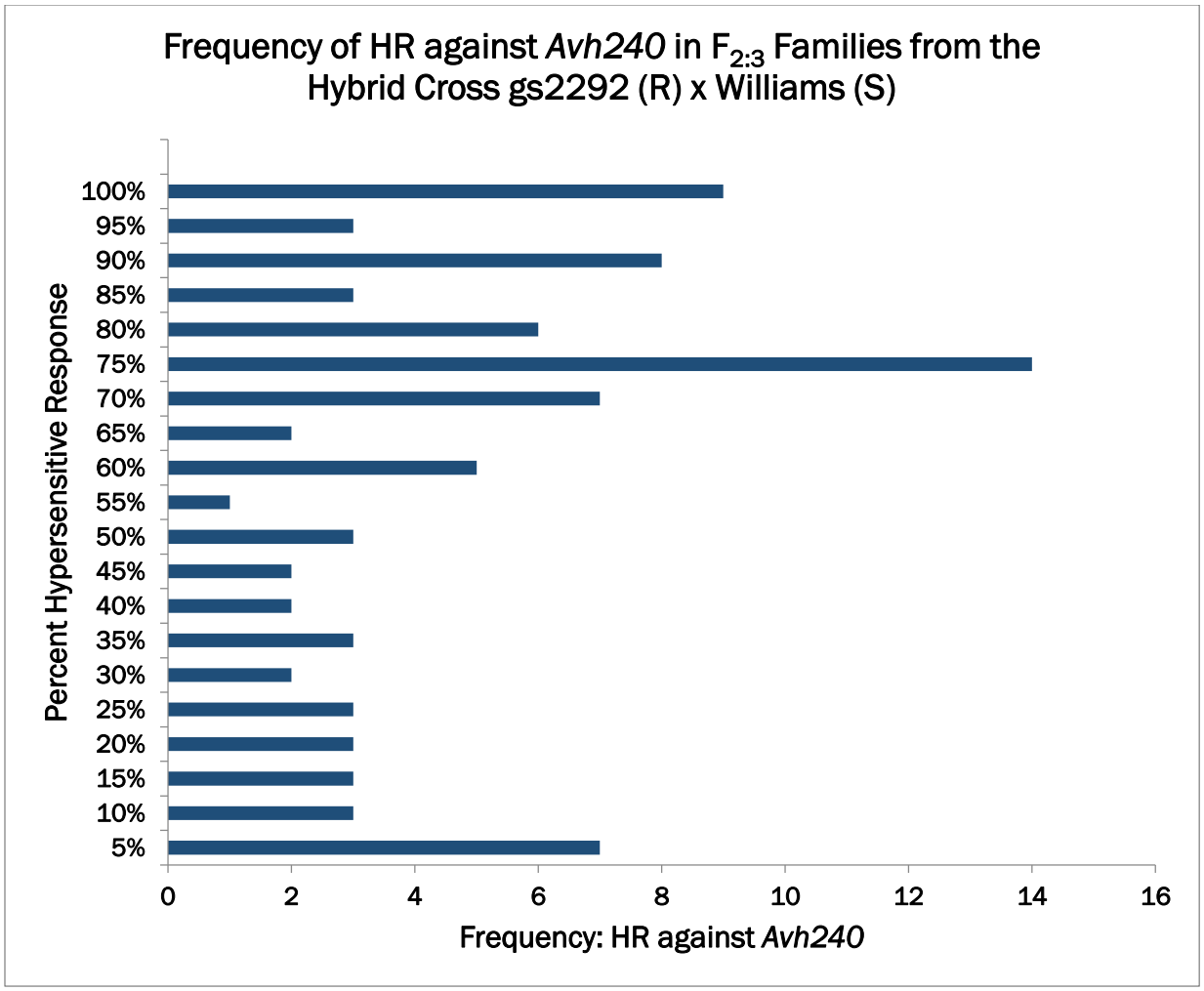


Figure 5.7: Histogram of effector-based screening on 88 $F_{2:3}$ hybrid families.

The distribution of effector response in segregating populations suggests the presence of a single dominant *R* gene against *Avh*240.

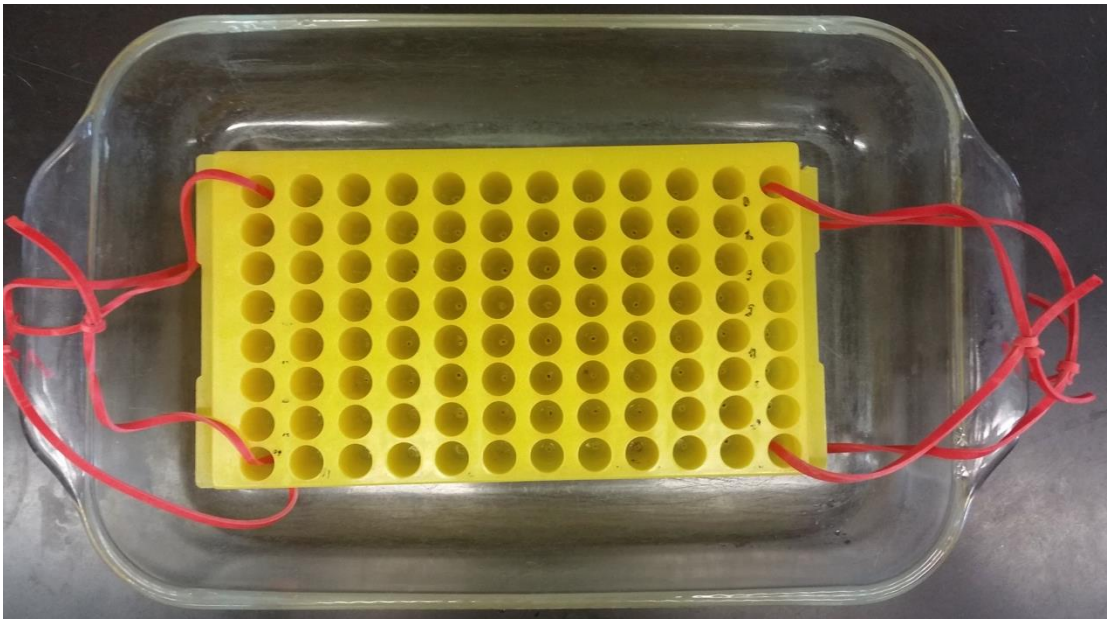


Figure 5.8: An apparatus for seed coat removal is shown in a photograph. Batch style acid scarification was used to remove the thick seed coats of *G. soja* seeds in a high throughput fashion.

Small holes were bored through the bottom of a 1.5 mL micro-centrifuge tube holder for scarification in 100% H_2SO_4. After 5 minutes, the apparatus is carefully picked up and residual acid is drained back into the bath. Washes were performed in diH_2O to remove residual acid. The appropriate safety precautions are essential due the volatility of this strong acid. All material must be acid resistant and care must be taken to avoid splashing or dipping.

Table 5.1: Accession Information for all tested *G. soja* germplasm is listed.

PI #	Accession ID	Origin
PI464935	gs2410	China: Jiangsu
PI549046	gs2761	China: Ningxia
PI549048	gs2763	China: Changping
PI464889A	gs727	China: Jilin
PI479746B	gs56	China Jilin
PI562538	gs2771	South Korea: Gyeon Gi Do
PI562539	gs2772	South Korea: Gyeon Gi Do
PI562542	gs725	South Korea: Gyeon Gi Do
PI407162	gs730	South Korea: Gyeon Gi Do
PI562544	gs2777	South Korea: Chung Ch'ong Nam Do
PI562547	gs2780	South Korea: Chung Ch'ong Nam Do
PI562549	gs2782	South Korea: Chung Ch'ong Nam Do
PI507582	gs2474	Japan: Aomori
PI507587	gs2479	Japan: Aomori
PI507597	gs2490	Japan: Nakajo
PI507621	gs2514	Japan: Nakajo
PI407076	gs2292	Japan: Unknown
PI507767	gs2601	Russia: Primorskaya Province
PI507788	gs2618	Russia: Primorskaya Province
PI507794	gs2623	Russia: Primorskaya Province
PI507814	gs714	Russia: Primorskaya Province
PI522215	gs719	Russia: Primorskaya Province
PI522216	gs720	Russia: Primorskaya Province

Table 5.2: Cumulative effector-based screening of wild soybean accessions.

Shaded boxes indicate which *G. soja* accession responds to which effector. Percentages are included with standard deviation when possible.

Accession ID	*Avh*488	SNEL1	*Avh*241	*Avh*53	*Avh*137	*Avh*16	*Avh*180	*Avh*240	Geographic Subgroup
gs2410	100%								1
gs2761	75%								2
gs727									3
gs728									3
gs2771			75%	80%					4
gs2772									4
gs725					75%				4
gs2777			71%		100%	87% ± 6			4
gs2780					67%				4
gs2782		100%							4
gs2474									5
gs2479					67%				5
gs2490									5
gs2514							90% ± 10		5
gs2292								95% ± 5	5
gs2601				50%	75%				6
gs2618				83%	100%				6
gs2623				67%	100%				6
gs714			100%	80%					6
gs719				67%	100%				6

gs720					100%				6

Table 5.3: Screening *G. soja* germplasm for a HR in response to *Psg* race 4.

HRs against *Psg* race 4 were observed in *G. soja* accessions gs730, gs2763, and gs727.

***G. soja* Line ID**	**% HR against *Psg* race 4**	**% Standard Deviation**
gs56	0%	0%
gs714	0%	0%
gs719	0%	0%
gs720	0%	0%
gs725	0%	0%
gs727	43%	7%
gs730	97%	4%
gs2292	0%	0%
gs2410	0%	0%
gs2474	0%	0%
gs2479	0%	0%
gs2490	0%	0%
gs2514	0%	0%
gs2601	0%	0%
gs2618	0%	0%
gs2623	0%	0%
gs2761	0%	0%
gs2763	97%	4%
gs2771	0%	0%
gs2772	0%	0%
gs2777	0%	0%
gs2780	0%	0%

gs2782	0%	0%

Table 5.4: Total *Avh16, Avh180,* and *Avh240* effector-response in *G. soja* germplasm.

Percent hypersensitive response and percent standard deviation is reported for the effectors *Avh16, Avh180,* and *Avh240*.

G. soja Line ID	*Avh*16 % HR	*Avh*16 % StDev	*Avh*180 % HR	*Avh*180 % StDev	*Avh*240 % HR	*Avh*240 % StDev
gs56	0%	0%	0%	0%	0%	0%
gs714	0%	0%	0%	0%	0%	0%
gs719	0%	0%	0%	0%	0%	0%
gs720	0%	0%	0%	0%	0%	0%
gs725	0%	0%	0%	0%	0%	0%
gs2292	0%	0%	0%	0%	95%	5%
gs2410	0%	0%	0%	0%	0%	0%
gs2474	0%	0%	0%	0%	0%	0%
gs2479	0%	0%	0%	0%	0%	0%
gs2490	0%	0%	0%	0%	0%	0%
gs2514	0%	0%	90%	10%	0%	0%
gs2601	0%	0%	0%	0%	0%	0%
gs2618	0%	0%	0%	0%	0%	0%
gs2623	0%	0%	0%	0%	0%	0%
gs2761	0%	0%	0%	0%	0%	0%
gs2771	0%	0%	0%	0%	0%	0%
gs2772	0%	0%	0%	0%	0%	0%
gs2777	87%	6%	0%	0%	0%	0%
gs2780	0%	0%	0%	0%	0%	0%
gs2782	0%	0%	0%	0%	0%	0%

Table 5.5: A-G Effector-based screening data for *Avh53, Avh137, Avh488,* SNEL1, *Avh241, Avh23,* and *Avh110*. Response to a given effector (*Avh* HR) divided by total plants screened (Total *Avh*) gives percent hypersensitive response (*Avh* % HR).

A **B**

G. soja Line ID	*Avh*53 HR	Total *Avh*53	*Avh*53 % HR	*G. soja* Line ID	*Avh*137 HR	Total *Avh*137	*Avh*137 % HR
gs56	-	-	NT	gs56	-	-	NT
gs714	4	5	80%	gs714	0	2	0%
gs719	4	6	67%	gs719	4	4	100%
gs720	0	3	0%	gs720	2	2	100%
gs725	0	5	0%	gs725	3	4	75%
gs2292	0	4	0%	gs2292	0	2	0%
gs2410	0	4	0%	gs2410	0	3	0%
gs2474	0	6	0%	gs2474	0	2	0%
gs2479	0	7	0%	gs2479	2	3	67%
gs2490	0	3	0%	gs2490	0	2	0%
gs2514	0	2	0%	gs2514	0	1	0%
gs2601	3	6	50%	gs2601	3	4	75%
gs2618	5	6	83%	gs2618	5	5	100%
gs2623	2	3	67%	gs2623	4	4	100%
gs2761	0	2	0%	gs2761	0	5	0%
gs2771	4	5	80%	gs2771	0	3	0%
gs2772	0	5	0%	gs2772	1	4	25%
gs2777	0	6	0%	gs2777	5	5	100%

gs2780	1	5	20%	gs2780	2	3	67%
gs2782	0	9	0%	gs2782	0	4	0%

C

G. soja **Line ID**	***Avh*488 HR**	**Total *Avh*488**	***Avh*488 % HR**
gs56	0	3	0%
gs714	0	2	0%
gs719	2	7	29%
gs720	1	5	20%
gs725	0	0	NT
gs2292	0	2	0%
gs2410	3	3	100%
gs2474	0	3	0%
gs2479	0	0	NT
gs2490	0	3	0%
gs2514	0	2	0%
gs2601	0	3	0%
gs2618	0	5	0%
gs2623	3	3	100%
gs2761	3	4	75%
gs2771	0	4	0%
gs2772	0	3	0%
gs2777	0	5	0%
gs2780	0	1	0%

D

G. soja **Line ID**	**SNEL1 HR**	**Total SNEL1**	**SNEL1 % HR**
gs56	0	3	0%
gs714	0	3	0%
gs719	0	6	0%
gs720	1	3	33%
gs725	0	0	NT
gs2292	0	1	0%
gs2410	1	5	20%
gs2474	0	3	0%
gs2479	0	0	NT
gs2490	0	1	0%
gs2514	0	1	0%
gs2601	0	0	NT
gs2618	0	5	0%
gs2623	0	0	NT
gs2761	0	5	0%
gs2771	0	2	0%
gs2772	0	2	0%
gs2777	0	4	0%
gs2780	0	1	0%

gs2782	0	2	0%	gs2782	3	3	100%

E

G. soja Line ID	*Avh*241 HR	Total *Avh*241	*Avh*241 % HR
gs56	0	6	0%
gs714	4	4	100%
gs719	1	8	13%
gs720	0	2	0%
gs725	0	3	0%
gs2292	0	6	0%
gs2410	0	1	0%
gs2474	0	11	0%
gs2479	0	3	0%
gs2490	0	4	0%
gs2514	0	2	0%
gs2601	2	7	29%
gs2618	2	8	25%
gs2623	0	6	0%
gs2761	0	7	0%
gs2771	3	4	75%
gs2772	0	11	0%
gs2777	5	7	71%
gs2780	0	2	0%
gs2782	2	6	33%

F

G. soja Line ID	*Avh*23 HR	Total *Avh*23	*Avh*23 % HR
gs56	0	4	0%
gs714	0	5	0%
gs719	0	2	0%
gs720	0	3	0%
gs725	0	3	0%
gs2292	0	2	0%
gs2410	0	3	0%
gs2474	0	2	0%
gs2479	0	4	0%
gs2490	0	5	0%
gs2514	0	3	0%
gs2601	0	3	0%
gs2618	0	3	0%
gs2623	0	2	0%
gs2761	0	5	0%
gs2771	0	2	0%
gs2772	0	2	0%
gs2777	0	3	0%
gs2780	0	2	0%
gs2782	0	3	0%

G

G. soja Line ID	*Avh*110 HR	Total *Avh*110	*Avh*110 % HR
gs56	0	3	0%
gs714	0	3	0%
gs719	0	3	0%
gs720	0	4	0%
gs725	0	3	0%
gs2292	0	2	0%
gs2410	0	2	0%
gs2474	0	5	0%
gs2479	0	2	0%
gs2490	0	3	0%
gs2514	0	3	0%
gs2601	0	2	0%
gs2618	0	2	0%
gs2623	0	4	0%
gs2761	0	4	0%
gs2771	0	2	0%
gs2772	0	2	0%
gs2777	0	2	0%
gs2780	0	2	0%
gs2782	0	3	0%

Table 5.6: *Avh240* effector-based screening data. - Cross gs2292 (R) x Williams (S).

Replicate 1 *Avh*240 responding plants are labeled by the column header (R1P). Replicate 1 total plants are labeled by the column header (R1T). The remaining replicates were labeled by the above scheme. Responders (P) divided by total plants screened (T) gives percent hypersensitive response (%HR) for all biological replicates.

Cross	R1P	R2P	R3P	R4P	R5P	R1T	R2T	R3T	R4T	R5T	P	T	%HR
1	0	3	0	2	0	0	3	3	3	0	5	9	56%
2	0	1	0	0	0	0	3	1	1	2	1	7	14%
3	0	4	2	0	0	0	4	5	0	0	6	9	67%
4	0	0	0	0	0	0	0	5	2	3	0	10	0%
5	0	1	1	1	3	0	1	1	2	4	6	8	75%
6	0	3	2	2	3	0	4	3	4	4	10	15	67%
7	0	1	0	0	0	0	3	1	3	0	1	7	14%
8	0	0	1	1	0	0	0	1	1	2	2	4	50%
9	0	3	0	0	3	0	4	2	0	4	6	10	60%
11	0	1	2	0	0	0	3	5	1	0	3	9	33%
12	0	0	1	1	3	0	4	3	2	5	5	14	36%
13	0	0	0	0	0	0	2	2	0	5	0	9	0%
14	0	0	1	0	1	0	2	3	0	4	2	9	22%
15	0	4	3	3	3	0	4	5	5	4	13	18	72%
17	0	0	0	0	0	0	3	2	0	0	0	5	0%
18	0	2	3	3	0	0	4	3	4	0	8	11	73%
19	0	0	2	1	3	0	2	4	2	3	6	11	55%
20	0	0	1	4	3	0	2	1	4	4	8	11	73%

21	0	0	2	1	0	0	0	2	1	0	3	3	100%
22	0	3	1	4	0	0	3	2	5	0	8	10	80%
23	0	3	2	3	0	0	3	2	3	1	8	9	89%
24	0	0	0	2	3	0	0	1	4	3	5	8	63%
25	0	4	2	2	5	0	4	5	4	5	13	18	72%
26	0	5	3	4	2	0	5	3	4	3	14	15	93%
27	0	3	2	1	0	0	3	2	1	0	6	6	100%
28	0	0	5	4	5	0	0	5	4	5	14	14	100%
29	0	2	5	5	3	0	3	5	5	4	15	17	88%
30	0	3	1	0	2	0	3	2	0	3	6	8	75%
31	0	5	2	0	4	0	5	5	0	4	11	14	79%
32	0	4	2	0	0	0	4	4	0	2	6	10	60%
33	0	3	3	0	2	0	4	4	0	3	8	11	73%
34	0	2	1	0	0	0	2	5	0	0	3	7	43%
35	0	1	1	4	0	0	1	1	5	1	6	8	75%
36	0	4	2	1	3	0	4	4	1	3	10	12	83%
37	0	4	2	1	0	0	5	4	1	0	7	10	70%
39	0	4	1	0	3	0	4	2	2	3	8	11	73%
40	0	0	0	0	1	0	0	3	1	5	1	9	11%
41	0	0	2	1	0	0	4	4	1	2	3	11	27%
42	0	0	0	0	1	0	4	2	0	4	1	10	10%
44	0	0	0	0	0	0	2	3	0	3	0	8	0%
45	0	0	0	1	0	0	2	2	2	0	1	6	17%
46	0	2	3	4	0	0	4	4	4	1	9	13	69%
49	0	0	1	2	0	0	2	1	2	0	3	5	60%

51	2	0	2	0	0	4	2	4	0	3	4	13	31%
52	3	3	0	0	0	4	3	0	0	0	6	7	86%
53	2	3	3	0	3	3	3	3	1	3	11	13	85%
54	4	0	3	0	2	4	0	3	0	2	9	9	100%
55	4	4	5	0	4	4	4	5	0	4	17	17	100%
56	3	0	5	4	5	5	0	5	4	5	17	19	89%
57	0	2	0	0	0	1	2	1	1	0	2	5	40%
59	0	1	1	1	1	1	2	2	0	1	4	6	67%
60	3	1	2	0	2	4	2	3	0	4	8	13	62%
61	0	0	1	0	0	6	2	4	0	2	1	14	7%
63	2	0	0	0	2	4	1	1	0	2	4	8	50%
64	0	0	0	0	0	3	2	0	0	0	0	5	0%
65	0	0	1	0	2	3	4	4	1	5	3	17	18%
66	2	1	0	0	1	5	4	0	0	3	4	12	33%
67	0	1	2	0	1	3	1	2	0	1	4	7	57%
68	3	0	2	1	0	4	0	2	1	0	6	7	86%
70	4	1	1	0	0	5	1	1	0	1	6	8	75%
71	5	2	0	0	0	5	2	0	0	1	7	8	88%
72	2	0	0	0	0	4	2	4	0	3	2	13	15%
73	2	2	2	1	2	2	2	4	1	2	9	11	82%
74	4	5	3	1	2	4	5	5	3	3	15	20	75%
75	3	2	2	1	3	4	2	3	1	4	11	14	79%
76	2	0	1	0	2	3	4	2	0	2	5	11	45%
77	1	0	1	0	0	1	0	1	0	0	2	2	100%
78	1	0	0	0	1	5	6	5	3	5	2	24	8%

79	3	1	3	3	1	4	2	4	4	2	11	16	69%
80	1	0	0	0	0	2	1	0	0	1	1	4	25%
81	3	0	0	2	0	3	0	0	2	0	5	5	100%
82	4	3	4	1	1	5	4	4	3	3	13	19	68%
83	3	3	2	2	1	5	3	2	3	1	11	14	79%
85	3	2	3	1	1	3	2	3	1	1	10	10	100%
86	5	0	3	4	0	5	0	5	4	0	12	14	86%
87	5	4	4	4	2	5	4	4	5	2	19	20	95%
88	5	0	3	1	0	5	0	3	1	0	9	9	100%
89	2	1	0	0	1	4	2	0	0	3	4	9	44%
90	3	4	2	0	3	4	4	2	0	3	12	13	92%
91	5	1	0	1	0	5	2	0	1	0	7	8	88%
92	2	0	0	0	0	4	2	1	0	0	2	7	29%
93	4	1	2	0	3	5	3	3	0	3	10	14	71%
94	2	1	1	0	0	2	2	1	0	0	4	5	80%
95	2	2	1	0	0	4	2	1	0	0	5	7	71%
96	0	0	0	0	0	1	0	1	0	0	0	2	0%
97	0	1	0	1	0	2	2	1	3	0	2	8	25%
98	0	0	0	0	0	1	0	1	0	0	0	2	0%
99	4	2	0	3	3	5	3	1	3	4	12	16	75%
100	5	1	3	0	2	5	2	3	2	2	11	14	79%

Chapter 6: Concluding Remarks

Summary of Research

This dissertation is focused on the identification and validation of disease resistance genes (*R*-genes) against two economically important pathogens of soybean; *Phytopthora sojae* (*P. sojae*) and *Soybean mosaic virus* (SMV), using a combination of molecular, genetic, and bioinformatic tools.

The deployment of host genetic resistance is essential for the management of devastating soybean pathogens. Complex molecular mechanisms underlie host resistance and a better understanding of the ongoing molecular arms race between plant pathogens and their hosts will allow for successful management of these diseases. *R*-genes commonly encode NLR proteins that operate on a gene-for-gene basis with pathogen avirulence (*Avr*) genes and form the basis for much of the deployed genetic resistance for both SMV and *P. sojae*. This research is focused on gaining a better understanding of host resistance and providing information to breeders that will allow for the generation of elite soybean cultivars with increased disease resistance qualities.

Chapter 2 describes the screening of segregating and recombinant inbred line (RIL) populations with *P. sojae* mycelium and effectors. The populations being screened were developed from a cross between parents with potential novel sources of *P. sojae* and the *P. sojae* susceptible population Williams. A bacterial delivery system was utilized to individually deliver effectors into soybean cells and phenotype the host plants for the presence or absence of a hypersensitive response (HR). A mycelium-based trifoliate disease assay was developed and utilized to determine if response to effector and resistance correlate. Four segregating

populations were scored in the $F_{2:3}$ generation and two populations were scored in the RIL generation for their response to core *P. sojae* effectors. Segregation ratios for each population tested indicate that resistance segregates independently, as single dominant genes as expected for *R*-genes. One RIL population was scored for resistance to *P. sojae* mycelium and the phenotypic data from this screen was used to produce a preliminary genetic map of the locus responsible for resistance.

Chapter 3 describes the validation of the SMV *R*-gene *Rsv3* using a transient expression assay and protein homology modeling. The *Rsv3* locus has been previously mapped to a region that contains ten genes, five of which encode nucleotide-binding domain leucine-rich repeat (NBD-LRR) proteins that commonly encode *R*-genes. Previous work has provided bioinformatic evidence of the gene Glyma14g38533 (G533) is the specific gene that encodes *Rsv3*. This project sought to build on that bioinformatic evidence with protein homology modeling and also provide functional validation through the use of a transient expression assay. The transient expression assay utilized *Agrobacterium tumefaciens* (*A. tumefaciens*) to deliver the avirulence component that *Rsv3* has been shown to recognize into *Nicotiana benthamiana* (*N. benthamiana*) leafs along with G533. Our hypothesis was that if G533 recognized the SMV avirulence component it would promote an immune response culminating in a robust HR. Results of this experiment failed to produce macroscopic HRs, but ion leakage assays have revealed an increase in cell death in lines carrying G533 from the resistant cultivar L29 when compared to G533 from the susceptible cultivar Williams82. Homology modeling experiments provided evidence that the amino acid differences between G533 from resistant and susceptible cultivars result in secondary structure

mutations. As protein structure dictates function, these differences could be significant. Gene silencing for each of the five NBD-LRR genes in the *Rsv3* locus is underway.

Chapter 4 describes the development and validation of an effector-directed breeding system to identify novel *P. sojae R*-genes in soybean. Thirty core *P. sojae* effectors were identified from the approximate 400 total effectors by B. Tyler's group at Oregon State University. Over 1000 *G. max* and *G. soja* isolates were screened to identify a total of 48 lines with potential sources of novel resistance by A. Robertson's group at Iowa State University. A *Pseudomonas fluorescens* EtHAN system was developed using the pEDV6 effector detector vector to deliver individual effectors into soybean cells and was validated with known *Avr/R* combinations. *G. max* parental lines identified as having potential novel resistance were screened with core effectors using EtHAN to identify the best candidates for durable *P. sojae* resistance. The combination of core effectors, resistant germplasm, and EtHAN effector delivery system were used to pilot effector-directed breeding in soybean and formed the basis for chapter 2.

Chapter 5 describes much of the same work that is covered in chapter 4 but with a focus on screening of the *G. soja* parents that were identified as having potential novel resistance. *G. soja* required special planting and growing condition optimizations due to the difficulty of working with the wild soybean relative.

Future Directions

Suggested follow-up experiments for *Phytophthora* Project

Population development of crosses between *P. sojae* resistant and susceptible parents should continue to move additional populations to the RIL generation. This will allow the

examination of populations that respond to core effectors that have not yet been screened since the initial parental screens potentially identifying additional sources of resistance. Lines from RIL populations, gm320 and gm326, should be res-screened to increase the data available for gene mapping. Effector screening data should be utilized to map the response to effectors *avh180* and *avh240* and determine if *avh180* response correlates with the genetic map of gm326 based on disease data. The preliminary genetic map of resistance in population gm326 should be validated.

Suggested follow-up experiments for *Rsv3* Project

Additional work in transient expression could provide functional validation of G533. This includes cloning the avirulence component from additional strains of SMV that *Rsv3* does not provide resistance to and see if cell death is still increased. Ideally, each of the five genes in the Rsv3 locus should be cloned and tested individually to ensure that G533 encodes *Rsv3*. However, I believe the most important experiments for the future of this project is gene complementation experiments for functional validation. Gene silencing is underway and early results look promising. We should work to support our collaborators in this effort. Complementation of G533 could also be done through overexpression experiments in susceptible soybean lines to determine if the susceptible cultivar becomes resistant.

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The Phytophthora sojae genome sequence: foundation for a revolution

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