Identification and characterization of brown stem rot resistance in soybean

by

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DEDICATION

To my husband Tyler for always believing in me, supporting my dreams, and constantly inspiring me to be my best.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DEDICATION</td>
<td>ii</td>
</tr>
<tr>
<td></td>
<td>ACKNOWLEDGEMENTS</td>
<td>v</td>
</tr>
<tr>
<td></td>
<td>ABSTRACT</td>
<td>vi</td>
</tr>
<tr>
<td></td>
<td>CHAPTER ONE: GENERAL INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Organization of Dissertation</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>References</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>CHAPTER TWO: IDENTIFYING NEW SOURCES OF RESISTANCE TO BROWN STEM ROT</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>SOYBEAN</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Abstract</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Introduction</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>Materials and Methods</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Results and Discussion</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>Conclusions</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>Acknowledgements</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>References</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>Figures and Tables</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>Supplemental Files</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>CHAPTER 3: HISTOPATHOLOGY OF SOYBEAN RESISTANT GENOTYPES IN RESPONSE TO</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td>INOCULATION BY <em>PHIALOPHORA GREGATA</em>, THE FUNGUS CAUSING BROWN STEM ROT</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td>Abstract</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td>Introduction</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>Materials and Methods</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>Results</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>Discussion</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>Acknowledgements</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>References</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td>Figures and Tables</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>CHAPTER 4: CHARACTERIZATION OF RESISTANT AND SUSCEPTIBLE RESPONSES TO</td>
<td>67</td>
</tr>
<tr>
<td></td>
<td><em>PHIALOPHORA GREGATA</em> IN SOYBEAN USING RNA-SEQ</td>
<td>67</td>
</tr>
<tr>
<td></td>
<td>Abstract</td>
<td>67</td>
</tr>
<tr>
<td></td>
<td>Introduction</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>Materials and Methods</td>
<td>69</td>
</tr>
<tr>
<td></td>
<td>Results</td>
<td>79</td>
</tr>
<tr>
<td></td>
<td>Discussion</td>
<td>97</td>
</tr>
</tbody>
</table>
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ABSTRACT

Breeding for pathogen resistance is an important objective to improve and protect soybean yields. In 2010, 14.4% of total soybean yield was suppressed by diseases. Brown stem rot (BSR), caused by the fungus *Phialophora gregata*, reduces yield by as much as 38%. To date, three dominant BSR resistance genes have been identified: *Rbs1, Rbs2*, and *Rbs3*. The objectives of my research were 1) to determine if plant introductions contained novel BSR resistance genes, 2) to determine the correlation between *P. gregata* hyphae growth and foliar symptoms as well as characterize the response of the three BSR resistance genes to *P. gregata* infection, and 3) to identify and characterize the gene networks regulating defense responses to BSR using RNA-Seq and Virus Induced Gene Silencing (VIGS).

To identify novel sources of resistance, four plant introductions with unknown sources of resistance (PI 594637, PI 594638B, PI 594650A, and PI 594858B) were each crossed to three genotypes with the three known BSR resistance genes, developing 12 populations for allelism studies. BSR symptoms were assessed under growth chamber conditions five weeks after inoculation by measuring foliar and stem severities, and recovery of *P. gregata* from stem sections. Allelism tests of plants from the crosses PI 594638B, PI 594858B, and PI 594650A with each of the resistant sources fit a 15:1 ratio, indicating that the resistant gene possessed by each of the PIs was non-allelic to *Rbs1, Rbs2*, and *Rbs3*. The three PIs contain at least one novel BSR-resistance gene, and have the potential to serve as donors to elite germplasm increasing stability of host resistance to *P. gregata*.

To understand the relationship between *P. gregata* hyphae growth and foliar symptom development, BSR severity was recorded for individual leaves on infected plants of five
different genotypes: L78-4097 (Rbs1), PI 437833 (Rbs2), PI 437970 (Rbs3), Corsoy 79
(susceptible control), and BSR 101 (resistant control). Microscopy was used to count hyphae in longitudinal sections made from stem and petiole segments corresponding to the rated leaves. Based on observed hyphal colonization patterns and significant correlations between the amount of hyphae present and the severity rating of the leaf, it was determined that Rbs1 has a different mechanism of resistance than Rbs2 and Rbs3. Further, the presence of hyphae is not a prerequisite for foliar BSR symptom development suggesting something other than hyphae, such as a toxin, may be responsible for foliar symptoms. This research provides insight into the mechanism of resistance to P. gregata for each of the three known BSR resistance genes.

To identify the gene networks regulating defense responses to P. gregata, we conducted RNA-Seq analyses of P. gregata infected and mock infected leaf, stem, and root tissues of both a resistant (PI 437970, Rbs3) and a susceptible (Corsoy 79) soybean genotype. Samples collected one-week post infection were used for RNA-Seq analyses. Results indicate that resistant and susceptible genotypes respond differently when infected with P. gregata. In all tissues, the resistant genotype induced greater numbers of defense-related genes with greater differential gene expression while the susceptible genotype induced a large number of general stress response genes. From the RNA-Seq results, novel SNPs were identified in the Rbs3 locus on Chromosome 16. Virus Induced Gene Silencing (VIGS) was used to characterize the candidate resistance genes and downstream defense responses. These results provide additional information about mechanisms of BSR resistance and will allow us to develop markers to screen lines for resistance earlier than current phenotyping protocols allow.
CHAPTER ONE: GENERAL INTRODUCTION

Soybean [*Glycine max* (L.) Merr.] is an important source of oil and protein used for both livestock and human consumption. The United States is the world’s leading soybean producer, producing 3.93 billion bushels on 82.7 million acres in 2015 (SoyStats, 2015). In 2015, this represented 61% of the world’s oilseed production and valued the soybean crop at $34.5 billion (SoyStats, 2015). As the monetary value for soybeans increases, the demand for healthy and productive soybean cultivars also increases.

Factors that reduce soybean yield, such as pests and pathogens, can influence the economic and general welfare of entire countries and individuals (Wrather et al., 2001). Throughout the world, there are 40 known soybean fungal pathogens. These pathogens reduce yield by varying degrees depending on the environment, cultivar, and geographic location (Grau et al., 2004). Brown stem rot (BSR), caused by the soil borne fungal pathogen *Phialophora gregata* f. sp. *sojae* (Allington & D.W. Chamberlain) W. Gams (syn. *Cadophora gregata*; Harrington and McNew, 2003), was responsible for a yield loss of 13,636,691 bushels in the United States in 2014 (Koenning and Wrather, 2010).

BSR was first identified in 1944 in central Illinois (Allington and Chamberlain, 1948). Since then, it has been identified in the majority of key soybean producing regions of the United States and Canada (Koenning and Wrather, 2010). Disease incidence and expression is greatest in environments with cool temperatures (Allington and Chamberlain, 1948). Because of these temperature requirements, BSR is more prevalent in the north central United States and Canada, and less important in the southern United States.
Two types of *P. gregata* have been identified: type A and type B. *P. gregata* type A causes leaf necrosis and chlorosis in addition to stem vascular discoloration, whereas *P. gregata* type B only causes vascular discoloration (Gray, 1972; Harrington et al., 2003). Type A and type B can be distinguished based on an insertion or deletion in the intergenic spacer region of ribosomal DNA (Hughes et al., 2009). Stunting, leaf deformity, reduced seed number and size, and plant lodging are also associated with BSR (Allington and Chamberlain, 1948).

*P. gregata* is an imperfect soil borne fungus; neither a sexual stage nor specialized survival structures, like chlamydospores or sclerotia, have ever been identified (Allington and Chamberlain, 1948). *P. gregata* overwinters as mycelium in soybean residue and conidia are produced. The conidia serve as the source of inoculum in the spring (Allington and Chamberlain, 1948). This lifecycle allows *P. gregata* to survive in soybean residue in the soil for several years.

The life cycle, pathogenic diversity, and distribution of BSR make the control of the disease difficult. Methods for controlling BSR include cultural practices such as tilling and crop rotation (Bachman and Nickell, 2000). These methods reduce the survival rate of *P. gregata* by reducing the amount of soybean residue in the field. However, common crop rotation practices in the Midwest are not long enough to significantly reduce BSR inoculum and the trend towards reduced tillage methods does not burry residue sufficiently enough to aid in decomposition (Adee et al., 1995). Therefore, there is a need for genetic resistance to BSR.

Three BSR resistance genes have been identified: *Rbs1, Rbs2*, and *Rbs3*. Each of the genes has been mapped to the same region on chromosome 16 in the soybean genome (Bachman et al., 2001; Klos et al., 2000; Lewers et al., 1999; Patzoldt et al., 2003; Webb, 1997).
These three genes provide incomplete resistance to BSR, defined as a delay or lack of symptom expression (Sebastian et al., 1985). The \textit{Rbs} resistant alleles also do not provide immunity from the disease, there are reports of BSR symptoms developing on soybean lines containing \textit{Rbs} alleles (Bachman et al., 1997b; Hanson et al., 1988; Nelson et al., 1989).

The overall objectives of this work were to identify new sources of resistance to brown stem rot as well as phenotypically and molecularly characterize the current sources of resistance. Thus, the first objective (Chapter 2) in this project was to identify and characterize novel sources of BSR resistance present in plant introductions by conducting allelism tests. Throughout this work, three different BSR disease assessment criteria were evaluated. The second objective (Chapter 3) was to characterize physiological responses to BSR in resistant and susceptible germplasm by microscopically examining hyphal growth patterns in stems and petioles in relation to foliar BSR symptom development. The third objective (Chapter 4) was to characterize molecular responses to BSR in resistant and susceptible germplasm using bioinformatics approaches.

\textbf{Organization of Dissertation}

The dissertation contains one published research article (Chapter 2) and two manuscripts in preparation (Chapters 3 and 4). Chapter 5 summarizes conclusions from the three studies. As each chapter contains its own introduction, the general introduction was kept brief. Literature for each individual experiment and procedure is introduced and discussed within the respective chapters. All individuals listed as authors have made contributions to the manuscripts. Author contributions in chapter two include: experimental design (S.R.C., C.E.M.,

References:


CHAPTER TWO: IDENTIFYING NEW SOURCES OF RESISTANCE TO BROWN STEM ROT IN SOYBEAN

A paper published in Crop Science

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and Michelle A. Graham

Abstract

Brown stem rot (BSR), caused by the fungus *Phialophora gregata*, causes yield losses up to 38%. Three dominant BSR resistant genes have been identified: *Rbs1*, *Rbs2*, and *Rbs3*. Additional BSR resistance loci will complement breeding efforts by expanding the soybean genetic base. The objective of this research was to determine if PI 594637, PI 594638B, PI 594650A, and PI 594858B contained novel BSR resistance genes. The accessions were crossed to three genotypes with known BSR resistance genes and populations were developed for allelism studies. A minimum of sixty F$_{2:3}$ families tracing to individual F$_2$ plants in each population were used, and six seeds from each F$_{2:3}$ family were tested. Resistant and susceptible controls and parents were also included. BSR symptoms were assessed under growth chamber conditions five weeks after inoculation by measuring foliar and stem severities, and recovery of *P. gregata* from stem sections. Allelism tests of F$_{2:3}$ plants from crosses of PI 594638B, PI 594858B, and PI 594650A with the resistant sources fit a 15:1 ratio, indicating that the resistant gene possessed by each of the PIs was non-allelic to *Rbs1*, *Rbs2*, and *Rbs3*. The three PIs contain at least one novel BSR-resistance gene, and have the potential to serve as donors to elite germplasm increasing stability of host resistance to *P. gregata*. Allelism tests of PI 594637 segregated in a 3:1 ratio and no significant difference was found between PI 594637 and the susceptible controls, indicating that PI 594637 is susceptible to BSR.
Introduction

Soybean \([Glycine\ max\ (L.\)\ Merr.]\) yields in the Northern United States are adversely affected by numerous diseases and pests, including brown stem rot (BSR) caused by the fungus \(Phialophora\ gregata\) f. sp. \(sojae\) (Allington & D.W. Chamberlain) W. Gams (syn. \(Cadophora\ gregata\); Harrington and McNew, 2003). When environmental conditions, such as cool temperatures (Allington and Chamberlain, 1948), favor disease development yield losses of up to 38% have been reported (Bachman and Nickell, 2000). BSR was first identified in 1944 in central Illinois (Allington and Chamberlain, 1948). Since then, it has also been found in the majority of key soybean producing regions of the United States and Canada (Koenning and Wrather, 2010). In surveys conducted in 2008, the disease was found in 68-73% of fields in Illinois, Iowa, and Minnesota (Malvick and Grunden, 2008).

Soybean plants are infected by \(P.\ gregata\) at the root level, after which the fungus colonizes the pith and vascular system, moving through the stem to the leaves of susceptible plants (Allington and Chamberlain, 1948). Two genotypes of \(P.\ gregata\) have been identified, A and B, both causing internal stem browning of the pith and vascular system (Gray, 1972; Harrington et al., 2003). In addition to stem rot, genotype A also produces leaf chlorosis and necrosis on infected plants. Genotype B does not produce leaf symptoms, and is only detectable when soybean stems are split at harvest and the pith and vascular system discoloration becomes visible (Gray, 1972; Harrington et al., 2003).

A combination of management practices, such as tilling and rotation with non-host crops, reduces the amount of soybean residue in the field which consequently reduces the survival rate of \(P.\ gregata\) and assists in protecting soybean yields (Tabor et al., 2003).
However, genetic resistance to BSR is the most effective tool to reduce BSR damage in soybeans (Bachman et al., 1997b; Mengistu et al., 1986). Three independent BSR resistance genes, \textit{Rbs1}, \textit{Rbs2}, and \textit{Rbs3}, have been identified, all mapping to chromosome 16, Molecular Linkage Group (MLG) J (Bachman et al., 2001; Klos et al., 2000; Lewers et al., 1999; Patzoldt et al., 2003; Webb, 1997). Each of the genes were identified in plant introductions (PI) of the USDA National Soybean Germplasm Collection (http://www.ars-grin.gov/). Resistance at the \textit{Rbs1} locus traces to PI 84946-2 (Chamberlain and Bernard, 1968) and the experimental line L78-4094 (Sebastian et al., 1985). Resistance at the \textit{Rbs2} locus traces to PI 437833 (Hanson et al., 1988), and the \textit{Rbs3} locus traces to both PI 84946-2 (Chamberlain and Bernard, 1968) and PI 437970 (Hanson et al., 1988). A resistant soybean reaction to BSR has been associated with the presence of a dominant allele at any of the three loci (Hanson et al., 1988; Willmont and Nickell, 1989). In addition to these three known resistance genes, other loci in the soybean genome may contribute to BSR resistance (Bachman and Nickell, 2000).

Despite the presence of BSR resistance genes, threats to future soybean production are still present. In breeding BSR-resistant soybean cultivars, the gene \textit{Rbs1} has been most commonly used in commercial cultivars (Tabor et al., 2003; Willmont and Nickell, 1989). It was first incorporated into line L78-4094 and later introgressed in the high-yielding cultivar ‘BSR 101’ (Eathington et al., 1995), which has become one of the most widely used donor sources of resistance to BSR leading to a narrow genetic base of BSR resistance. This narrow genetic base of resistance to BSR in current soybean cultivars, in addition to the narrow genetic base of the soybean crop in general (Gizlice et al., 1994) is a cause of great concern to soybean breeders and producers. There already are several reports of BSR symptoms on soybean lines containing
the three known \textit{Rbs} genes (Bachman et al., 1997b; Hanson et al., 1988; Nelson et al., 1989).

Further, possible pathogenic variability (Phillips, 1973) and physiological specialization of the fungus \textit{P. gregata} (Gray, 1971; Willmont and Nickell, 1989) have already been reported. These facts reinforce the crucial need to identify new sources of BSR resistance to continue the protection of the soybean yield from this disease.

In the search for new sources of BSR resistance, Perez et al. (2010) evaluated four PIs from south-central China that had shown resistance reactions to BSR (Patzoldt et al., 2003). In conducting the work, Perez et al. (2010) crossed each accession to the universal BSR susceptible cultivar ‘Century 84’ (Walker et al., 1986). The progenies of each cross were then screened for BSR resistance under growth chamber conditions. The resistance to \textit{P. gregata} was evaluated using foliar disease criteria (Perez et al., 2010) in addition to the stem colonization screening method developed by Tabor et al. (2003).

The objective of the work reported here was to determine if the BSR resistance in the four PIs was due to novel BSR-resistance genes. To this purpose, the four PIs (PI 594637, PI 594638B, PI 594650A, and PI 594858B) identified by Perez et al. (2010) were crossed with the three known sources of BSR resistance genes to develop 12 different genetic study populations to conduct allelism tests. Three different BSR disease assessment criteria were used to evaluate the populations: foliar severity, stem severity, and recovery of \textit{P. gregata} from stem segment.
Materials and Methods

Plant Material

Twelve soybean populations were developed by crossing PI 594637, PI 594638B, PI 594650A, and PI 594858B containing unknown sources of BSR resistance (Perez et al., 2010) to each of the three known sources of BSR resistance genes. The resistant source for gene \textit{Rbs1} was L78-4094 (Sebastian and Nickell, 1985), the source for \textit{Rbs2} was PI 437833 (Hanson et al., 1988), and the source for \textit{Rbs3} was PI 437970 (Hanson et al., 1988).

Population development was conducted at the Iowa State University research site located at the Isabela Substation, Univ. of Puerto Rico, Isabela, PR. Crossing blocks were planted in June 2009, creating F\textsubscript{1} seed for each of the 12 populations. The F\textsubscript{1} seed were planted in January 2010 and F\textsubscript{2} seed from ten individual F\textsubscript{1} plants from each cross combination were harvested. The individual F\textsubscript{1} plant identification created a total of ten distinct subpopulations for each hybrid combination. The identities were preserved in the planting of F\textsubscript{2} seed in June 2010. In October 2010, from each cross combination, a minimum of six F\textsubscript{2} plants from each of the 10 F\textsubscript{1} plants were harvested generating F\textsubscript{2:3} lines. This design resulted in a minimum of 60 F\textsubscript{2:3} families per population to conduct the allelism test.

Growth Chamber Experiments

To assess population resistance or susceptibility to the pathogen \textit{P. gregata}, experiments were conducted in growth chambers at the Agronomy Department at Iowa State University. On the basis of previous results (Perez et al., 2010), which suggested the presence of single BSR resistance genes in each of the PIs, and as per Sedcole (1977) calculations, 60 F\textsubscript{2:3} families were needed for evaluation in each population. To ensure that at least 60 families from
each cross were represented in the tests, a 75% germination percentage was assumed. On this basis, a total of 80 families were selected and used in the growth chamber experiments. Germination varied among crosses; therefore the number of families evaluated was different depending on the cross.

From each of the $F_{2:3}$ families, six seeds were transplanted. For each population six replications of the parents of the cross, two resistant controls ‘BSR 101’ (Tachibana et al., 1987) and ‘IA 1006’ (Fehr et al., ISURF Disclosure Docket # 02098), and two susceptible controls ‘Corsoy 79’ (Bernard and Cremeens, 1988) and PI 437654 (http://www.ars-grin.gov/), were also included in the experiments. To ensure adequate numbers of individual plants per population, seeds were sown in germination paper prior to transplanting into potting media in the growth chamber.

Seeds were germinated on germination paper following Burris and Fehr’s (1971) method number 8. Paper rolls were placed in a 62.45 liter (66 quart), clear plastic container with 1.5 cm of water. The container was sealed with a plastic lid and stored in the lab at 19°C under 16 hours of fluorescent and incandescent light per day. One week after sowing in germination paper, the germinated seedlings were transplanted into 3.8 x 21 cm cone-tainers (Stuewe and Sons, Corvallis, Oregon) in a randomized complete block design in the growth chamber. Each seedling was planted in a separate cone-tainer with Metro-Mix 900 (Sun Grow Horticulture, Agawam, Massachusetts) potting soil formulated with bark, Canadian Sphagnum peat moss, perlite, starter nutrient charge with Gypsum and slow release nitrogen, and dolomitic limestone. The temperature in the growth chamber was set to a constant 19°C +/-
1.5°C with a photoperiod of 16 hours of light per day, provided by fluorescent and incandescent light bulbs.

Inoculation Protocol

Two-week old plants were inoculated with *P. gregata* following the protocol described by Perez et al. (2010) which was a modification of the protocol described by Tabor et al. (2003). The *P. gregata* isolate used for all inoculations, *Oh*$_{2-3}$, was derived from the same isolates used in Tabor et al. (2003). Isolate *Oh*$_{2-3}$ (Lewers et al., 1999) is a single spore isolate of *Oh*$_2$ (Eathington et al., 1995) provided by Cecil Nickell at the University of Illinois. This isolate was chosen because it is well characterized and has the ability to consistently produce both stem and foliar symptoms under growth chamber conditions. The cultures were grown on green bean extract medium containing 35 g/liter of ground frozen *Phaseolus vulgaris* L. green pods, 20 g/liter of bacto agar (Becton, Dickinson and Company, Franklin Lakes, NJ), and 50 mg/liter of ampicillin (Sigma-Aldrich Corp., St. Louis, MO). Cultures were incubated for 60 days at room temperature and ambient light.

Spores were harvested from the green bean agar plates by washing the surface with sterile distilled water and gently scraping the cultures with a spatula. Spore concentration was determined with a hemocytometer. Spores were suspended in a 1.2% water agar paste to obtain a final spore concentration of $2.7 \times 10^7$ spores/ml. Approximately 20µl of the suspension was injected into the soybean stems 2 cm above the soil line with an 18 gauge needle. Soil in cone-tainers was kept moist by watering the pots daily until saturation. Plants were fertilized with a 24-8-16 fertilizer mixture on a weekly basis.
Disease Severity Assays

BSR severity was assessed five weeks after inoculation at plant growth stages V4-V5 (Fehr and Caviness, 1977), based on three measurements: foliar severity, stem severity, and stem recovery of *P. gregata*. BSR foliar severity was assessed on each plant using a severity scale (Perez et al., 2010) from 1-7 (Figure 1), where 1 represents the most infected. Perez et al. (2010) referred to the rating of foliar symptoms as vigor; here we will define the expression of BSR foliar symptoms as foliar severity.

To prepare plants for measuring stem symptoms (stem severity and pathogen recovery), plants were defoliated and cut at the soil line. Plant stems were surface sterilized by first submerging in 70% ethanol for 3 minutes, then submerging them in 10% sodium hypochlorite for 5 minutes, and finally rinsing twice with sterile distilled water. Plant height from the soil to the top of the main stem was also measured at the time of assessment and recorded in cm. To measure stem severity, plant stems were cut lengthwise from top to bottom. The amount of tissue discoloration and damage inside the vascular tissue was measured in cm upward from the inoculation point as indicated by Tabor et al. (2003). A plant was considered discolored if there was any visible dark brown discoloration on the vascular tissue or the pith. The length of discoloration was then divided by the total plant height and multiplied by 100, to obtain stem severity as a percentage.

To measure recovery of *P. gregata* from the stem, a modification of the procedure described by Tabor et al. (2003) was used. Five evenly spaced, 1-cm long, stem segments were cut from the plant. The first stem segment was harvested at the inoculation point, the second segment was 2/5 of the way up the stem, the third segment was in the mid portion of the stem,
the fourth segment was 4/5 of the way up the stem, and the fifth segment was the top of the plant. The five stem segments were plated in sequential order on green bean extract agar and allowed to grow in the dark at 5°C. Plates were evaluated for the presence of *P. gregata* colonies growing around the stem segments at two and four weeks after plating. Each plant was given a rating from 1 to 5, with 1 if *P. gregata* was only recovered from the inoculation point, 2 if *P. gregata* was recovered from the inoculation point and the second segment, 3 if recovered from the inoculation point and the next two segments, 4 if recovered from the inoculation point and the next three segments, and as a 5 if *P. gregata* was recovered from each of the five segments. The average of the measurements after two and four weeks of fungal growth was used to assess resistance/susceptibility of the plant.

**Data Analyses**

Following BSR disease assessment, the means for foliar severity, stem severity, and recovery of *P. gregata* were calculated for each F$_{2:3}$ family using PROC MEANS of SAS v. 9.1 (SAS Institute, 2003). The CONTRAST statement in PROC GLM was used to compare each F$_{2:3}$ family to the resistant and susceptible standards. All terms, F$_{2:3}$ family and plants per family, were treated as random in the model. Lines were declared resistant or susceptible if they were not significantly different from the resistant or susceptible standards, respectively (Eathington et al., 1995; Nelson et al., 1989). Histograms in Supplemental Figure S1 depict the cutoff used for determining resistance or susceptibility for each disease assessment criteria in each of the twelve populations. Lines that were significantly different from both the resistant and susceptible standards were classified as intermediate (Chen et al., 2001). Tabor et al. (2003) had observed that even resistant cultivars exhibit some BSR symptoms, and these intermediate
symptoms could be interpreted as incomplete resistance to *P. gregata*. Intermediate phenotypes not as resistant as the resistant class but superior in resistance to the susceptible class and susceptible controls were also identified in the present study, also indicating incomplete resistance. Therefore in this study the resistant and intermediate categories were pooled together for the analysis (Chen et al., 2001).

Using the two categories of resistant/intermediate counts and susceptible counts, a chi-square test was applied to each population to determine the goodness-of-fit of the observed segregation ratios to the expected genetic ratios. Theoretical inheritance ratios were tested for the one gene hypothesis of 3:1 (resistant: susceptible), the two gene hypothesis 15:1 (resistant: susceptible), and the dominant and resistant epistatic two gene hypothesis 13:3 (resistant: susceptible). Based on segregation ratios, we hypothesized four scenarios: 1) if the PI of interest contained a novel gene, the segregation ratio of 15:1 (resistant: susceptible) would be seen in crosses between the PI and L78-4094 (*Rbs1*), PI 437833 (*Rbs2*), and PI 437970 (*Rbs3*); 2) if the PI of interest contained one of the known BSR resistance genes, then no segregation would be seen when crossed to one of the three lines confirming that the gene in the PI is allelic to that known gene, and a 15:1 (resistant: susceptible) ratio would be seen with progeny from crosses with the other two non-allelic genes; 3) if the PI of interest contained a recessive resistance gene, the segregation ratio of 13:3 (resistant: susceptible) would be seen in crosses between the PI and *Rbs1, Rbs2,* and *Rbs3;* 4) if the progeny showed a good fit to a ratio of 3:1 (resistant: susceptible) which is expected for a single gene segregation, the PI in question was susceptible.
In order to determine if there was redundancy between the three measurements for disease screening, phenotypic rank correlations were computed for lines in each of the 12 populations using a Spearman rank correlation analysis. The three disease assessment criteria were used as variables in the correlation analysis. The mean performances of the F$_{2:3}$ families were ranked from the most resistant to most susceptible for each of the three phenotypic measurements. PROC CORR of SAS v. 9.1 (SAS Institute, 2003) was used to conduct the Spearman rank analysis to determine if there were significant correlations between foliar severity and stem severity, foliar severity and recovery of *P. gregata*, as well as correlations between stem severity and recovery of *P. gregata* in each population.

**Marker Analysis**

Previous work by Perez et al. (2010) tested for an association between the four PIs and the previously identified BSR loci. Using two markers from chromosome 16, Perez et al. (2010) reported a significant association between the markers and BSR resistance in PI 594638B, PI 594650A, and PI 594858B. To confirm this finding, we evaluated individuals from 595 available F$_{2:3}$ families in the L78-4094 (*Rbs1*) x PI 594650A population with simple sequence repeat markers (SSRs) spanning the length of chromosome 16 (linkage group J, 12 to 90 centiMorgans).

One week after infection with *P. gregata* using the above protocol, the first trifoliate was collected from one plant in each of the 595 F$_{2:3}$ families in the L78-4094 (*Rbs1*) x PI 594680A population. Tissue was flash frozen in liquid nitrogen and stored at -80°C. Flash frozen tissue was ground with a mortar and pestle in liquid nitrogen. A Qiagen® DNeasy® Plant Mini Kit (Qiagen®, Germantown, MD) was used to extract DNA following the manufacturer’s recommended protocol. Five weeks post inoculation foliar and stem severity ratings were
taken. Plants were scored resistant or susceptible based on their relationship to the resistant controls, BSR 101 and IA 1006, and the susceptible controls, Corsoy 79 and PI 437654.

Nineteen SSR markers from chromosome 16 were tested for polymorphism against the parents L78-4094 and PI 594650A (Table S2). Markers were amplified using polymerase chain reaction (PCR) and the PCR product was run on a 3% agarose gel with ethidium bromide. Bands were visualized under UV light. The seven markers that were polymorphic between the parents were then screened against DNA from each of the 595 F_{2:3} families. Using PROC CORR of SAS v. 9.1, single factor ANOVA tests were performed to detect associations between the SSR markers and BSR resistance.

**Results and Discussion**

Some degree of BSR symptoms were observed on all lines tested, which is expected as resistance to BSR is incomplete as mentioned previously (Tabor et al., 2003). Foliar severity was most severe on the susceptible control lines Corsoy 79 and PI 437654, with average foliar severity ratings of 4.4 and 3.0 respectively (Table 1). High foliar severity ratings indicating a more resistant reaction were observed on the resistant controls of BSR 101 and IA 1006, with average foliar severity ratings of 5.2 and 5.4 respectively.

BSR stem symptoms, with both stem severity and recovery of *P. gregata* measurements, were more severe in the susceptible controls than the resistant controls. The resistant controls had low stem severity and recovery of *P. gregata* ratings, indicating a resistant reaction (Table 1). Because the control lines had appropriate BSR phenotypic reactions, this gave reliability to the phenotypic reactions of the F_{2:3} progeny.
Even though the Oh<sub>2</sub> isolate has been in continuous culture since the 1970’s (Eathington et al., 1995; Hughes and Grau, 2010), control lines behaved appropriately when inoculated, indicating that Oh<sub>2,3</sub> is virulent under growth chamber conditions. Although little genetic variation has been found in the north-central population of <i>P. gregata</i> (Harrington et al., 2003), the need to expand the results requires characterization of these lines with other <i>P. gregata</i> isolates. Research is in progress to determine resistance reactions to other isolates of <i>P. gregata</i>.

**PI 594638B Allelism Tests**

After inoculation with <i>P. gregata</i>, the F<sub>2:3</sub> progeny of the cross L78-4094 (<i>Rbs1</i>) x PI 594638B did not deviate from a ratio of 15:1 for both foliar severity and recovery of <i>P. gregata</i> disease assessment criteria (Table 2). This indicated that the resistance gene in PI 594638B was not similar to <i>Rbs1</i>. When stem severity was used to assess BSR resistance in this cross, the F<sub>2:3</sub> progeny did not deviate from a ratio of 13:3. This ratio suggested resistance measured by stem severity was due to the interaction of a dominant and a recessive resistance gene, in contradiction with the ratios obtained from the foliar severity and recovery of <i>P. gregata</i> measurements.

There are several explanations for the contradictory 13:3 ratio results obtained with the stem severity measurement. Sebastian et al. (1985) found that even under controlled greenhouse conditions, heritability of foliar symptoms was two to three times greater than heritability of stem symptoms. Resistance to BSR decreases both the height and intensity of discoloration in the stem. Because the stem severity measurement only quantifies the height of discoloration, the overall complexity of the stem symptoms is not reflected. This may lead to a
high rate of error in quantifying stem severity which consequently leads to a low heritability and an overestimation of the number of susceptible plants. Further, Tabor et al. (2001) reported that infected plants can be heavily colonized by \textit{P. gregata} with little detectable stem browning, leading to the misclassification of resistant and susceptible plants. Together, these findings suggest that stem severity may be a poor indicator of colonization by \textit{P. gregata}.

In genetic populations from PI 437833 (\textit{Rbs2}) x PI 594638B and PI 437970 (\textit{Rbs3}) x 594638B, the progeny did not deviate from a ratio of 15:1 with any of the three disease assessment criteria (Table 2). This provided evidence that the resistance gene in PI 594638B was dissimilar to \textit{Rbs2} and \textit{Rbs3}. Genetic analysis conducted with PI 594638B indicated non-allelism with \textit{Rbs1, Rbs2,} and \textit{Rbs3}, indicating that PI 594638B contains a new resistance allele or gene to this pathogen.

\textbf{PI 594650A Allelism Tests}

The F$_{2:3}$ progeny of the crosses L78-4094 (\textit{Rbs1}) x PI 594650A and PI 437970 (\textit{Rbs3}) x PI 594650A did not deviate from a ratio of 15:1 for both foliar severity and recovery of \textit{P. gregata} disease assessment criteria (Table 3) which indicated that the resistance gene in PI 594650A was not similar to \textit{Rbs1} and \textit{Rbs3}. When evaluated for stem severity, the F$_{2:3}$ progeny from the cross of L78-4094 (\textit{Rbs1}) x PI 594650A did not deviate from a ratio of 13:3 and the F$_{2:3}$ progeny from the PI 437970 (\textit{Rbs3}) x PI 594650A population did not deviate from a 13:3 or 3:1 ratio. The fit to the 3:1 ratio, as evaluated by the chi square test, was better (P-value, 0.74) than for the 13:3 ratio (P-value, 0.30). These ratios suggest resistance, as measured by stem severity, was due to the interaction of a dominant and a recessive resistance gene (13:3 ratio) or single gene segregation (3:1 ratio). As mentioned, stem severity is a poor indicator of colonization by \textit{P.}
This may lead to the misclassification of resistant and susceptible plants and consequently fitting incorrect ratios.

When inoculated with *P. gregata* the progeny from the cross PI 437833 (*Rbs2*) x PI 594650A did not deviate from a ratio of 15:1 for all three disease assessment criteria (Table 3). This indicated that the resistance gene in PI 594650A was also dissimilar to *Rbs2*. Because genetic analysis conducted with PI 594650A indicated non allelism with *Rbs1, Rbs2,* and *Rbs3*, it is concluded that PI 594650A contains a new resistance allele or gene for BSR resistance.

**PI 594858B Allelism Tests**

When inoculated with *P. gregata*, the F$_{2:3}$ progeny in all crosses containing PI 594858B did not deviate from a ratio of 15:1 for the foliar severity disease assessment criteria (Table 4). This indicated that the resistance gene in PI 594858B was dissimilar to *Rbs1, Rbs2,* and *Rbs3*. However, stem disease severity measurements did not follow this trend. Stem disease severity measurements for all crosses containing PI 594858B did not deviate from either a 3:1 ratio, which indicated a susceptible reaction, and/or a 13:3 ratio, which indicated one dominant and one recessive gene are controlling resistance in the population. When rating stem symptoms, Perez et al. (2010) also found a higher number of susceptible progeny in PI594858B.

In order to differentiate between a 3:1 ratio and 13:3 ratio larger population sizes, approximately 800-900 observations, would be required (Hanson, 1959). Further studies will be needed to resolve the allelic nature of PI 594858B. However, since leaf symptoms are more reliable in screening for resistance than stem symptoms (Harrington et al., 2003; Sebastian et al., 1985), we hypothesize that the mechanism of resistance in PI 594858B is probably novel as indicated by the foliar severity measurement. It is also possible that PI 594858B is expressing
different mechanisms of resistance for leaf and stem symptoms (Waller et al., 1991). In this model leaf symptoms are controlled by one dominant resistance gene and stem symptoms are controlled by dominant and recessive epistasis, where the dominant allele masks the expression of both the alleles at a second locus, and a recessive allele masks expression of alleles at the first locus also resulting in a resistant phenotype. Bachman and Nickell (2000) have also proposed a genetic model for BSR resistance where resistance results from the epistatic interaction between pairs of loci.

**PI 594637 Allelism Tests**

Foliar severity ratings of the F$_{2:3}$ progeny from the genetic population L78-4094 ($Rbs1$) x PI 594637 did not fit any genetic ratio tested (Table 5). The genetic population PI 437833 ($Rbs2$) x PI 594637 did not deviate from the two gene ratio of 15:1 for the recovery of *P. gregata* measurement. The remaining seven chi-square tests conducted with PI 5494637 did not deviate from both the single gene segregation ratio (3:1) and the dominant-recessive epistatic segregation ratio (13:3). Segregation ratios for populations containing PI 594637 were not consistent and many ratios did not deviate from a 3:1 ratio, which indicated that PI 594637 was susceptible.

These results are consistent with those of Perez et al. (2010) who noticed that PI 594637 had greater susceptibility to BSR and was not statistically different from Century 84, the susceptible control. Although PI 594637 was chosen as a resistant parent based on a previous screening (Patzoldt et al., 2003), the screening in this research of both the line (Table 1) as well as the F2:3 population (Table 5) indicate that PI 594637 is BSR-susceptible and does not contain a novel resistance allele or gene to this pathogen.
**Marker Analysis**

Segregation ratios based on phenotypic data indicated that PI 594638B, PI 594858B, and PI 594650A may contain a novel resistance allele or gene to *P. gregata*. Genotyping by single marker analysis was conducted on the L78-4094 (*Rbs1*) x PI 594650A population (Supplemental Table S1). The population derived from *Rbs1* x PI 594650A was chosen for marker analysis because it was the largest population available, allowing for greater statistical power, and because *Rbs1* is the main source of resistance used in soybean breeding programs (Tabor et al., 2003; Willmont and Nickell, 1989).

Seven polymorphic SSR markers spanning chromosome 16 were screened on 595 F$_{2:3}$ lines in the population. A significant association was found between foliar severity and one SSR marker on chromosome 16, Satt621 (P-value, 0.021). However, this marker only explains 1.3% of the phenotypic variation ($R^2$, 0.013). A significant association was also found between stem severity and four of the SSR markers on chromosome 16. The $R^2$ values were low ranging from 1.2% to 5%.

Previously Perez et al. (2010) tested four resistant x susceptible populations, derived from PI 594638B, PI 594858B, PI 594637, and PI 594650A and the susceptible cultivar Century 84, for an association with two markers from the BSR loci on chromosome 16. Marker Satt547 was significantly associated with BSR resistance in populations PI 594638B x Century 84 and PI 594858B x Century 84, with $R^2$ values ranging 25 to 48%. While we also identified a significant association with Satt547 (P-value, < .0001), however, this marker only explained 5% of the phenotypic variation in stems. For foliar severity, this marker was not significant.
Differences between the findings of these studies could be due to differences in genetic backgrounds, environments and evaluation methods. Environmental and genotypic effects can alter disease severity. While both groups found similar average foliar disease scores (5.15 in Perez et al. (2010) and 5.6 here), different parameters were used for assessing disease severity in the stems. Further, testing of multiple markers spanning the length of chromosome 16 with similar results validate our findings. Our analyses suggest there may be a small effect gene at the Rbs locus, however the main resistance gene in PI 594650A is not located on chromosome 16. Future mapping studies will determine the exact location of BSR disease resistance genes in PI 594650A and the other lines tested in this study.

**Determination of Appropriate BSR Screening Methods**

BSR resistance is expressed in both the stems and leaves of susceptible soybean cultivars (Tabor et al., 2003). Therefore one foliar measurement (foliar severity) and two stem measurements (stem severity and recovery of *P. gregata*) were used to determine plant resistance or susceptibility. A Spearman-rank correlation analysis was conducted with each population to determine if there was redundancy between the three measurements for disease screening and the corresponding classification as resistant or susceptible genotypes (Table 6). The two stem measurements were found to be positively correlated in all populations tested. The foliar measurement had a higher correlation with the recovery of *P. gregata* measurement than with the stem severity measurement (Table 6) The findings are consistent with those of Tabor et al. (2003), who noted that stem discoloration was frequently absent even in stems that were heavily colonized by *P. gregata*. Therefore, based on the results of this study, measuring stem symptoms by the recovery of *P. gregata* measurement appears to be more
reliable than measuring internal stem discoloration. It is also worth noting that the foliar severity measurement was not strongly correlated to either stem measurement. If there were a toxin produced by *P. gregata* causing leaf necrosis and chlorosis, as has been previously suggested (Gray and Chamberlain, 1975; Kobayashi and Ui, 1977), stem measurements would not account for the damage caused by the toxin. Therefore, for the most accurate characterization of soybean resistance or susceptibility, both foliar severity and recovery of *P. gregata* measurements need to be recorded.

**Conclusions**

Through this experiment, PI 594638B and PI 594650A were identified as potentially containing a novel resistance allele or gene to *P. gregata* on the basis of the 15:1 segregation ratio obtained when the PIs were crossed to the three known sources of BSR resistance (*Rbs1, Rbs2*, and *Rbs3*). Because of the discrepancies of segregation ratios found in crosses with PI 594858B, it was determined that PI 594858B could contain a novel source of resistance or it could be expressing one mechanism of resistance for leaf symptoms and a different one for stem symptoms. Single marker analysis using the L78-4094 (*Rbs1*) x PI 594650A population revealed that there may be a gene with a small effect at the Rbs locus on chromosome 16, while the main novel resistance gene is located elsewhere in the soybean genome. Additional studies will be need to fine map the location of the novel gene(s). The new sources of resistance identified have the potential to serve as donor genes or alternate sources of resistance in breeding for resistant elite germplasm, thereby increasing the stability of the soybean host resistance to *P. gregata*. This research is one of the first conclusive pieces of
evidence that confirm that the genetic basis of resistance to *P. gregata* can be increased by introducing new sources of resistance in breeding programs.

In this study three disease assessment criteria, foliar severity, stem severity, and recovery of *P. gregata* from stems of artificially inoculated soybean plants were used to determine the severity of the BSR symptoms in the plant. The results of the Spearman-rank correlation analysis indicated that accurately classifying lines as resistant or susceptible to *P. gregata* requires both foliar severity and stem recovery of *P. gregata* measurements.

**Acknowledgements**

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**References**


Fehr, W.R., and C.E. Caviness. 1977. Stages of soybean development. Cooperative Extension Service; Agriculture and Home Economics Experiment Station, Iowa State University of Science and Technology.


**Figure 1.** Visual assessment scale of brown stem rot (BSR) severity in soybean plants 5 weeks after inoculation. A score of 1 was assigned if the plant was dead, 2 if the stem was green and had no leaves, 3 indicated predominantly chlorotic and necrotic leaves, 4 indicated some stunting with mosaic chlorosis and necrosis on leaves, 5 if the plant had normal leaf area with some leaves showing yellowing, 6 the plant appeared small but healthy, and 7 the plant appeared healthy and normal in height.
Table 1. BSR severity means of control plants and parents of genetic crosses. Lines were rated for foliar severity, stem severity, and recovery of *P. gregata*.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Foliar Severity $^1$</th>
<th>Stem Severity $^1$</th>
<th>Recovery of <em>P. gregata</em> $^5$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corsoy 79 (S)</td>
<td>4.4 e$^8$</td>
<td>36.5 c, d</td>
<td>3.8 a, b</td>
</tr>
<tr>
<td>PI 437654 (S)</td>
<td>3.0 f</td>
<td>58.2 a</td>
<td>3.9 a, b</td>
</tr>
<tr>
<td>BSR 101 (R)</td>
<td>5.2 a, b</td>
<td>34.4 d</td>
<td>3.3 c, d</td>
</tr>
<tr>
<td>IA 1006 (R)</td>
<td>5.4 a</td>
<td>35.2 d</td>
<td>2.8 e</td>
</tr>
<tr>
<td>L78-4094 (<em>Rbs1</em>)</td>
<td>4.8 c, d, e</td>
<td>36.1 c, d</td>
<td>3.0 c, d, e</td>
</tr>
<tr>
<td>PI 437833 (<em>Rbs2</em>)</td>
<td>5.3 a, b</td>
<td>41.6 b, c, d</td>
<td>4.0 a</td>
</tr>
<tr>
<td>PI 437970 (<em>Rbs3</em>)</td>
<td>5.1 a, b, c</td>
<td>40.2 b, c, d</td>
<td>2.7 e</td>
</tr>
<tr>
<td>PI 594637</td>
<td>4.7 d, e</td>
<td>46.4 b</td>
<td>3.5 b, c</td>
</tr>
<tr>
<td>PI 594638B</td>
<td>4.9 b, c, d</td>
<td>23.7 e</td>
<td>3.0 d, e</td>
</tr>
<tr>
<td>PI 594650A</td>
<td>5.5 a</td>
<td>42.9 b, c</td>
<td>2.2 f</td>
</tr>
<tr>
<td>PI 594858B</td>
<td>5.3 a, b</td>
<td>45.4 b</td>
<td>3.5 b, c</td>
</tr>
</tbody>
</table>

$^1$Foliar severity: measured on a scale of 1-7; 1 represents the most infected.

$^2$Stem severity: amount of internal stem browning divided by total plant height, and multiplied by 100 to obtain a percentage.

$^5$Recovery of *P. gregata*: measured on a scale of 1-5; 1 represents recovery of the fungus from the inoculation point only, 5 represents recovery of the fungus from the entire plant stem.

$^8$Means followed by the same letter in a column are not significantly different ($P < 0.05$; LSD).
Table 2. Segregation ratios for F2:3 plants of three populations screened for brown stem rot reaction. The three populations consisted of PI 594638B crossed to L78-4094 (Rbs1), PI 437833 (Rbs2), and PI 437970 (Rbs3). Lines were declared resistant (R), susceptible (S), or intermediate (I) for foliar severity, stem severity, and recovery of P. gregata. For the Chi-square analysis, R and I classes were pooled together. The expected segregation ratios of 15:1 (R:S) for two genes, 3:1 (R:S) for one gene, and 13:3 (R:S) for one dominant and one recessive gene, were tested.

<table>
<thead>
<tr>
<th>Cross/Allelism test</th>
<th>Disease assessment criteria</th>
<th>R</th>
<th>I</th>
<th>S</th>
<th>Ratio Tested</th>
<th>$\chi^2$ value</th>
<th>P value</th>
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<td><strong>Rbs1 gene</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>L78-4094 x PI 594638B</td>
<td>Foliar Severity</td>
<td>48</td>
<td>23</td>
<td>6</td>
<td>15:1</td>
<td>0.31</td>
<td>0.58</td>
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<td>57</td>
<td>12</td>
<td>13:3</td>
<td>0.51</td>
<td>0.48</td>
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<td>L78-4094 x PI 594638B</td>
<td>Recovery of P. gregata</td>
<td>17</td>
<td>58</td>
<td>2</td>
<td>15:1</td>
<td>1.75</td>
<td>0.19</td>
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<tr>
<td><strong>Rbs2 gene</strong></td>
<td></td>
<td></td>
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<td></td>
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<td>Foliar Severity</td>
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<td>15</td>
<td>8</td>
<td>15:1</td>
<td>3.05</td>
<td>0.08</td>
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<td>3</td>
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<td>2</td>
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<td>1.70</td>
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<td>Stem Severity</td>
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<td>60</td>
<td>3</td>
<td>15:1</td>
<td>0.69</td>
<td>0.41</td>
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<tr>
<td>PI 437970 x PI 594638B</td>
<td>Recovery of P. gregata</td>
<td>32</td>
<td>42</td>
<td>2</td>
<td>15:1</td>
<td>1.70</td>
<td>0.19</td>
</tr>
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</table>

$^1$Lines were classified as resistant (R) or susceptible (S) if not significantly different from the resistant or susceptible standards, respectively. Lines were classified as intermediate (I) if significantly different from both the resistant and susceptible standards.
Table 3. Segregation ratios for $F_{2:3}$ plants of three populations screened for brown stem rot reaction. The three populations consisted of PI 594650A crossed to L78-4094 ($Rbs1$), PI 437833 ($Rbs2$), and PI 437970 ($Rbs3$). Lines were declared resistant (R), susceptible (S), or intermediate (I) for foliar severity, stem severity, and recovery of *P. gregata*. For the Chi-square analysis, R and I classes were pooled together. The expected segregation ratios of 15:1 (R:S) for two genes, 3:1 (R:S) for one gene, and 13:3 (R:S) for one dominant and one recessive gene, were tested.

<table>
<thead>
<tr>
<th>Cross/Allelism test</th>
<th>Disease assessment criteria</th>
<th>Foliar Severity</th>
<th>Stem Severity</th>
<th>Recovery of <em>P. gregata</em></th>
<th>Observed Plant Ratios$^1$</th>
<th>R</th>
<th>I</th>
<th>S</th>
<th>Ratio Tested</th>
<th>$\chi^2$ value</th>
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<tr>
<td>L78-4094 x PI 594650A</td>
<td>Foliar Severity</td>
<td>59</td>
<td>11</td>
<td>7</td>
<td>15:1</td>
<td>1.06</td>
<td>0.30</td>
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<td>Stem Severity</td>
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<td></td>
<td>Recovery of <em>P. gregata</em></td>
<td>16</td>
<td>55</td>
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<td>15:1</td>
<td>0.31</td>
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<td><strong>Rbs2 gene</strong></td>
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<tr>
<td>PI 437833 x PI 594650A</td>
<td>Foliar Severity</td>
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<td>58</td>
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<td>60</td>
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<td>15:1</td>
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<td>0.58</td>
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<tr>
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<td>Recovery of <em>P. gregata</em></td>
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<tr>
<td>PI 437970 x PI 594650A</td>
<td>Foliar Severity</td>
<td>57</td>
<td>17</td>
<td>3</td>
<td>15:1</td>
<td>0.73</td>
<td>0.39</td>
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<td>56</td>
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<td>3:1</td>
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<td>Recovery of <em>P. gregata</em></td>
<td>20</td>
<td>49</td>
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<td>15:1</td>
<td>2.25</td>
<td>0.13</td>
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</tbody>
</table>

$^1$Lines were classified as resistant (R) or susceptible (S) if not significantly different from the resistant or susceptible standards, respectively. Lines were classified as intermediate (I) if significantly different from both the resistant and susceptible standards.
### Table 4. Segregation ratios for F2:3 plants of three populations screened for brown stem rot reaction. The three populations consisted of PI 594858B crossed to L78-4094 (Rbs1), PI 437833 (Rbs2), and PI 437970 (Rbs3). Lines were declared resistant (R), susceptible (S), or intermediate (I) for foliar severity, stem severity, and recovery of P. gregata. For the Chi-square analysis, R and I classes were pooled together. The expected segregation ratios of 15:1 (R:S) for two genes, 3:1 (R:S) for one gene, and 13:3 (R:S) for one dominant and one recessive gene, were tested.

<table>
<thead>
<tr>
<th>Cross/Allelism test</th>
<th>Disease assessment criteria</th>
<th>Observed Plant Ratios</th>
<th>χ² value</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Rbs1 gene</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L78-4094 x PI 594858B</td>
<td>Foliar Severity</td>
<td>R: 60, I: 10, S: 4</td>
<td>15:1</td>
<td>0.90</td>
</tr>
<tr>
<td>L78-4094 x PI 594858B</td>
<td>Stem Severity</td>
<td>R: 10, I: 51, S: 13</td>
<td>13:3</td>
<td>0.07</td>
</tr>
<tr>
<td>L78-4094 x PI 594858B</td>
<td>Recovery of P. gregata</td>
<td>R: 14, I: 49, S: 11</td>
<td>13:3</td>
<td>0.73</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>χ²</strong></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td><strong>value</strong></td>
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<td></td>
<td></td>
<td><strong>P</strong></td>
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</tr>
<tr>
<td><strong>Rbs2 gene</strong></td>
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<tr>
<td>PI 437833 x PI 594858B</td>
<td>Foliar Severity</td>
<td>R: 52, I: 15, S: 6</td>
<td>15:1</td>
<td>0.48</td>
</tr>
<tr>
<td>PI 437833 x PI 594858B</td>
<td>Stem Severity</td>
<td>R: 8, I: 56, S: 9</td>
<td>13:3</td>
<td>1.98</td>
</tr>
<tr>
<td>PI 437833 x PI 594858B</td>
<td>Recovery of P. gregata</td>
<td>R: 22, I: 40, S: 11</td>
<td>13:3</td>
<td>0.65</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>χ²</strong></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td><strong>value</strong></td>
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<td><strong>P</strong></td>
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<tr>
<td><strong>Rbs3 gene</strong></td>
<td></td>
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<tr>
<td>PI 437970 x PI 594858B</td>
<td>Foliar Severity</td>
<td>R: 63, I: 3, S: 2</td>
<td>15:1</td>
<td>1.27</td>
</tr>
<tr>
<td>PI 437970 x PI 594858B</td>
<td>Stem Severity</td>
<td>R: 2, I: 51, S: 15</td>
<td>13:3</td>
<td>0.49</td>
</tr>
<tr>
<td>PI 437970 x PI 594858B</td>
<td>Recovery of P. gregata</td>
<td>R: 16, I: 34, S: 18</td>
<td>13:3</td>
<td>2.67</td>
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</tbody>
</table>

†Lines were classified as resistant (R) or susceptible (S) if not significantly different from the resistant or susceptible standards, respectively. Lines were classified as intermediate (I) if significantly different from both the resistant and susceptible standards.
Table 5. Segregation ratios for F$_{2:3}$ plants of three populations screened for brown stem rot reaction. The three populations consisted of PI 594637 crossed to L78-4094 (Rbs1), PI 437833 (Rbs2), and PI 437970 (Rbs3). Lines were declared resistant (R), susceptible (S), or intermediate (I) for foliar severity, stem severity, and recovery of *P. gregata*. For the Chi-square analysis, R and I classes were pooled together. The expected segregation ratios of 15:1 (R:S) for two genes, 3:1 (R:S) for one gene, and 13:3 (R:S) for one dominant and one recessive gene, were tested.

<table>
<thead>
<tr>
<th>Cross/Allelism test</th>
<th>Disease assessment criteria</th>
<th>R</th>
<th>I</th>
<th>S</th>
<th>Ratio Tested</th>
<th>$\chi^2$ value</th>
<th>P value</th>
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<tbody>
<tr>
<td><strong>Rbs1 gene</strong></td>
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<td>Foliar Severity</td>
<td>27</td>
<td>18</td>
<td>27</td>
<td>3:1</td>
<td>6.00</td>
<td>0.01</td>
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<tr>
<td></td>
<td>Stem Severity</td>
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<td>55</td>
<td>14</td>
<td>13:3</td>
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<td></td>
<td></td>
<td>3:1</td>
<td></td>
<td></td>
<td></td>
<td>1.19</td>
<td>0.28</td>
</tr>
<tr>
<td></td>
<td>Recovery of <em>P. gregata</em></td>
<td>4</td>
<td>58</td>
<td>10</td>
<td>13:3</td>
<td>1.12</td>
<td>0.29</td>
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<tr>
<td><strong>Rbs2 gene</strong></td>
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</tr>
<tr>
<td>PI 437833 x PI 594637</td>
<td>Foliar Severity</td>
<td>21</td>
<td>41</td>
<td>18</td>
<td>13:3</td>
<td>0.74</td>
<td>0.39</td>
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<tr>
<td></td>
<td>Stem Severity</td>
<td>4</td>
<td>57</td>
<td>19</td>
<td>13:3</td>
<td>1.32</td>
<td>0.25</td>
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<td></td>
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<td></td>
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<td>Recovery of <em>P. gregata</em></td>
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<td>21</td>
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<td>Stem Severity</td>
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<td>50</td>
<td>16</td>
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<tr>
<td></td>
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<td>3:1</td>
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<td>0.45</td>
<td>0.50</td>
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<td>Recovery of <em>P. gregata</em></td>
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<td>42</td>
<td>21</td>
<td>3:1</td>
<td>0.45</td>
<td>0.50</td>
</tr>
</tbody>
</table>

†Lines were classified as resistant (R) or susceptible (S) if not significantly different from the resistant or susceptible standards, respectively. Lines were classified as intermediate (I) if significantly different from both the resistant and susceptible standards.
Table 6. Spearman rank correlation analysis. Rank correlation and P-value of the mean performances of lines for each population across three different phenotypic measurements.

<table>
<thead>
<tr>
<th>Arrangements Correlated</th>
<th>Foliar Severity : Stem Severity</th>
<th>Foliar Severity : Recovery of <em>P. gregata</em></th>
<th>Stem Severity : Recovery of <em>P. gregata</em></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Rank Correlation</td>
<td>Rank Correlation</td>
<td>Rank Correlation</td>
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<td>Population</td>
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<tr>
<td>L78-4094 x PI 594858B</td>
<td>0.174</td>
<td>0.272*</td>
<td>0.609*</td>
</tr>
<tr>
<td>PI 437833 x PI 594858B</td>
<td>0.136</td>
<td>0.305*</td>
<td>0.561*</td>
</tr>
<tr>
<td>PI 437970 x PI 594858B</td>
<td>-0.076</td>
<td>-0.072</td>
<td>0.186</td>
</tr>
<tr>
<td>L78-4094 x PI 594637</td>
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<td>0.267*</td>
</tr>
<tr>
<td>PI 437833 x PI 594637</td>
<td>0.344*</td>
<td>0.100</td>
<td>0.416*</td>
</tr>
<tr>
<td>PI 437970 x PI 594637</td>
<td>0.448*</td>
<td>0.621*</td>
<td>0.594*</td>
</tr>
<tr>
<td>L78-4094 x PI 594638B</td>
<td>0.047</td>
<td>0.157</td>
<td>0.491*</td>
</tr>
<tr>
<td>PI 437833 x PI 594638B</td>
<td>0.350*</td>
<td>0.342*</td>
<td>0.492*</td>
</tr>
<tr>
<td>PI 437970 x PI 594638B</td>
<td>0.130</td>
<td>0.192</td>
<td>0.430*</td>
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<td>0.252</td>
<td>0.461*</td>
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<tr>
<td>PI 437833 x PI 594650A</td>
<td>0.248*</td>
<td>0.266*</td>
<td>0.400*</td>
</tr>
<tr>
<td>PI 437970 x PI 594650A</td>
<td>0.043</td>
<td>0.124</td>
<td>0.599*</td>
</tr>
</tbody>
</table>

*Values significant at the 0.05 probability level*
Supplemental Files

Supplemental Figure S1. Histograms of three disease assessment criteria (foliar severity, stem severity, and recovery of *P. gregata*) in each of the 12 populations tested. Solid blue line indicates the cut off value for plants categorized as susceptible.
**Supplemental Table S1.** Phenotypic means of genotypic classes, P-values, and $R^2$ values for SSR markers on chromosome 16.

<table>
<thead>
<tr>
<th>SSR Marker</th>
<th>cM Position</th>
<th>Severity Rating</th>
<th>Phenotypic means for genotypic classes</th>
<th>P-Value</th>
<th>$R^2$</th>
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<tbody>
<tr>
<td>Satt674</td>
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<td>Stem Severity</td>
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<td>0.263</td>
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<tr>
<td>Satt596</td>
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<td>5.58</td>
<td>5.59</td>
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<td>Stem Severity</td>
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<td>0.27</td>
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<td>Foliar Severity</td>
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<td>Stem Severity</td>
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<tr>
<td>Sat_366</td>
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<td>Stem Severity</td>
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<td>Satt547</td>
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<td>Stem Severity</td>
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<tr>
<td>Sat_395</td>
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<td></td>
<td></td>
<td>Stem Severity</td>
<td>0.33</td>
<td>0.27</td>
</tr>
</tbody>
</table>

† 7 out of 19 simple sequence repeat (SSR) markers tested were polymorphic and were used for genotyping. Non polymorphic markers include: Sat_225, Sat_259, Sat_339, Sat_350, Sat_361, Satt244, Satt287, Satt380, Satt456, Satt620, Satt622, and Satt654. Marker position is based on the SoyBase 2003 consensus map (http://www.soybase.org/sbt/).

‡ Rbs1, Rbs2, and Rbs3 are located in the region 44.08 – 78.57 (Bachman et al. 1999). Markers within this interval are shaded in grey.

§ Foliar severity: measured on a scale of 1-7; 1 represents the most infected.

¶ Stem severity: amount of internal stem browning divided by total plant height, and multiplied by 100 to obtain a percentage.

*Values significant at the 0.05 probability level
CHAPTER 3: HISTOPATHOLOGY OF SOYBEAN RESISTANT GENOTYPES IN RESPONSE TO INOCULATION BY *PHIALOPHORA GREGATA*, THE FUNGUS CAUSING BROWN STEM ROT

Chantal E. McCabe, Leonor F. Leandro, Silvia R. Cianzio, and Michelle A. Graham

Abstract

Breeding for pathogen resistance is an important objective to improve and protect soybean yields. In 2014, 13,636,691 bushels of soybean were lost in the United States to the disease brown stem rot (BSR), caused by the fungus *Phialophora gregata*. Three dominant BSR resistance genes *Rbs1*, *Rbs2*, and *Rbs3*, have been identified and mapped to soybean chromosome 16. However, the mechanism(s) of resistance for these genes remains unknown. A microscopic analysis was conducted to examine fungal growth patterns in a susceptible cultivar and cultivars containing *Rbs1*, *Rbs2*, and *Rbs3* following infection with *P. gregata*. To understand *P. gregata* disease progression and the internal soybean plant responses, external foliar symptoms were compared with internal hyphal colonization. Our results suggest that *Rbs1* has a different mechanism of resistance than *Rbs2* and *Rbs3*, due to the fact differences in hyphae petiole colonization. BSR foliar symptoms were identified on some trifoliates lacking hyphae in the respective stem and petiole segments. Further, xylem tissue was degraded in the susceptible control. This indicated that the presence of hyphae is not a prerequisite for foliar symptom development, suggesting a toxin is involved in *P. gregata* pathogenesis.
Introduction

Soybean \([\text{Glycine max (L.) Merr.}]\) is one of the main oilseed crops grown throughout the world, representing 61% of oilseed production worldwide (SoyStats, 2015). The United States is the world’s leading soybean producer, producing 3.93 billion bushels in 2015 (SoyStats, 2015). Given the economic value of soybean, it is important to maintain its productivity. Each year soybean yield is reduced due to damage from pests and pathogens. In 2014, 13,636,691 bushels of soybean were lost in the United States to the disease brown stem rot (Koenning and Wrather, 2010).

Brown stem rot (BSR) is caused by the fungal pathogen \(\text{Phialophora gregata f. sp. sojae}\) (Allington & D.W. Chamberlain) W. Gams (syn. \text{Cadophora gregata}; Harrington and McNew, 2003). This soil borne fungus infects soybean plants at the root level, after which the fungus colonizes the pith and vascular system, moving through the stem of susceptible plants (Allington and Chamberlain, 1948). Two types of \(P. gregata\) have been identified: type A and type B. \(P. gregata\) type A causes leaf necrosis and chlorosis in addition to stem vascular discoloration, whereas \(P. gregata\) type B only causes vascular discoloration (Gray, 1972; Harrington et al., 2003).

Resistance genes prevent yield loss in soybeans grown in environments conducive to BSR (Bachman et al., 1997a). Three genes for resistance to \(P. gregata\) have been identified: \(Rbs1\), \(Rbs2\), and \(Rbs3\). Each of these genes has been mapped to chromosome 16 in the soybean genome (Bachman et al., 2001; Klos et al., 2000; Lewers et al., 1999; Patzoldt et al., 2003; Webb, 1997). Previous studies have suggested different cultivars possess different resistance
mechanisms (Eathington et al., 1995) and that foliar and stem symptom expression could be controlled by different mechanisms (McCabe et al., 2016; Waller et al., 1991).

Studying pathogen establishment and development in resistant and susceptible genotypes can improve our understanding of symptom development, disease progression, and resistance mechanisms. Microscopic analyses allow for visualization of both disease progression and internal plant anatomical responses. A microscopic analysis comparing anatomical responses during latent and pathogenic stages of soybean plants infected with \emph{P. gregata} was conducted by Impullitti and Malvick (2014) using \emph{Rbs2} and \emph{Rbs3} resistant cultivars, as well as a susceptible cultivar. The authors proposed two mechanisms of resistance to \emph{P. gregata}. One was that the soybean vascular system could regenerate after infection by producing more vessels to compensate for loss of vessel function due to \emph{P. gregata} colonization. Another possibility was that resistant plants have the ability to restrict or exclude the pathogen from the vascular system, an ability susceptible plants do not have. Despite these proposed mechanisms of resistance, many unanswered questions still remain regarding hyphae colonization and development of foliar symptoms.

The purpose of our study was to gather insights into the mechanism of resistance to \emph{P. gregata} for each of the three known BSR resistance genes. Hyphal growth patterns in stems and petioles of resistant and susceptible cultivars in relation to foliar BSR symptom development were observed. This work was conducted in three genotypes each possessing one of three known BSR resistance genes as well as resistant and susceptible controls. To our knowledge, this study is the first attempt using a targeted approach to compare external foliar symptom development with the internal hyphal growth patterns.
Materials and Methods

Growth conditions and inoculation protocol

The experiment was conducted using genotypes possessing the three known sources of BSR resistance: $Rbs_1$, $Rbs_2$, and $Rbs_3$. The resistant source for gene $Rbs_1$ was L78-4094 (Sebastian and Nickell, 1985), the source for $Rbs_2$ was PI 437833 (Hanson et al., 1988), and the source for $Rbs_3$ was PI 437970 (Hanson et al., 1988). The resistant cultivar ‘BSR 101’ (Tachibana et al., 1987) containing $Rbs_1$ and $Rbs_3$, and the susceptible cultivar ‘Corsoy 79’ (Bernard and Cremeens, 1988), were also included in the study as controls.

Twelve seeds of each of the five genotypes were planted in a completely randomized design in the growth chamber. Each seed was planted in a separate 3.8 x 21 cm cone-tainer (Stuewe and Sons, Corvallis, OR) with Metro-Mix 900 potting soil (Sun Grow Horticulture, Agawam, MA). The temperature in the growth chamber was set at 19°C +/- 1.5°C with a photoperiod of 16 hours of light per day provided by fluorescent and incandescent light bulbs. All plants were watered daily until saturation, and fertilized with a 24-8-16 fertilizer mixture weekly.

Two weeks after planting, when plants were at approximately the V2 growth stage (Fehr and Caviness, 1977), ten plants in each of the five genotypes were inoculated with $P. gregata$ type A. The $P. gregata$ spore suspension was prepared using isolate $Oh_{2.3}$ (Lewers et al., 1999). $Oh_{2.3}$ is a single spore isolate of $Oh_2$ (Eathington et al., 1995) provided by Cecil Nickell at the University of Illinois. This isolate was chosen because it is well characterized and has the ability to consistently produce both stem and foliar symptoms under growth chamber conditions. The cultures were grown on green bean extract medium containing 35 g/L of ground frozen
Phaseolus vulgaris L. green pods, 20 g/L of water agar, and 50 mg/L of ampicillin. Cultures were incubated for 60 days at room temperature and ambient light. Spores were harvested from the agar plates by washing the agar surface with sterile distilled water and gently scraping the surface of the agar with a spatula. Spore concentration was determined with a hemocytometer. Spores were suspended in a 1.2% water agar paste to obtain a final spore concentration of 2.7x10^7 spores/ml (Perez et al., 2010; Tabor et al., 2003). Approximately 20 µl of the spore suspension was injected into the soybean stem 2 cm above the soil line using an 18 gauge needle.

The remaining two plants in each of the five genotypes were injected with a sterile solution of 1.2% water agar, serving as the mock-inoculated control. Approximately 20 µl of the sterile solution was injected into the soybean stems 2 cm above the soil line using an 18 gauge needle.

**Tissue collection**

Five weeks after inoculation, at plant growth stages V4 and V5 (Fehr and Caviness, 1977), two plants (one inoculated with *P. gregata* and one mock-inoculated) from each genotype were selected for microscopic analysis. For the purpose of the analysis, selected plants could not have any missing leaves. Each leaf on the plant was given a BSR individual leaf severity rating: 1- healthy and green leaf, 2- partially yellow leaf, 3- entirely yellow leaf, 4- entirely yellow leaf and curling, 5- brown and dead leaf.

Stem and petiole tissue segments corresponding to each individually rated leaf were collected from each plant in each genotype. Plant tissue was submerged in formalin-acetic acid-alcohol (FAA) during the collection process. Tissues were collected by harvesting the 1 cm
segment of the leaf’s petiole nearest to the plant stem and the 1 cm stem segment directly below that leaf. Collected tissue was immediately placed in a 20mL vial containing FAA, maintaining the identity of each tissue segment. From each genotype, all stem and petiole segments from the inoculated plant and the bottom stem and petiole segments from the mock-inoculated plant were prepared for light microscopy.

**Tissue preparation**

For light microscopy, the stem and petiole segments were fixed in FAA for a minimum of 48 hours at 4°C by the Microscopy and NanoImaging Facility at Iowa State University. Segments were dehydrated in a graded ethanol series (30%-100%), cleared with 100% xylene, and infiltrated and embedded using Paraplast paraffin (Fisher Scientific, Pittsburgh, PA). Tissue segments were serial sectioned at 10µm thickness from the epidermis to the approximate middle of the pith. The longitudinal sections were cut using an A/O 820 rotary microtome (Fisher Scientific, Pittsburgh, PA). Sections were mounted onto glass slides, deparaffinized, stained with 1% toluidine blue-O, dehydrated in graded ethanol solutions, cleared with xylene, and cover slipped. A sequence of approximately 22 serial sections was observed for each tissue segment, an average depth of 220µm for each tissue. Digital images were collected using a Zeiss Axiocam HRC on an Olympus BX-40 compound microscope (Olympus Scientific, Melville, NY).

**Microscope measurements**

The amount of *P. gregata* hyphae in each stem and petiole tissue segment was quantified using two measurements: percent of longitudinal sections with hyphae and number of groups of hyphae in the tissue segment. The first measurement, percentage of longitudinal
sections containing hyphae, was calculated for both stem and petiole segments by observing each 10µm longitudinal section for the presence or absence of *P. gregata* hyphae. The number of longitudinal sections that contained hyphae in each tissue segment was then divided by the total number of sections observed and multiplied by 100 to express this observation as percentage. This measurement only recorded presence or absence of hyphae and did not take into account hyphae that extended through multiple 10µm longitudinal sections.

The second measurement, number of groups of hyphae in each tissue segment, allowed us to quantify the amount of hyphae present in each stem and petiole segment. Due to the difficulty of counting individual hyphal strands, the number of clusters or groups present in all sections was instead recorded. Hyphae that extended through multiple 10µm longitudinal sections were taken into account and therefore were not recorded twice.

**Correlation analysis**

A correlation analysis was conducted to determine if there were significant correlations between the individual leaf severity rating and the four microscope measurements: percent of longitudinal sections with hyphae in stem, percent of longitudinal sections with hyphae in petiole, number of groups of hyphae in stem, and number of groups of hyphae in petiole. A Pearson correlation analysis was conducted using PROC CORR of SAS v. 9.1 (SAS Institute, 2003).

**Whole plant severity analysis**

To verify expected BSR symptom development, five weeks after inoculation BSR severity was assessed on the eight remaining plants of each genotype based on foliar and stem severity. BSR foliar severity was evaluated on each plant using the following whole plant severity scale
(Perez et al., 2010): 1- dead plant, 2- green stem with no leaves, 3- predominantly chlorotic and necrotic leaves, 4- stunted plant with mosaic chlorosis and necrosis on leaves, 5- normal leaf area with some leaf yellowing, 6- small but healthy plant, 7- healthy plant normal in size.

To measure the stem severity, plant height from the soil to the top of the main stem was measured and recorded in cm. Plant stems were then cut lengthwise from top to bottom. The amount of tissue discoloration and damage inside the vascular tissue was measured in cm upward from the inoculation point as indicated by Tabor et al. (2003). A plant was considered discolored if there was any visible dark brown discoloration on the vascular tissue or the pith. Stem severity was calculated as a ratio of the length of discoloration to total plant height expressed as a percentage.

**Results**

**Verification of BSR symptom expression**

Eight plants of each genotype were evaluated with foliar severity and stem severity measurements five weeks after inoculation to verify expected BSR symptoms (Table 1). The most severe foliar disease reactions were observed on Corsoy79, the susceptible genotype with a foliar severity rating of 4.5 (Table 1). Foliar disease measurements were not significantly different between any of the four resistant genotypes with foliar severity ratings ranging from 5.4 to 5.9 (Table 1). No significant difference was observed between the five genotypes for stem severity. In all genotypes, mock-inoculated plants did not display any BSR foliar or stem disease symptoms (data not shown), indicating they were healthy and were not injured by mock inoculation.
During microscopic analysis, hyphae were not observed in stem or petiole tissue of any mock infected plant. In all *P. gregata* infected plants, hyphae were observed in stem and petiole tissue near the inoculation point, regardless of whether the genotype was resistant or susceptible (Table 2). This confirmed that inoculation was successful. Because hyphae were observed in all resistant genotypes, this indicated that resistance to *P. gregata* is not complete. Since each genotype had appropriate BSR phenotypic reactions and microscopic hyphae observations for the treatment they received, this provides confidence for further analyses.

**Colonization patterns of *P. gregata* hyphae**

When stained with toluidine blue-O, hyphae appear a deep purple color. In each genotype, the fungal hyphae of *P. gregata* were present mainly in the xylem vessels of the vascular system. The amount of mycelium in the vessels varied from a few strands to a solid mass of hyphae, sometimes occupying almost the entire volume of the xylem vessel (Figure 1). Xylem vessels in infected and mock-infected plants were similar in size (data not shown). This indicated that *P. gregata* may not alter the xylem vessel diameter.

As expected, in all genotypes inoculated with *P. gregata* the largest quantity of hyphae was detected near the inoculation point, in stem tissue segments associated with the unifoliate leaf. Colonization of hyphae in tissue segments was uninterrupted from the inoculation point and upwards, and the intensity of colonization in the stem segments always decreased progressively towards the stem apex (Table 2).

BSR foliar symptoms were present only on the lower four leaves of the resistant plant BSR 101, containing the *Rbs1* and *Rbs3* resistance genes (Figure 2). The 3rd and 4th trifoliate leaves of BSR 101 had a green and healthy appearance (leaf severity rating of 1). Similar
numbers of groups of hyphae were observed in stem and petiole sections corresponding to the same leaf. In BSR 101, hyphae were only observed in tissue segments corresponding to leaves displaying BSR foliar symptoms. The upper two leaves did not have foliar symptoms and also did not have *P. gregata* colonization in their respective stem or petiole segments. Significant (P-value < 0.05) correlations were identified (Table 3) between BSR foliar symptoms and three internal microscope measurements (percent of longitudinal sections with hyphae in the stem, groups of hyphae in the stem, and groups of hyphae in the petiole).

The lower five leaves on the susceptible plant Corsoy 79 displayed BSR foliar symptoms, only the 4th trifoliate remained green (Figure 2). Corsoy 79 had more hyphae (109 groups) in the bottom stem segment, near the inoculation point than any of the resistant genotypes (Table 2). Hyphae were never observed in the uppermost two stem segments and the uppermost three petiole segments, despite the presence of foliar symptoms on the second and third trifoliate. Therefore a significant correlation (Table 3) was only found between BSR foliar symptoms and percent of longitudinal sections with hyphae in the stem measurement.

Foliar symptoms were only present on the unifoliate of L78-4094 (*Rbs1*). Despite the healthy appearance of leaves, stem and petiole segments of L78-4094 were heavily colonized by *P. gregata* hyphae (Figure 2, Table 2). Colonization was so severe that many tissue segments had hyphal counts greater than the susceptible plant. For example, 58 groups of hyphae were detected in the stem segment associated with the trifoliolate of L78-4094, but only 13 groups of hyphae were detected in the same segment of Corsoy 79. Surprisingly, hyphae were detected in tissue segments associated with the trifoliolate, first trifoliate, and second trifoliate, even though leaves associated with those tissue segments appeared healthy. Significant correlations
(Table 3) were identified between BSR foliar symptoms and three internal microscope measurements (percent of longitudinal sections with hyphae in the petiole, groups of hyphae in the stem, and groups of hyphae in the petiole).

Despite possessing a resistance gene, PI 437833 (Rbs2) displayed BSR foliar symptoms on all leaves (Figure 2). Hyphae colonization was severe and many tissue segments had hyphal counts greater than the susceptible plant. For example, 152 groups of hyphae were observed in the stem segment associated with the trifoliolate of PI 437833, compared to only 13 groups in this stem segment in Corsoy 79 (Table 2). Colonization of *P. gregata* increased in petioles from the unifoliate to the second trifoliate. Hyphae were not observed in the uppermost stem and petiole segments despite the presence of foliar symptoms on the third trifoliate. This resulted in negative correlations (Table 3) between BSR foliar symptoms and petiole microscope measurements (percent of longitudinal sections with hyphae in the petiole and groups of hyphae in the petiole).

PI 437970 (Rbs3) displayed foliar symptoms only on the lower three leaves, while the upper three leaves remained green and healthy (Figure 2). The upper three leaves without foliar symptoms also did not have *P. gregata* colonization in their respective stem or petiole segments (Table 2). Colonization of hyphae in both the stem and petiole of PI 437970 were similar to those seen in PI 437833. Colonization in stems decreased progressively from the inoculation point to the stem apex, yet increased in petioles from the unifoliate to the first trifoliate. In PI 437970 a significant correlation (Table 3) was identified between BSR foliar symptoms and percent of longitudinal sections with hyphae in the stem.
Xylem anatomy after infection

In addition to differences in hyphae counts, the microscopic analysis also revealed differences in plant cell architecture. In both BSR 101 and Corsoy 79, *P. gregata* hyphae were identified in stem and petiole xylem vessels near the inoculation point. These xylem vessels appeared undamaged and cell architecture was similar between the two genotypes (Figure 3). However, towards the top of the plant, xylem vessels appeared degraded and collapsed in Corsoy 79, yet remained intact in BSR 101.

**Discussion**

The purpose of this observational study was to gain an understanding of the resistance mechanisms to *P. gregata* in five different soybean genotypes. *P. gregata* hyphal colonization patterns were microscopically analyzed in both stems and petioles, relating colonization to BSR foliar symptoms. Large sample sizes are time consuming and labor intensive, thus impractical for microscopic work. Therefore, instead of analyzing multiple replications of one genotype, only one replication of five different genotypes were used for this analysis. As a result, from each of the five genotypes, only one *P. gregata* infected plant and one mock-infected plant was used for microscopic analysis. However, the lack of replication was supplemented by whole plant severity analyses.

Foliar disease measurements on Corsoy 79, the susceptible genotype, were significantly more severe than the four resistant genotypes. However, no significant differences were detected for stem severity between the five genotypes. Tabor et al. (2003) and McCabe et al. (2016), reported that stem discoloration was frequently absent even in stems that were heavily colonized by *P. gregata*, suggesting that the amount of pith browning may not always indicate
the extent of colonization by *P. gregata*. In our analysis, the greatest amount of internal stem browning was seen in PI 437970 (*Rbs3*) yet this genotype was not as heavily colonized by hyphae as others. These findings, taken together, suggest that measuring BSR severity by relying on internal stem discoloration is not a reliable indicator of BSR symptom expressions and classification of resistance/susceptibility of genotypes.

Hyphae were observed in the stems and petioles of all inoculated genotypes, regardless of the BSR resistance gene present. Tabor et al. (2003) reported that BSR foliar symptoms as well as stem browning were often observed on BSR resistant genotypes, and *P. gregata* could be isolated from both resistant and susceptible genotypes. The authors proposed that resistance to *P. gregata* in soybean is incomplete, and BSR resistance could be defined as the delay or lack of symptom expression after an incubation period. Our research was the first to observe hyphae in xylem tissue of resistant genotypes containing *Rbs1*, *Rbs2*, and *Rbs3*. Therefore, it can be concluded that the current three sources of resistance to *P. gregata* (*Rbs1*, *Rbs2*, and *Rbs3*) provide incomplete resistance to BSR.

The colonization pattern of the hyphae in the petioles of BSR 101 and L78-4094 (*Rbs1*) decreased towards the stem apex, whereas hyphae in the petioles of Corsoy 79, PI 437833 (*Rbs2*), and PI 437970 (*Rbs3*) increased towards the stem apex. This suggests that different mechanisms of resistance to *P. gregata* exist in soybean. Various *P. gregata* resistance mechanisms have previously been proposed: 1) resistant plants have the ability to restrict or exclude the pathogen from the vascular system (Impullitti and Malvick, 2014), 2) resistant plants have the ability to regenerate their vascular system following infection (Impullitti and Malvick, 2014), or 3) resistant plants are insensitive to a toxin produced by *P. gregata* (Gray and
Chamberlain, 1975). On the basis of our results, we hypothesize that there are at least two different mechanisms of resistance to *P. gregata*, however the exact mechanism causing colonization differences in the petiole cannot be determined using light microscopy.

Impullitti and Malvick (2014) observed fungal hyphae in areas of the soybean stem with and without evidence of stem browning, proposing that infection by *P. gregata* in xylem tissue is required before stem browning occurs. Similarly, we also found fungal hyphae in stem and petiole segments with and without BSR foliar symptoms. However, some trifoliates of Corsoy 79 and PI 437833 (*Rbs2*) exhibited characteristic BSR foliar symptoms but lacked hyphae in the respective stem and petiole segments. This indicates that the presence of hyphae is not a prerequisite for foliar symptom development. Something other than the hyphae, such as blocked xylem vessels or a phytotoxin, could explain foliar symptom development in the absence of *P. gregata* hyphae. The amount of mycelium in the vessels varied from a few strands to a solid mass of hyphae, sometimes occupying almost the entire volume of the xylem vessel. It is possible that hyphae colonization restricts transport in xylem vessels which consequently reduces the amount of nutrients transported to leaves resulting in foliar symptoms.

We also observed degraded xylem vessels in susceptible plants infected with *P. gregata*. The degraded vessels were present in upper stem and petiole tissue segments of Corsoy 79 (susceptible) but not BSR 101 (resistant). It is possible that the damage of xylem vessels following the invasion of *P. gregata* is due to a toxin or cell degrading enzyme produced by the pathogen. The presence of the toxin and amount of hyphal colonization in the stems appear to be independent.
Gray and Chamberlain (1975) proposed that a toxin is involved in BSR pathogenesis, responsible for the foliar symptoms associated with *P. gregata* type A. Kobayashi and Ui (1980) later reported that gregatins, a family of compounds produced by *P. gregata*, were found to be toxic to adzuki bean (*Vigna angularis*), and that gregatins may also have an important role in pathogenesis in soybean. To our knowledge, the relationship between gregatins and BSR symptom development has not been investigated further. The BSR susceptible plants sensitivity to a toxin could explain the observed degradation of the vascular tissue in the susceptible plant and the occurrence of foliar symptoms in the absence of hyphae.

The foliar symptoms associated with *P. gregata* type A are nearly identical to those produced by *Fusarium viguliforme*, the pathogen causing sudden death syndrome (SDS) in soybean. FvTox1 toxin produced by *F. viguliforme* is known to be a major pathogenicity factor involved in SDS foliar symptom development (Brar et al., 2011). Only SDS susceptible soybean lines are sensitive to FvTox1 proteins, resulting in loss of chlorophyll as well as necrosis symptoms on leaves (Brar et al., 2011). It is possible that a toxin produced by *P. gregata* might be responsible for foliar symptoms on BSR susceptible lines. HC-toxin is the major pathogenicity factor of *Cochilobous carbonum*, the fungus causing leaf spot and ear mold of maize. Resistant maize plants are able to produce the enzyme HC-toxin reductase, which inactivates HC-toxin. Therefore, detoxification of HC-toxin is specific to resistant lines (Meeley et al., 1992). BSR resistant soybeans could also produce an enzyme that detoxifies the *P. gregata* toxin, therefore avoiding xylem vessel and foliar symptom damage.

Our microscopy study revealed that the resistance mechanism may differ among each of the three BSR resistance genes: *Rbs1, Rbs2,* and *Rbs3*. The exact mode of gene action, however,
remains unknown and merits future study. Understanding the differences in resistance mechanisms between the current sources of BSR resistance genes will allow breeders to choose the most appropriate combination of resistance genes to pyramid into soybean cultivars, thus increasing the stability of soybean yields and host resistance to *P. gregata*. Using a novel experimental design, we have gained insights into the mechanisms of soybean resistance to *P. gregata*. This establishes a new method for looking at internal plant damage, relating the presence and quantity of hyphae to external foliar symptoms. This designed approach can be useful for studying plant and pathogen interactions on a broader scale.

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**Figures and Tables**

**Figure 1:** Hyphae in stem and petiole tissue segments appear a deep purple color after staining with toluidine blue-O. Fungal hyphae of *P. gregata* were present mainly in the xylem vessels of the soybean vascular system.
Figure 2. For all selected plants, each leaf was individually given a severity rating of 1-5 with 5 being the most infected. (1- healthy and green leaf, 2- partially yellow leaf, 3- entirely yellow leaf, 4- entirely yellow leaf and curling, 5- brown and dead leaf.)
Figure 3: Vascular tissue architecture was compared between Corsoy 79 (susceptible) and BSR 101 (resistant) after infection with *P. gregata*. Towards the top of the plant, xylem vessels appear degraded and collapsed in Corsoy 79, yet remain intact in BSR 101.
**Table 1:** BSR severity means of plants five weeks post infection with *P. gregata*. Plants were rated for foliar severity and stem severity.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Foliar Severity(^1)</th>
<th>Stem Severity(^1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSR 101 (Res)</td>
<td>5.9 A(^5)</td>
<td>44.7 A</td>
</tr>
<tr>
<td>Corsoy79 (Sus)</td>
<td>4.5 B</td>
<td>39.5 A</td>
</tr>
<tr>
<td>L78-4094 <em>(Rbs1)</em></td>
<td>5.4 A</td>
<td>48.1 A</td>
</tr>
<tr>
<td>PI 437833 <em>(Rbs2)</em></td>
<td>5.8 A</td>
<td>39.4 A</td>
</tr>
<tr>
<td>PI 437970 <em>(Rbs3)</em></td>
<td>5.9 A</td>
<td>52.4 A</td>
</tr>
</tbody>
</table>

\(^1\)Foliar severity: measured on a scale of 1-7; 1 represents the most infected.

\(^2\)Stem severity: amount of internal stem browning divided by total plant height, and multiplied by 100 to obtain a percentage.

\(^5\)Means followed by the same letter in a column are not significantly different (*P* < 0.05; LSD).
Table 2. Leaf score and microscopic measurements were recorded for each genotype. Stem and petiole sections were examined microscopically for the presence of *P. gregata* hyphae based on percentage of longitudinal sections containing hyphae and groups of hyphae.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Leaf Location</th>
<th>Leaf Symptoms</th>
<th>Leaf Score&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Percent Stem Sections with Hyphae&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Percent Petiole Sections with Hyphae&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Groups of Hyphae in Stem</th>
<th>Groups of Hyphae in Petiole</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSR 101</td>
<td>Unifoliate</td>
<td>Yellow, Curl</td>
<td>4</td>
<td>100% (16/16)</td>
<td>59% (10/17)</td>
<td>21</td>
<td>21</td>
</tr>
<tr>
<td>BSR 101</td>
<td>Trifoliolate</td>
<td>Yellow</td>
<td>3</td>
<td>77% (17/22)</td>
<td>100% (21/21)</td>
<td>18</td>
<td>19</td>
</tr>
<tr>
<td>BSR 101</td>
<td>1st Trifoliate</td>
<td>Partial Yellow</td>
<td>2</td>
<td>94% (16/17)</td>
<td>100% (20/20)</td>
<td>15</td>
<td>13</td>
</tr>
<tr>
<td>BSR 101</td>
<td>2nd Trifoliate</td>
<td>Partial Yellow</td>
<td>2</td>
<td>21% (4/19)</td>
<td>19% (4/21)</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>BSR 101</td>
<td>3rd Trifoliate</td>
<td>Green</td>
<td>1</td>
<td>0% (0/19)</td>
<td>0% (0/22)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>BSR 101</td>
<td>4th Trifoliate</td>
<td>Green</td>
<td>1</td>
<td>0% (0/18)</td>
<td>0% (0/18)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Corsoy 79</td>
<td>Unifoliate</td>
<td>Yellow</td>
<td>3</td>
<td>100% (18/18)</td>
<td>100% (26/26)</td>
<td>109</td>
<td>68</td>
</tr>
<tr>
<td>Corsoy 79</td>
<td>Trifoliolate</td>
<td>Yellow</td>
<td>3</td>
<td>100% (12/12)</td>
<td>5% (1/22)</td>
<td>13</td>
<td>1</td>
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<tr>
<td>Corsoy 79</td>
<td>1st Trifoliate</td>
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<td>2</td>
<td>85% (17/20)</td>
<td>9% (2/23)</td>
<td>22</td>
<td>1</td>
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<tr>
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<td>2nd Trifoliate</td>
<td>Partial Yellow</td>
<td>2</td>
<td>36% (5/14)</td>
<td>0% (0/19)</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Corsoy 79</td>
<td>3rd Trifoliate</td>
<td>Partial Yellow</td>
<td>2</td>
<td>0% (0/27)</td>
<td>0% (0/25)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Corsoy 79</td>
<td>4th Trifoliate</td>
<td>Green</td>
<td>1</td>
<td>0% (0/17)</td>
<td>0% (0/22)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>L78-4094</td>
<td>Unifoliate</td>
<td>Partial Yellow</td>
<td>2</td>
<td>100% (13/13)</td>
<td>100% (22/22)</td>
<td>96</td>
<td>87</td>
</tr>
<tr>
<td>L78-4094</td>
<td>Trifoliolate</td>
<td>Green</td>
<td>1</td>
<td>100% (18/18)</td>
<td>57% (20/35)</td>
<td>58</td>
<td>20</td>
</tr>
<tr>
<td>L78-4094</td>
<td>1st Trifoliate</td>
<td>Green</td>
<td>1</td>
<td>55% (12/22)</td>
<td>15% (4/26)</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>L78-4094</td>
<td>2nd Trifoliate</td>
<td>Green</td>
<td>1</td>
<td>33% (6/18)</td>
<td>0% (0/12)</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>L78-4094</td>
<td>3rd Trifoliate</td>
<td>Green</td>
<td>1</td>
<td>0% (0/26)</td>
<td>0% (0/21)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>L78-4094</td>
<td>4th Trifoliate</td>
<td>Green</td>
<td>1</td>
<td>0% (0/29)</td>
<td>0% (0/25)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PI 437833</td>
<td>Unifoliate</td>
<td>Yellow</td>
<td>3</td>
<td>100% (16/16)</td>
<td>30% (9/31)</td>
<td>102</td>
<td>6</td>
</tr>
<tr>
<td>PI 437833</td>
<td>Trifoliolate</td>
<td>Yellow</td>
<td>3</td>
<td>100% (30/30)</td>
<td>46% (11/24)</td>
<td>152</td>
<td>3</td>
</tr>
<tr>
<td>PI 437833</td>
<td>1st Trifoliate</td>
<td>Yellow</td>
<td>2</td>
<td>100% (24/24)</td>
<td>78% (18/23)</td>
<td>38</td>
<td>10</td>
</tr>
<tr>
<td>PI 437833</td>
<td>2nd Trifoliate</td>
<td>Partial Yellow</td>
<td>2</td>
<td>83% (2/24)</td>
<td>100% (24/24)</td>
<td>27</td>
<td>38</td>
</tr>
<tr>
<td>PI 437833</td>
<td>3rd Trifoliate</td>
<td>Partial Yellow</td>
<td>2</td>
<td>0% (0/22)</td>
<td>0% (0/30)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PI 437970</td>
<td>Unifoliate</td>
<td>Partial Yellow</td>
<td>2</td>
<td>82% (9/11)</td>
<td>7% (1/15)</td>
<td>37</td>
<td>2</td>
</tr>
<tr>
<td>PI 437970</td>
<td>Trifoliolate</td>
<td>Partial Yellow</td>
<td>2</td>
<td>58% (11/19)</td>
<td>53% (10/19)</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>PI 437970</td>
<td>1st Trifoliate</td>
<td>Partial Yellow</td>
<td>2</td>
<td>35% (9/26)</td>
<td>81% (17/21)</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>PI 437970</td>
<td>2nd Trifoliate</td>
<td>Green</td>
<td>1</td>
<td>0% (0/20)</td>
<td>0% (0/19)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PI 437970</td>
<td>3rd Trifoliate</td>
<td>Green</td>
<td>1</td>
<td>0% (0/20)</td>
<td>0% (0/22)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PI 437970</td>
<td>4th Trifoliate</td>
<td>Green</td>
<td>1</td>
<td>0% (0/39)</td>
<td>0% (0/29)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>a</sup>Each individual leaf was given a severity rating of 1-5, with 5 being the most infected.

<sup>b</sup>The number of longitudinal sections that contained hyphae in each stem and petiole segment was divided by the total number of sections observed.
Table 3. Pearson correlation coefficients were calculated between leaf score and all four microscope measurements: percent sections with hyphae in stem, percent sections with hyphae in petiole, groups of hyphae in stem, or groups of hyphae in petiole.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>BSR 101 (Rbs1, Rbs3)</th>
<th>Corsoy 79 (Susceptible)</th>
<th>L78-4094 (Rbs1)</th>
<th>PI 437833 (Rbs2)</th>
<th>PI 437970 (Rbs3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percent Hyphae Stem</td>
<td>0.93*</td>
<td>0.82*</td>
<td>0.56</td>
<td>0.49</td>
<td>0.91*</td>
</tr>
<tr>
<td>Percent Hyphae Petiole</td>
<td>0.63</td>
<td>0.87</td>
<td>0.84*</td>
<td>-0.3</td>
<td>0.73</td>
</tr>
<tr>
<td>Groups of Hyphae Stem</td>
<td>0.91*</td>
<td>0.61</td>
<td>0.83*</td>
<td>0.93*</td>
<td>0.65</td>
</tr>
<tr>
<td>Groups of Hyphae Petiole</td>
<td>0.9*</td>
<td>0.55</td>
<td>0.97*</td>
<td>-0.41</td>
<td>0.81</td>
</tr>
</tbody>
</table>

* P < 0.05
CHAPTER 4: CHARACTERIZATION OF RESISTANT AND SUSCEPTIBLE RESPONSES TO PHIALOPHORA GREGATA IN SOYBEAN USING RNA-SEQ

Chantal E. McCabe, Silvia R. Cianzio and Michelle A. Graham*

Abstract

Breeding for pathogen resistance is an important objective to improve and protect soybean yields. In 2010, 14.4% of total soybean yield was suppressed by diseases. Brown stem rot (BSR), caused by the fungus *Phialophora gregata*, reduces yield by as much as 38%. To date, three dominant BSR resistance genes have been identified: *Rbs1*, *Rbs2*, and *Rbs3*, however the gene networks regulating defense responses to BSR remain unknown. Further, identifying resistant germplasm by genotyping or phenotyping remains difficult due to complexities of soybean/*P. gregata* interactions. To better understand resistance mechanisms, we conducted RNA-Seq of *P. gregata* infected and mock infected leaf, stem, and root tissues of both a resistant (PI 437970, *Rbs3*) and a susceptible (Corsoy 79) soybean genotype. Samples were collected one-week post infection. Our bioinformatic analyses focused on treatment, genotype, and treatment by genotype effects on gene expression. Our results indicate that resistant and susceptible genotypes respond differently when infected with *P. gregata*. Further, regardless of infection, the resistant and susceptible genotypes differ in their innate defense responses. These data indicate that molecular markers could be developed to allow identification of resistant and susceptible individuals at 1 week post infection, four weeks earlier than current protocols. Given that *Rbs3* has been mapped to soybean chromosome 16, the RNA-Seq data was also used to generate novel SNPs within the *Rbs3* locus that could be used for fine mapping.
Virus induced gene silencing (VIGS) was also used to characterize the candidate resistance genes and downstream defense responses.

**Introduction**

In 2015, the United States produced 3.93 billion bushels of soybeans on 82.7 million acres, valuing the crop at $34.5 billion (SoyStats, 2015). In order to maintain high profit margins when producing soybeans, high yields are crucial. Pests and pathogens, however, can drastically reduce soybean yield. In 2014, 13,636,691 bushels of soybean were lost to the disease brown stem rot (Koenning and Wrather, 2010).

Brown stem rot (BSR) is caused by the fungus *Phialophora gregata* f. sp. *sojae* (Allington and Chamberlain, 1948) W. Gams (syn. *Cadophora gregata* (Harrington and McNew, 2003)). Two types of *P. gregata* have been identified: type A and type B, both producing internal stem browning of the pith and vascular tissue. In addition to stem browning, *P. gregata* type A also produces foliar symptoms appearing as leaf necrosis and chlorosis, while leaves of plants infected with *P. gregata* type B appear healthy (Gray, 1972; Harrington et al., 2003). Both types of this soil borne fungus infect soybean plants at the root level (Allington and Chamberlain, 1948), yet little attention has been paid to disease expression below ground.

Three dominant BSR resistance genes have been identified: *Rbs1*, *Rbs2*, and *Rbs3*. All three genes have been mapped to chromosome 16 (Bachman et al., 2001; Klos et al., 2000; Lewers et al., 1999; Webb, 1997). Genetic resistance to BSR is the most effective tool to reduce yield loss due to BSR in soybeans. Current screening methods for BSR resistance are conducted in the growth chamber five weeks after infection. Screening methods are time consuming, requiring both foliar severity measurements and recovery of *P. gregata* from stem sections.
McCabe et al., 2016). Identifying molecular markers would allow disease screening to be conducted prior to the onset of BSR symptoms. Examining gene expression after *P. gregata* infection in leaf, stem, and root tissues will provide better understanding of resistance signaling pathways induced by *P. gregata* infection and will aid in the development of molecular markers tightly linked with resistance, improving screening efficiency. To our knowledge, no studies have investigated the molecular responses to BSR in resistant and susceptible germplasms using expression analyses.

The objectives of this study were to use bioinformatics approaches to identify genes in the BSR resistance pathway. Analysis of leaf, stem, and root tissues in the resistant soybean line PI 437970 (*Rbs3*) and the susceptible soybean line Corsoy 79, inoculated or mock-inoculated with *P. gregata*, was conducted to characterize the molecular responses to BSR. Novel SNPs were identified on soybean chromosome 16 within the *Rbs3* locus to be used for disease screening and fine mapping resistance. In addition, virus induced gene silencing (VIGS) has been used to characterize the candidate resistance genes and downstream defense responses.

**Materials and Methods**

**Experimental Design and Inoculation**

Two soybean lines were used in this study: the resistant line PI 437970 (Hanson et al., 1988) is the source for BSR resistance gene *Rbs3*, and a susceptible line Corsoy 79 (Bernard and Cremeens, 1988). From each genotype, forty seeds were planted into separate 3.8 x 21 cm cone-tainers (Stuewe and Sons, Corvallis, Oregon) with Metro-Mix 900 potting soil (Sun Grow Horticulture, Agawam, Massachusetts) in a growth chamber. The temperature in the growth chamber was set to a constant 19°C +/- 1.5°C with a photoperiod of 16 hours of light per day,
provided by fluorescent and incandescent light bulbs. Soil was kept moist by watering the pots daily until saturation. Plants were fertilized weekly with a 24-8-16 fertilizer mixture.

Two weeks after planting, 20 resistant plants and 20 susceptible plants were inoculated with *P. gregata*. The *P. gregata* spore suspension was prepared using isolate Oh<sub>2-3</sub> (Lewers et al., 1999). Oh<sub>2-3</sub> is a single spore isolate of Oh<sub>2</sub> (Eathington et al., 1995) originally provided by Cecil Nickell at the University of Illinois. This isolate was chosen because it is well characterized and has the ability to consistently produce both stem and foliar symptoms under growth chamber conditions. The cultures were grown on green bean extract medium (35 g/L of ground frozen *Phaseolus vulgaris* L. green pods, 20 g/L of water agar, and 50 mg/L of ampicillin), and incubated for 60 days at room temperature and ambient light. The *P. gregata* spores were suspended in a 1.2% water agar paste and adjusted to obtain a final spore concentration of 2.7x10<sup>7</sup> spores/ml by means of a hemocytometer (Perez et al., 2010; Tabor et al., 2003). Plants were inoculated by injecting the soybean stem with approximately 20 µl of the suspension 2 cm above the soil line using an 18 gauge needle.

Twenty resistant plants and 20 susceptible plants were inoculated with a spore-free suspension consisting solely of 1.2% water agar two weeks after planting, serving as the mock inoculated control. Using an 18 gauge needle, 20 µl of the spore-free suspension was injected into the soybean stems.

**Tissue Collection**

Leaf, stem, and root tissues were collected from three plants in each of the four treatments: resistant-infected, resistant-mock, susceptible-infected, and susceptible-mock. From each plant, tissues were collected by harvesting the first fully expanded trifoliate, the
stem section between the cotyledon and the unifoliate, and the whole root. Three biological replicates were harvested for each sample at one, two, three, and four weeks after inoculation. Tissue was flash frozen in liquid nitrogen and stored at -80°C.

**Disease Severity Assays**

As samples were collected before the onset of BSR symptoms, the eight remaining Corsoy 79 and PI 437970 plants were monitored to verify expected BSR symptoms. Five weeks after inoculation, BSR severity was assessed based on three measurements: foliar severity, stem severity, and stem recovery of *P. gregata*.

BSR foliar severity was evaluated on each plant using the following severity scale (Perez et al., 2010): 1- dead plant, 2- green stem with no leaves, 3- predominantly chlorotic and necrotic leaves, 4- stunted plant with mosaic chlorosis and necrosis on leaves, 5- normal leaf area with some leaf yellowing, 6- small but healthy plant, 7- healthy plant normal in size. Plant height from the soil to the top of the main stem was recorded in cm.

Plants were then defoliated and cut at the soil line. Plant stem sterilization was accomplished by submerging in 70% ethanol for 3 minutes, 10% sodium hypochlorite for 5 minutes, and rinsing twice with sterile distilled water. Plant stems were cut open lengthwise from top to bottom to measure stem severity. Brown tissue discoloration in the pith or vascular tissue was measured in cm upward from the inoculation point (Tabor et al., 2003). Stem severity was calculated as a ratio of the length of discoloration to total plant height expressed as a percentage.

Recovery of *P. gregata* from the stem was measured using a modification of the procedure described by Tabor et al. (2003). Five evenly spaced, 1-cm long, stem segments were
cut from the plant, plated in sequential order on green bean extract agar, and allowed to grow in the dark at room temperature. Segments were harvested from the following locations on the plant: inoculation point, 2/5 of the way up the stem, the mid portion of the stem, 4/5 of the way up the stem, and the top of the plant. The stem segments on each plate were evaluated after two weeks of incubation at room temperature for the presence of *P. gregata* colonies. Evaluation was based on recovery of *P. gregata* colonies from specific stem segments using the following ratings: 1 - inoculation point only, 2 - inoculation point and the second segment, 3 - inoculation point and the next two segments, 4 - inoculation point and the next three segments, and 5 - each of the five segments.

**RNA Isolation and RNA-Seq**

Flash frozen tissue was ground with a mortar and pestle in liquid nitrogen. A Qiagen® RNeasy® Plant Mini Kit (Qiagen®, Germantown, MD) was used to extract RNA following the manufacturer’s recommended protocol with the following modifications. For stems and roots, approximately 300 mg of ground tissue was lysed using the RLT buffer. All samples were incubated at 56°C for two minutes with 800 rpm shaking to aid in tissue disruption, and columns were incubated at room temperature for 10 min during elution.

Contaminating DNA was then removed with an Ambion® TURBO DNA-free kit™ (Ambion®, Austin, TX). Finally, a Qiagen® RNeasy® MiniElute Cleanup Kit (Qiagen®, Germantown, MD) was used to clean and concentrate the RNA. RNA quality and concentration was assessed using a Thermo Fisher Scientific® NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific®, Waltham, MA, USA). RNA was considered to be of good quality if the 260/280 ratio was greater than 2.0. Due to the fast advance of BSR symptoms in infected
plants, RNA from samples collected at later time points (2, 3, and 4 weeks post inoculation) was
degraded and not suitable for RNA-Seq analysis. Therefore, only tissue from one week post
inoculation was used for analysis, a total of 36 samples. RNA quality of the 36 samples was
analyzed using an Agilent® 2100 Bioanalyzer™ (Agilent®, Santa Clara, CA). RNA was considered
to be acceptable quality if the RNA was not degraded or was only marginally degraded with a
RNA integrity number (RIN) greater than seven.

Sequencing was performed at the Iowa State University DNA facility. Library preparation
and single end sequencing was conducted with 4 µg of total RNA using the Illumina HiSeq 2500
platform (Illumina®, San Diego, CA). Library preparation was unsuccessful for one susceptible
mock-infected root sample. Therefore, 35 samples were sequenced in total.

**Bioinformatics and Statistical Analyses**

A total of 35 libraries were sequenced and the resulting 100 base pair reads were
trimmed prior to alignment using the bioinformatic programs Scythe
(https://github.com/vsbuffalo/scythe) to remove adaptor sequences, FASTX trimmer
(http://hannonlab.cshl.edu/fastx_toolkit/) to remove 15 bases of sequencing artifacts from
each read, and Sickle (https://github.com/najoshi/sickle) to remove bases with low quality
scores and short reads. Single end reads were then aligned to version 2 of the Williams 82
reference genome sequence (Wm82.a2.v2, downloaded from phytozome.net) (Schmutz et al.,
2010) using TopHat version 2.0.3 (Trapnell et al., 2009) with the default settings. Unreliably
mapped reads were removed using the program Samtools (Li et al., 2009) and mapping files
(bamfiles) were imported into the statistical program R (R Development Core Team, 2014) using
Rsamtools (Morgan and Pages, 2013). The gene feature file corresponding to the Wm82.a2.v2
(Schmutz et al., 2010) genome was imported using rtracklayer (Lawrence et al., 2009). The number of reads per sample aligning to each gene were counted using GenomicAlignments (Lawrence et al., 2013). Only genes with log counts per million (cpm) > 1 in at least two replicates were used in the analysis.

Data was normalized using the Trimmed Mean of M (TMM) values (Robinson and Oshlack, 2010) in the Bioconductor package edgeR (McCarthy et al., 2012; Robinson et al., 2010; Robinson and Smyth, 2007; Robinson and Smyth, 2008; Zhou et al., 2014). The R graphics program ggplot2 (Wickham, 2009) was used to generate principal component and biological coefficient of variance plots to visually compare sample replicates for technical reproducibility. During this quality control stage, five samples (one resistant-infected stem, one resistant-mock infected stem, one resistant-infected root, one resistant-infected leaf, and one resistant-mock infected leaf) were removed because graphical analyses indicated that these samples were substantially different relative to the other 12 samples of the same tissue type. Analyses conducted with and without these five samples confirmed they affected tagwise dispersion estimation. Analysis by tissue type was conducted on the remaining ten samples of each tissue using edgeR to identify differentially expressed (DE) genes.

Data was visualized at multiple FDR, and DE genes were considered significant with a false discovery rate (FDR) < 0.001. To distinguish between genotype and treatment effects, our statistical analyses identified DE genes responding to *P. gregata* treatment in both genotypes, genotype-specific responses to *P. gregata* infection, genotype x treatment interactions, and general genotype differences for each tissue type.
Hierarchical Clustering Heat Maps

In order to identify groups of genes involved in defense responses to *P. gregata*, we performed hierarchical clustering analysis based on expression profiles of the DE genes for treatment, genotype by treatment interaction, and genotype responses. Clustering was conducted with gene log counts per million (cpm) which allowed datasets from different tissues to be compared directly and to identify trends that may not be apparent with fold change. Dendrograms of DE genes were generated using hclust (R Development Core Team, 2014) function with the default complete linkage method in R.

Annotation and Analysis of DE Genes in Clusters

The lists of significant DE genes for each cluster were annotated using the SoyBase Genome Annotation Report page (www.soybase.org/genomeannotation) which provided UniRef100 (Apweiler et al., 2004) hit information, best *A. thaliana* homologs, and gene ontology (GO) information inferred from *A. thaliana* (The Arabidopsis Information Resource [TAIR] version 10, www.arabidopsis.org). Significantly (P-value <0.05) overrepresented biological process GO terms were identified using a Fisher’s Exact Test (Fisher et al., 1960) and Bonferroni correction (Bonferroni, 1935), to compare gene ontology terms within a cluster to their genome representation. To reduce the number of significant GO terms and to eliminate redundancy, any GO terms whose genes completely overlapped were mapped to the largest significantly overrepresented GO term.

Identification of Differentially Expressed Transcription Factors

To identify transcription factors that might play a role in disease resistance/susceptibility signaling, we used the SoyDB transcription factor database ((Wang et
al., 2010), http://casp.rnet.missouri.edu/soydb/ to identify transcription factors within our DE gene clusters. The gene identifiers of transcription factors present in the database were updated to reference the new genome assembly and annotation (Wm82.a2.v2) (Schmutz et al., 2010).

Identification of Overrepresented Transcription Factor Binding Sites

We used Clover (cis element over representation) (Frith et al., 2004) and the JASPAR transcription factor binding site database (Mathelier et al., 2015), to identify transcription factor binding sites significantly (t <0.05) overrepresented within promoters of DE genes in a cluster relative to all promoters in the soybean genome. Promoter size was limited to 500 bases upstream of the start methionine. Promoters less than 500 bases in length, containing gaps, or ambiguous bases were excluded from the analysis.

Single Linkage Clustering

Single linkage clustering, as described by Graham et al. (2006), was used to identify gene families that could be acting in defense responses. Protein sequences corresponding to all DE genes were compared against themselves using BLASTP (E<10^-10) (Altschul et al., 1997). Proteins with overlapping BLAST reports were assigned to groups representing potential gene families.

SNP Identification

Bamfiles for each genotype and treatment were sorted and merged using samtools (Li et al., 2009). This included merging files across tissues. Variant calling between the resistant and susceptible genotypes was performed using the programs samtools mpileup (Li et al., 2009) and bcftools (Li, 2011) functions with default parameters. Only SNPs within the Rbs3 interval of interest on chromosome 16 (Chr16:33,333,000-33,819,000) were called. The resulting variant
call format (VCF) file was sorted manually and SNPs with a quality score less than 25 were removed (Additional File 12). In addition, we identified genes within this region that were differentially expressed in response to treatment or genotype that could be candidates for the *Rbs3* resistance gene.

**Virus Induced Gene Silencing**

Nine significant (FDR < 0.001) downstream defense genes with an absolute log fold change greater than five were identified from the DE gene datasets as genes of interest. Glyma.01G021000, Glyma.02G063600, and Glyma.09G107600 were significant in leaf tissue, Glyma.02G042500, Glyma.14G102900, and Glyma.17G030400 were significant in stem tissue, and Glyma.01G046900, Glyma.16G175100, and Glyma.20G029600 were significant in root tissue. Primers were designed (Additional File 13) and BPMV VIGS constructs were created following the protocol in Whitham et al. (2016). The constructs were bombarded into 10 day old Williams 82 seedlings using the biolistic particle bombardment method described in Whitham et al. (2016). Trifoliates displaying virus symptoms were collected four weeks after bombardment, tissue was lyophilized and stored at -20°C.

VIGS constructs were also designed from R genes located in the *Rbs1*, *Rbs2* and *Rbs3* regions of the soybean genome. The sequences of molecular markers used for identifying *Rbs1*- *Rbs3* (Bachman et al., 2001; Klos et al., 2000; Lewers et al., 1999; Webb, 1997), were used for BLASTN (Altschul et al., 1997) against the soybean genome. BLASTX against the Uniref100 database was used to identify predicted genes within in these regions with homology to known R genes. Since many of the R genes within this region were clustered, CLUSTALW (Thompson et al., 2002) was used to align candidate R gene sequences. VIGS constructs were designed to
silence multiple homologs. Eleven R gene constructs were developed: NBSLRRRA (Glyma.16G215400), RLPA (Glyma.16G170900), RLPB (no Wm82.a2.v2 correspondence), RLPC (Glyma.16G188000), S143_R197 (Glyma.16G153800), S143_R62 (Glyma.15G253000), S58_R276 (Glyma.16G122100), S58_R32 (Glyma.02G050300), S58_R449 (Glyma.16G118600), S58_R487 (Glyma.16G117700), and S58_R888 (Glyma.09G196800).

Following the protocol described in Whitham et al. (2016), infected tissue from each of the 20 BPMV vector constructs (9 downstream defense genes and 11 R genes) was rub inoculated onto 10 day old seedlings of PI 437970 (Rbs3). NBSLRRRA, RLPA, RLPB, and RLPC were also rub inoculated onto L78-4094 (Rbs1) and PI 437833 (Rbs2). In addition to the 20 BPMV vectors, both empty vector controls (BPMV vector lacking an insert) and mock VIGS controls (rub inoculated with buffer only) were also included in the experiment. Four seedlings were inoculated for each construct and control. Two days after rub inoculation, all seedlings were stab-inoculated with P. gregata as described above. To determine if a particular VIGS construct compromised resistance to P. gregata, foliar and internal stem phenotypes were observed five weeks after infection. Four downstream defense genes (Glyma.01G021000, Glyma.01G046900, Glyma.09G107600, and Glyma.16G175100) and six R-genes (RLPA, RLPB, RLPC, S58_R32, S58_R487, and S58_R888) displayed high levels of both BPMV and BSR symptoms and were selected for further investigation. The experiment was repeated twice, with two independent replicates, as described above using the ten BPMV vectors.
Results

Verification of BSR symptom expression

As tissues used for generating RNA-Seq samples were collected before the onset of BSR symptoms, eight plants of both Corsoy 79 and PI 437970 were monitored to verify expected BSR symptom development. Five weeks after inoculation, BSR severity was assessed based on three measurements: foliar severity, stem severity, and stem recovery of *P. gregata* (Table 1). The most severe disease reactions were observed on the susceptible-infected plants with foliar severity, stem severity, and stem recovery of *P. gregata* ratings of 3.9, 55.8, and 3.3, respectively. Resistant-infected plants were not significantly different from the mock-inoculated plants for foliar severity and recovery of *P. gregata* measurements. Neither resistant-mock nor susceptible-mock plants displayed any BSR disease symptoms, indicating they were healthy. Since each genotype had appropriate BSR phenotypic reactions for the treatment they received, this provides confidence for gene expression analyses.

Analysis of differentially expressed genes

RNA-Seq libraries were sequenced and mapped to the soybean genome. The 30 samples analyzed contained a total of 427,668,168 100 base pair (bp) single end reads: 146,495,103 from 10 leaf samples, 145,953,503 from 10 stem samples, and 135,219,562 from 10 root samples. These sequences have been uploaded to the National Center for Biotechnology Small Reads Archive under Bioproject Accession XXXXX.

Significant (FDR < 0.001) DE genes responding to *P. gregata* treatment, genotype-specific response to *P. gregata* treatment, genotype by treatment interaction, and genotype for each tissue type were identified using edgeR (Table 2). In response to *P. gregata* treatment,
regardless of genotype, a total of 295 DE genes were identified in leaf tissue, 913 in stem tissue, and 2 in root tissue. In the resistant-specific response to \textit{P. gregata} 2492, 530, and 16 DE genes were identified in leaf, stem, and root tissue respectively. In the susceptible-specific response to \textit{P. gregata}, 89 DE genes were identified in leaf tissue, 424 in stem tissue, and 163 in root tissue. For genotype differences regardless of treatment, 4131, 2590, and 2094 DE genes were identified in leaf, stem, and root tissue respectively. In the genotype by treatment interaction 974, 37, and 44 DE genes were identified in leaf, stem, and root tissue respectively. DE genes with a genotype by treatment interaction responded differently to \textit{P. gregata} infection, depending on their genotype.

For each tissue, DE genes responding to infection in a given genotype were compared for overlap between the resistant response to \textit{P. gregata} infection and the susceptible response to \textit{P. gregata} infection. In leaves, 25 DE genes were in common between the resistant and susceptible response to \textit{P. gregata}. The direction of expression changed between the resistant and susceptible responses for only three of these 25 genes. In stems, 173 genes were in common between the resistant and susceptible response to \textit{P. gregata}. The direction of expression was the same for all 173 genes. Due to the low number of DE genes, there was no overlap between the resistant and susceptible response to \textit{P. gregata} in root tissue. These results suggest that the resistant and susceptible genotypes have distinct responses to \textit{P. gregata} infection in all tissues.

**Hierarchical Clustering of \textit{P. gregata} Treatment Response**

To analyze trends due to treatment, we combined DE genes from the following gene lists: \textit{P. gregata} treatment, resistant response to \textit{P. gregata} infection, and susceptible response
to *P. gregata* infection. This resulted in 3,788 unique DE genes responding to treatment in one of the three tissues (Additional File 1). To allow visualization across tissues, we used log2 cpm data for each sample to conduct a hierarchical clustering analysis using the hclust command in R. Clustering was based on z-scores, which measures the number of standard deviations away a sample is relative to the mean for all samples for a given gene. The clustering order was also used to produce heatmaps based on fold change data (Additional File 2).

The treatment response hierarchical clustering heat map (Figure 1, Additional File 1) differentiated the three tissue types. As indicated by the dendrogram at the top of the heat map, gene expression in stems and roots was more similar to each other than to gene expression in the leaves. In roots, only 181 significant DE genes were identified, so expression patterns across all root samples was very similar. In stems, 1867 DE genes were identified and more expression differences could be observed than in roots. However, the largest difference corresponded to the 2876 significant DE genes identified in leaves, resulting in a distinct expression pattern for the resistant genotype infected with *P. gregata*. In contrast, the expression patterns of resistant mock infected, susceptible mock infected and susceptible infected with *P. gregata* are quite similar.

Hierarchical clustering also identified groups of genes with similar expression patterns. The 3,788 treatment DE genes grouped into seven distinct expression clusters, reflecting general trends in response to *P. gregata* infection (Figure 1). The genes in each treatment cluster are identified in Additional File 1. Clusters T1-T7 contained 678, 135, 683, 308, 281, 666, and 1037 genes, respectively. To assign function to these clusters, we used gene ontology (GO) overrepresentation to identify GO terms significantly overrepresented (P-value < 0.05) within a
cluster relative to all genes in the soybean genome (Figure 2, Additional File 3). Treatment clusters T1-T7 contained 7, 4, 5, 0, 1, 20, and 52 significant GO terms, respectively. GO term analyses revealed that clusters had distinct biological functions.

Clusters T1 and T3 had similar gene expression patterns (Figure 1) in the leaves, but opposite expression trends in stems and roots, which likely separated the clusters. When we examine fold change (P. gregata infected versus mock, Additional File 2) in resistant and susceptible interactions in the leaves, a strong response was observed in the resistant genotype relative to the susceptible. Surprisingly, in the stems the resistant and susceptible mirror expression observed in resistant leaves. Very little differential expression was observed in the roots. In response to treatment, four significantly overrepresented GO terms were identified in clusters T1 and T3 related to hormone signaling (Figure 2). This included response to ethylene (GO:0009723, cluster T1), response to jasmonic acid (GO:0009753, cluster T1), negative regulation of ethylene-activated signaling pathway (GO:0010105, cluster T3) and systemic acquired resistance, salicylic acid mediated signaling pathway (GO:0009862, cluster T3). Clusters T1 and T3 also contained five significantly overrepresented GO terms related to defense and stress responses. Defense and stress response terms in cluster T1 included response to wounding (GO:0009611), response to gravity (GO:0009629), and osmosensory signaling pathway (GO:0007231). Terms in cluster T3 included response to fungus (GO:0009620) and phosphorelay signal transduction system (GO:0000160).

In cluster T6, almost all genes were induced in response to P. gregata infection in the leaves in the resistant genotype (Figure 1, Additional File 2). Weaker induction was observed in the susceptible genotype. Cluster T6 contained seven GO terms related to both photosynthesis
and nine GO terms related to metabolism (Figure 2). GO terms associated with photosynthesis included: photosynthesis, light reaction (GO:0019684), photosystem II assembly (GO:0010207), and photosynthesis (GO:0015979). GO terms related to metabolism included: pentose-phosphate shunt (GO:0006098), starch biosynthetic process (GO:0019252), isopenenyl diphosphate biosynthetic process (GO:0019288), and positive regulation of catalytic activity (GO:0043085).

The largest cluster identified in response to treatment was cluster T7 (Figure 1). In the resistant genotype, all of the genes in cluster T7 were repressed in response to *P. gregata* infection. However, little differential expression was observed in the susceptible or in any other tissue. Gene ontology analyses revealed that genes in cluster T7 were associated with the cell cycle (33 terms) and gene silencing (15 terms, Figure 2). These GO terms include regulation of cell cycle (GO:0051726), regulation of DNA replication (GO:0006275), DNA replication (GO:0006260), DNA methylation (GO:0006306), cytokinesis by cell plate formation (GO:0000911), histone H3-K9 methylation (GO:0051567), and cell proliferation (GO:0008283).

Clusters T2, T4 and T5 were the smallest clusters identified (Figure 1). In cluster T2, strong differential gene expression was observed in the resistant leaves, but little differential expression was observed in the susceptible or in any other tissue (Figure 1, Additional File 2). Cluster T2 contained four GO terms related to transport (Figure 2) including organic cation transport (GO:0015695), methylammonium transport (GO:0015843), oligopeptide transmembrane transport (GO:0035672), and ammonium transmembrane transport (GO:0072488). Almost all genes in cluster T4 were induced in response to *P. gregata* infection in the resistant genotype and weak induction was observed in the susceptible genotype.
(Additional File 2). However, no significantly overrepresented GO terms were identified in this cluster. In contrast, in cluster T5, the majority of genes were repressed in response to *P. gregata* infection in the leaves with little differential expression observed in the susceptible or in any other tissue (Figure 1, Additional File 2). Cluster T5 had a single overrepresented GO term, maltose biosynthetic process (GO:0000024).

**Transcription Factor Analyses of *P. gregata* Treatment Response**

To identify transcription factors that play a role in regulating responses to *P. gregata* treatment, DE transcription factors were identified using the SoyDB transcription factor database (Wang et al., 2010). To compare changes in transcription factor expression, we observed expression of all transcription factors responding to *P. gregata* treatment across all tissues and genotypes (Figure 3, Additional File 4). Two hundred and fifteen transcription factors were identified. These transcription factors belong to 30 different transcription factor families. When we examined the resistance response (Figure 3A), the direction of transcription factor expression was the same in leaves and stems, however the magnitude of expression was often greater in stems. By plotting across transcription factor families, we observed families of transcription factors that appeared important in the resistant response. This included WRKY, MYB-HD, Homeodomain/HOMEOBOX, bHLH, and AP2-EREBP transcription factor families. While expression of these families in leaves and stems tended to be conserved, the AP2-EREBP family was largely repressed in leaves but induced in stems. Very little response to *P. gregata* infection was observed in resistant roots.

In the susceptible genotype (Figure 3B), the same families of transcription factors were differentially expressed however expression magnitude was greater in the resistant genotype.
Within the susceptible genotype, greater differential gene expression was observed in stem and roots. In the leaves, little significant differential expression was observed, but the majority of genes were repressed in response to pathogen infection.

In order to associate transcription factors with the clusters of genes they might regulate, we also analyzed the transcription factors by cluster. In leaves, 82 significant (P < 0.05) transcription factors were identified: 13, 1, 17, 7, 5, 25, and 14 in clusters T1 through T7, respectively. In stems 22, 2, 52, 26, 21, 4, and 5 significant transcription factors were identified in clusters T1 through T7, respectively for a total of 132 transcription factors. Only one significant (P < 0.05) transcription factor was identified in roots, belonging to cluster T4 (Figure 3). While this approach identified transcription factors functioning one week after *P. gregata* infection, we were also interested in identifying transcription factors that might act earlier during defense responses but were not captured in our RNA-Seq data. Using Clover (cis element over representation) (Frith et al., 2004) and the JASPAR transcription factor database (Mathelier et al., 2015), we identified transcription factor binding sites significantly (t < 0.01) overrepresented in promoters of DE genes in a cluster relative to promoters of all predicted genes in the soybean genome. Ninety-six unique transcription factor binding sites were identified across all clusters (P <0.05, Additional File 5). We identified 50, 27, 10, 51, 25, 3, and 20 transcription factor binding sites in clusters T1 through T7, respectively. Many of the transcription factor binding sites identified were known to be involved in the cell cycle and DNA replication as well as stress and defense responses. Several of the transcription factors identified within clusters have functions related to the biological processes assigned to the cluster. With this information, we could begin to build a framework for the different biological
processes identified in the RNA-Seq data and the transcription factors regulating their expression.

As previously discussed, GO analyses revealed that genes expressed in clusters T1 and T3 were related to both hormone signaling, defense and stress responses (Figure 2). Transcription factor analysis for these two clusters identified transcription factor families known to be associated with abiotic and biotic stress responses, such as the WRKY, MYB-HD and AP2-EREBP transcription factor families (Figure 3 and Additional File 4). For some transcription factors, similar fold changes in response to *P. gregata* infection could be observed in both resistant and susceptible genotypes. Two homologs of *AtMYB15* (Glyma.10G180800 and Glyma.10G00660) were significantly induced in stems. Three homologs of *AtMYB73* (Glyma.01G190100, Glyma.05G098200, and Glyma.11G052100) and one homolog of *AtMYB77* (Glyma.06G036800) were significantly repressed in stems and leaves. Members of AP2-EREBP transcription factor family, Glyma.10G186800 (*AtERF1* homolog) and Glyma.09G041500 (*AtERF72* homolog) were induced in stems. Other transcription factors in clusters T1 and T3 had very large differences between the resistant and susceptible genotype. For example, Glyma.17G222500 and Glyma.06G147100 (homologs of *AtWRKY40* and *AtWRKY51*) were induced 7-fold and 46-fold in the resistant genotype, respectively and only 4-fold and 21-fold in the susceptible genotype. Similarly, Glyma.03G162700 and Glyma.19G163900 (homologs of *AtERF15* and *AtERF1*) in the AP2-EREBP transcription factor family were induced 362-fold and 132-fold in the resistant genotype, respectively, but only 244-fold and 31-fold in the susceptible, respectively. *AtMYB15*, *AtMYB73*, and *AtMYB77* are associated with general stress responses and are induced by wounding (Cheong et al., 2002) and upregulated by cold stress in
Arabidopsis (Ding et al., 2009; Fowler and Thomashow, 2002). Over expression of AtERF1 enhances defenses against necrotrophic pathogens (Robert-Seilaniantz et al., 2011), AtERF1 and AtERF72 have been shown to mediate resistance to the soil borne fungus *Fusarium oxysporum* in Arabidopsis (Berrocal-Lobo and Molina, 2004; Chen et al., 2014). AtERF15 positively regulates immunity against *Pseudomonas syringae* and *Botrytis cinerea* (Zhang et al., 2015). WRKYs AtWRKY40 and AtWRKY51 negatively regulate jasmonic acid inducible defense responses (Gao et al., 2011). We also identified transcription factor binding sites corresponding to these TFs (Additional File 5). Binding sites for AtWRKY40 and AtWRKY63 were unique to cluster T1 and AtWRKY1 (AtZAP1) was unique to cluster T3. Similarly, we identified ERF transcription factor binding sites ERF7, ERF8, ERF69, ERF98, ERF105 and DREB1G significantly overrepresented in the promoters of DE genes corresponding to clusters T1 and T3, respectively. Interestingly, cluster T1 also contained six significantly overrepresented DOF transcription factor binding sites (AtDOF1.8, AtDOF 2.4, AtDOF2.5, AtDOF 5.3, AtDOF5.6, and AtDOF5.7). AtDOF2.5 is involved in the control of seed germination (Gualberti et al., 2002), AtDOF5.6 regulates vascular tissue development (Guo et al., 2009), and AtDOF5.7 regulates stomatal guard cell maturation (Negi et al., 2013).

Cluster T7 was associated with the cell cycle and gene silencing (Figure 2), with genes belonging to this cluster strongly repressed in resistant leaves. All but one of the transcription factors in cluster T7 were repressed in either leaf or stem tissue (Additional File 4). Further, the magnitude of repression was always greater in the resistant genotype. Many of these transcription factors had functions associated with DNA replication, the cell cycle and gene silencing. Glyma.07G250700 is a homolog of AtCHR17, a member of the imitation switch
subfamily in Arabidopsis important in the formation of nucleosome distribution patterns (Li et al., 2014). Glyma.01G175300 is homologous to AtDDM1, responsible for transposon and transgene silencing as well as maintaining DNA methylation levels (Gendrel et al., 2002). Glyma.05G189400 is homologous to AtTSK, which is required for cell cycle progression (Suzuki et al., 2005). Glyma.04G238400 and Glyma.06G125500 are homologous to AtSPCH, which initiates asymmetric cell divisions in the leaf and are essential for stomatal development (Danzer et al., 2015). Promoters of DE genes from cluster T7 were significantly overrepresented with transcription factor binding sites for transcription factors related to growth and development (Additional File 5) including AtTCP2, AtTCP19, AtTCP20, and AtTCP23. AtTCP19 and AtTCP20 which are involved in regulating leaf senescence (Danisman et al., 2013). Further, AtTCP20 regulates the expression of the DNA replication gene cyclin CYCB1;1 and several ribosomal proteins, regulating growth and/or cell division (Li et al., 2005).

No significantly overrepresented GO terms were identified in cluster T4 (Figure 2). However, a treatment effect was identified in leaves with the majority of the genes induced in response to P. gregata (Figure 1, Additional File 2). Many transcription factor families in cluster T4 were known to be associated with defense and biotic stress (Figure 3). In cluster T4 we identified homologs of the transcription factor AtWRKY40 (Glyma.14G102900 and Glyma.04G061400), a pathogen induced transcription factor that acts as a negative regulator in Arabidopsis defense (Xu et al., 2006). Both Glyma.14G102900 and Glyma.04G061400 were significantly induced in stems by P. gregata stress, but induced to a greater degree in the resistant response compared to the susceptible response. Two homologs of AtWRKY50 (Glyma.17G224800 and Glyma.04G054200) and one homolog of AtWRKY51...
(Glyma.05G184500) were also induced response to *P. gregata* in stems. AtWRKY50 and AtWRKY51 repress jasmonic acid derived defense responses (Gao et al., 2011). Several homologs of ethylene response factors were also identified (*AtERF1, AtERF15*, and *AtERF98*). Glyma.10G036700, Glyma.19G163900, and Glyma.20G203700 are homologous to *AtERF1*, a transcription factor that integrates both the ethylene and jasmonate signaling pathways, regulating the expression of pathogen response genes (Guttenson and Reuber, 2004). Glyma.03G162700 and Glyma.13G123100, are homologous to *AtERF15* which acts as a positive regulator of the immune response against the fungus *Botrytis cinera* in Arabidopsis (Zhang et al., 2015). Glyma.03G162400 and Glyma.19G163700 are homologs of *AtERF98*, which enhances salt tolerance by protecting plants from reactive oxygen species (ROS) damage (Zhang et al., 2012).

Cluster T4 also contained the largest number of significantly overrepresented transcription factor binding sites (Additional File 5). Many transcription factor binding sites identified had functions related to defense and stress responses. AtAHL20 negatively regulates PAMP-triggered immunity (PTI) in Arabidopsis (Lu et al., 2010). Binding sites for several transcription factors regulating abscisic acid signaling were overrepresented including ERF transcription factors AtERF096, AtERF098, AtERF105, AtERF112, AtERF7, and AtERF8, as well as a bHLH transcription factor, AtAIB (Lee et al., 2015; Song et al., 2005; Wang et al., 2015). AtBIM1, AtBIM2, and AtBIM3 and several TCP transcription factors binding sites were also overrepresented. AtBIM transcription factors are positive brassinosteroid signaling proteins that regulate plant growth and development as well as environmental stress responses (Song et al., 2005; Yin et al., 2005). Similarly, AtTCP2, AtTCP15, AtTCP16, AtTCP19, AtTCP20, and
AtTCP23 are involved in regulating leaf growth, leaf shape, and senescence (Kieffer et al., 2011). TCP20 also regulates root growth during nitrogen foraging (Guan et al., 2014) and regulates the expression of iron deficiency induced transcription factors bHLH38, 29, 100 and 101 (Andriankaja et al., 2014). ATHB7 acts in a signal transduction pathway that mediates drought responses (Söderman et al., 1996).

Hierarchical Clustering of *P. gregata* Genotype Response

In the genotype response 4131, 2590 and 2094 DE genes were identified in leaf, stem, and root tissue respectively. Hierarchical clustering was used to cluster the 6,938 unique DE genes responding to genotype based on common expression patterns. The resulting heat map (Figure 4) differentiated the three tissue types. Similar to the treatment effect, the dendrogram at the top of the genotype response heat map revealed gene expression in stems and roots were more similar to each other than to gene expression in the leaves. Within each of the three tissues, regardless of infection, genes cluster by genotype and the largest effect was identified in leaves.

Using z-scores, the 6938 genes were organized into clusters based on similar expression pattern. Eight distinct expression clusters emerged reflecting the general trends between genotypes (Figure 4). The genes in each cluster are listed in Additional File 6. Clusters G1 through G8 contained 1083, 1094, 717, 921, 177, 507, 903, and 1530 genes, respectively. To identify pathways due to genotype differences, biological process gene ontology (GO) terms significantly (P-value <0.05) overrepresented within each genotype cluster were identified (Additional File 7). Clusters G1 through G8 contained 36, 47, 3, 17, 3, 11, 4, and 13 significant GO terms, respectively.
Clusters G1, G4 and G8 had similar expression patterns with more DE genes identified with greater expression in the susceptible genotype in leaves, stems and roots. In Cluster G1, all but 29 of the 1083 DE genes were expressed more in the susceptible genotype relative to the resistant, regardless of the tissue examined (Figure 4, Additional File 6). However, the greatest repression was observed in resistant leaves after infection (Figure 4). Thirty-six significantly overrepresented GO terms were identified in cluster G1 relating to cell cycle and gene silencing (Additional File 7). GO terms in cluster G1 included regulation of DNA replication (GO:0006275), cell proliferation (GO:0008283), regulation of cell cycle (GO:0051726), and gene silencing (GO:0016572). Cluster G4 contained 309 of 519 (60%), 355 of 419 (84%), and 179 of 195 (91%) DE genes expressed more in the susceptible genotype in leaves, stems, and roots, respectively. Significantly overrepresented GO terms were largely associated with photosynthesis and ion homeostasis (photosystem II light assembly (GO:0010207), photosynthesis light reaction (GO:0019684), divalent metal ion transport (GO:0070838), regulation of proton transport (GO:0010155), and cellular cation homeostasis (GO:0030003)). Cluster G8 was overrepresented with GO terms associated with defense and wounding including: response to wounding (GO:0009611), defense response (GO:0006952), defense response to fungus (GO:0050832), and xylem development (GO:0010089). In leaves, stems and roots, 646 of 663 (97%), 654 of 668 (97%) and 508 of 534 (95%) of DE genes were expressed more in the susceptible genotype.

Clusters G3 and G7, had greater differential gene expression in the resistant genotype. In cluster G3, 240 of 391 (61%), 258 of 289 (89%), and 128 of 205 (62%) of DE genes in leaves, stems and roots were expressed more in the resistant genotype, respectively. Only three GO
terms were identified in cluster G3: aspartate transport (GO:0015810), tryptophan transport (GO:0015827), and pattern specific process (GO:0007389). In cluster G7, 511 of 516 (99%), 526 of 530 (99%), and 318 of 325 (97%) of DE genes in leaves, stems and roots, respectively, had greater expression in the resistant genotype. Cluster G7 was overrepresented with GO terms associated with ribosome biogenesis (GO:0042254), post-translation protein targeting to membrane (GO:0031204), translation (GO:0006412) and RNA methylation (GO:0001510).

While all genotype clusters described thus far had similar expression patterns across tissues and genotypes, clusters G2, G5 and G6 were quite different. Cluster G2 had 47 significantly overrepresented GO terms included GO terms related to photosynthesis, metabolism and response to oxidative stress (Additional File 7). Of the DE genes identified in cluster G2, DE genes expressed more in the resistant genotype were associated with leaves (821 of 946, 86%), while DE genes expressed more in the susceptible genotype were associated with the stems and roots (127 of 167 [76%] in stems and 48 of 87 [55%] in roots). Cluster G5 was associated with three significant GO terms including translation elongation (GO:0006414), translation (GO:0006412) and RNA methylation (GO:0001510). In cluster G5, 37 of 54 (68%) DE genes had greater expression in susceptible leaves. However, 82 of 83 (99%) DE genes in stems and all 96 DE genes in roots had greater expression in the resistant genotype. In cluster G6, we observed 92 of 175 (52%) DE genes and 50 of 65 (77%) DE genes expressed more in the susceptible genotype in leaves and stems, respectively. While in roots, 250 of 321 (78%) DE genes were expressed more in the resistant genotype. Eleven overrepresented GO terms were identified in cluster G6 relating to iron homeostasis (Additional File 7). GO terms included oxidation-reduction process (GO:0055114), cellular response to ethylene stimulus
Hierarchical Clustering of *P. gregata* Treatment x Genotype Response

Significant (FDR < 0.001) DE genes responding to the treatment by genotype interaction for each tissue type were identified using edgeR. In leaves, stems, and roots, 974, 37, and 44 DE genes were identified, respectively (Additional File 8). Hierarchical clustering of leaf treatment x genotype DE genes organized the genes into two expression clusters (Figure 5). Clusters LTG1 and LTG2 contained 169 and 805 genes, respectively. All 169 genes in cluster LTG1 were induced in response to infection in the resistant genotype but repressed in the susceptible genotype (Additional File 8). In contrast, all 805 genes in cluster LTG2 were repressed in response to *P. gregata* infection in the resistant genotype but induced in the susceptible genotype (Additional File 8). Two significant GO terms were identified in cluster LTG1: cellular iron ion homeostasis (GO:0006879) and response to zinc ion (GO:0010043). Cluster LTG2 contained 30 significant GO terms related to cell cycling, gene silencing, and metabolism (Additional File 9). These included cell proliferation (GO:0008283), DNA replication (GO:0006260), chromatin silencing by small RNA (GO:0031048) and very long-chain fatty acid biosynthetic process (GO:0042761).

The 37 treatment by genotype DE genes identified in stems organized into two expression clusters containing 33 and 4 genes (STG1 and STG2, Additional File 8, Additional File 10). All genes in the larger cluster were induced in response to infection in the resistant genotype, but not the susceptible. No significantly overrepresented GO terms were identified in either stem cluster (Additional File 9); however several genes were identified with functions
relating to defense (Additional File 8). Three homologs of \textit{AtMLP423} (Glyma.17G030200, Glyma17G030300, and Glyma.17G030400) were significantly induced. \textit{AtMLP423} is involved in the defense response and is induced by pathogen colonization (Plett et al., 2015).

The 44 treatment by genotype DE genes identified in roots also clustered into two groups containing 28 and 16 genes (RTG1 and RTG2, Additional File 8, Additional File 10). Cluster RTG1, containing 28 genes, was induced in response to infection in the susceptible genotype, while cluster RTG2, containing 16 genes was repressed in response to infection in the susceptible genotype. Though weak, the opposite patterns were observed in the resistant genotype. In cluster RTG2, only one significantly overrepresented GO term was identified, lignin metabolic process (GO:0009808). Even though only one GO term was identified as significant, several significant genes had functions related to defense. Three homologs of \textit{AtFBA2} (Glyma11G111100, Glyma11G111400, and Glyma.12G037400) were significantly induced. \textit{AtFBA2} is involved in abiotic stress responses (Lu et al., 2012). Homologs of \textit{AtUGT73B5} and \textit{AtUGT73B3} (Glyma01G046300 and Glyma15G221300), involved in the hypersensitive response in Arabidopsis (Simon et al., 2014), were also induced in roots.

\textbf{Single Linkage Clustering}

Single linkage clustering was used to group all differentially expressed genes, allowing us to highlight gene families responding to BSR resistance that may not have been identified in the hierarchical clustering analysis. 275 gene families were identified, each group containing 2 to 895 unique sequences (Additional File 11). Forty-six gene families were identified containing 20 or more sequences. Expression in the majority of these gene families had tissue specific expression patterns. Several of these groups were also largely specific to transcription factors.
Groups 14, 86, 34, 3, 61, and 22 were related to WRKY, NAC, MYB, homeodomain/HOMEOBOX, bHLH, and AP2-EREBP transcription factors, respectively. Group two, the largest group containing 895 unique sequences, was largely specific to protein kinases. 212 unique sequences were identified in group seven, largely specific to disease resistance proteins. Group 99 contained 31 glutathione S-transferase genes.

**Identification of *Rbs3* candidate genes**

In order to identify candidate BSR resistance genes, differential gene expression was analyzed in the *Rbs3* region on chromosome 16 (Chr16:33,333,000-33,819,000) (Klos et al., 2000; Lewers et al., 1999). Of the 37 genes in the locus, 21 genes were differentially expressed between genotypes (Figure 6). The strongest differential expression occurred in root tissue, particularly in twelve genes with homology to receptor-like proteins (RLP). Expression of the RLPs was greater in the resistant genotype compared to the susceptible genotype. RLP resistance genes play an important role in both the perception of and resistance to pathogens that grow extracellularly (Kruijt et al., 2005). Several UDP-glucose transferases were also differentially expressed in the *Rbs3* region. UDP-glucose transferases also play roles in plant defense. In Arabidopsis, AtUGT73B5 and AtUGT73B3 are involved in the hypersensitive response (Simon et al., 2014). AtUGT73C5 conjugates and detoxifies the fungal toxin deoxynivalenol mycotoxins produced by *Fusarium* species (Poppenberger et al., 2003).

**SNP identification**

SNPs were called between the resistant and susceptible genotype for the region corresponding to *Rbs3* (Klos et al., 2000; Lewers et al., 1999). Sequence data was used from both genotypes and all tissues. 258 SNPs with a quality score greater than 25 were identified.
The SNPs are listed in Additional File 12. These SNPs were identified one week after infection. Use of these molecular markers would allow disease screening to be conducted before the onset of BSR symptoms, improving screening efficiency.

**Virus Induced Gene Silencing**

VIGS constructs were developed for both downstream defense genes and for R gene constructs (Additional File 13). Downstream defense genes were chosen from the DE gene datasets and included Glyma01g021000 (homologous to *AtCAD8*, cluster T2), Glyma.01G046900 (homologous to *AT5G36930* a TIR/NBS/LRR resistance gene, G7), Glyma.02G063600 (homologous to *AtMES1*, cluster T1), Glyma.02G042500 (homologous to *AtPR3*, cluster T4), Glyma.09G107600 (homologous to *AT5G01720*, an RNI-like superfamily protein, cluster G7), Glyma.14G102900 (homologous to *AtWRKY40*, cluster T4), Glyma.16G175100 (homologous to *AtRLP33*, cluster G6), Glyma.17G030400 (homologous to *AtMLP423*, cluster T4) and Glyma.20G029600 (homologous *AtRBOHD*, cluster G7). R gene constructs were designed from R genes located in the *Rbs1*, *Rbs2* and *Rbs3* regions of the soybean genome (Additional File 13). VIGS constructs were designed to silence multiple homologs, accounting redundant function and soybean genome duplication events. To perform VIGS experiments, 10 day old seedlings of L78-4094 (*Rbs1*), PI 437833 (*Rbs2*), and PI 437970 (*Rbs3*) were rub inoculated with either a candidate defense gene VIGS construct, an empty vector control (BPMV vector lacking an insert), or mock VIGS controls (rub inoculated with buffer only). Two days after rub inoculation, all seedlings were stab-inoculated with *P. gregata*. In the first round of experiments, VIGS constructed developed from the RNA-Seq data were tested on five *Rbs3* plants. Similarly, R-gene constructs developed from the *Rbs1*, *Rbs2* and
Rbs3 loci were tested on five plants of each genotype (Rbs1, Rbs2 and Rbs3). Five weeks after infection, foliar and internal stem systems were recorded. Any constructs that resulted in altered defense phenotype were re-screened two additional times.

Silencing with RLPA, RLPB, and RLPC constructs caused PI 437833 (Rbs2) plants to exhibit a susceptible stem browning phenotype five weeks after inoculation with *P. gregata* (Figure 7). In addition, RLPA and RLPB silencing on PI 437833 plants were significantly (P-value < 0.05) taller than empty vector PI 437833 plants. Surprisingly, no other VIGS construct caused BSR foliar or stem phenotypes significantly different from empty vector plants. As expected, control plants did not experience any BSR symptoms. However, additional screenings are planned to optimize VIGS and *P. gregata* co-infection.

**Discussion**

Brown stem rot, caused by the fungus *Phialophora gregata*, is a yield limiting disease found in the majority of key soybean producing regions on the United States and Canada (Koenning and Wrather, 2010). In surveys conducted in 2008, the disease was found in 68-73% of fields in Illinois, Iowa, and Minnesota (Malvick and Grunden, 2008). In 2014, 13,636,691 bushels of soybean were lost to the disease brown stem rot (Koenning and Wrather, 2010). The development and use of disease resistant soybean cultivars is the most economical way to reduce soybean yield loss due to pests and pathogens.

In order to continue advancing progress in breeding for resistance, understanding the genetic mechanisms underlying resistance is crucial. To our knowledge, no studies have investigated the molecular responses to BSR in resistant and susceptible germplasm using expression analyses. To identify and understand the genetic basis of BSR resistance, RNA-Seq
expression profiling was conducted with leaf, stem, and root tissues of a resistant (PI 437970, Rbs3) and susceptible (Corsoy 79) soybean line, inoculated or mock-inoculated with P. gregata. Significant DE genes responding to P. gregata treatment in both genotypes, genotype-specific responses to P. gregata infection, genotype x treatment interactions, and general genotype differences were identified and analyzed for each tissue type.

This expression study identified genes differentially expressed one week after P. gregata infection. Disease screening for BSR resistance normally occurs in the growth chamber five weeks after infection. Identifying signatures of defense response earlier than phenotyping allows would increase the rate and efficiency of BSR screening, ultimately increasing genetic gain. Our results indicate that one week after infection strong differential expression occurred in genes functioning in defense, DNA replication, and iron homeostasis.

Defense

One week post infection, stronger gene expression, especially of defense genes, was correlated to P. gregata infection in the resistant genotype. In leaves, 2,492 genes were DE in the resistant response to P. gregata compared to only 89 in the susceptible genotype. In stems, 530 and 424 DE genes were expressed in resistant and susceptible genotypes, respectively. In roots, 16 and 163 DE genes were identified in the resistant and susceptible genotypes, respectively. Given that we see strongest DE gene expression in resistant leaves, and plants were inoculated in the stem, it is clear defense signaling has spread throughout the plant within one week of infection.

Plant innate immunity is first triggered by recognition of pathogen-associated molecular patterns (PAMP) (Göhre and Robatzek, 2008). LRR-RLKs (leucine-rich repeat receptor like
kinase) and receptor-like proteins (RLP) are well characterized PAMP recognitions (Göhre and Robatzek, 2008) that stimulate the defense signaling cascade leading to defense reactions. Plants also produce reactive oxygen species (ROS) that are harmful to the pathogen, strengthen cell walls to limit penetration by the fungus, and express of antimicrobial products in response to PAMP.

If pathogen effectors are able to successfully suppress the initial defense responses and colonize the host, plants then deploy R-gene mediated resistance (Göhre and Robatzek, 2008). This response is typically associated with the hypersensitive response (HR) and systemic acquired resistance (SAR) (Göhre and Robatzek, 2008). Signaling and regulation of downstream defense genes occurs through the actions of different hormones and transcription factors. In clusters T1 and T3, analysis of overrepresented gene ontology terms suggested hormonal regulation by ethylene, jasmonic acid and salicylic acid were important in regulating defense responses to *P. gregata*. These hormones are vital for regulating plant immune responses to a wide range of pathogens (Denancé et al., 2013). Similarly, we see a number of transcription factors families responding to pathogen attack including WRKYs, MYBs, and AP2-EREBPs. While these transcription factors were expressed in both resistant and susceptible genotypes, the number of DE transcription factors and expression magnitude was greater in the resistant response. Interestingly, while we observed greater differential gene expression in leaves, we observed greater transcription factor expression in the stems. Given the abundance of DE genes identified in our analyses, it is likely that earlier time point sampling could provide additional insight into the soybean/*P. gregata* interaction.
We were also particularly interested in examining the response of genes in the *Rbs3* locus to *P. gregata* infection. Surprisingly, many of the genes in this locus did not respond to infection. Instead, they were consistently expressed at higher levels in the resistant genotype, particularly in the root, where natural infection with *P. gregata* would occur. Of the 37 genes in the locus, 21 were differentially expressed between genotypes. This includes 12 RLPS with greater expression in the resistant genotype. The *Rbs3* RLPS are homologous to the apple *HcrVf* genes, which provide resistance to apple scab, the tomato *Cf, Ve* and *LeEIX* genes, which provide resistance to *Cladosporium fulvum, Verticillium* wilt and *Trichoderma viride*, and the Arabidopsis gene *RPP27*, which provides resistance to *Peronspora parasitica* (reviewed by Krujit et al., 2005). Each of these resistance genes can be found in closely linked clusters within their respective genomes, allowing unequal recombination and divergent selection to occur, leading to the evolution of novel pathogen specificities.

**DNA Replication**

In resistant infected leaves, genes with functions relating to DNA replication, regulation of the cell cycle, and gene silencing were generally repressed (clusters G1 and T7). Recognition of plant pathogens and initiation of defense responses requires reprogramming of the plant cell in order to stop pathogen growth. These responses are associated with increased demands for energy resulting in a fitness cost to the plant (Bolton, 2009). Heil and Baldwin (2002) reported that Arabidopsis mutants constitutively expressing defense responses had decreased growth and reproduction. In contrast, Arabidopsis mutant plants defective in defense signaling pathways were taller and had increased fitness in the absence of pathogen infection. The
energy the plant saves by downregulating cell growth can be diverted and used for defense responses and defense signaling networks.

Photosynthesis and metabolism are also influenced as the plant adjusts to meet the demands of defense response (Bolton, 2009). Surprisingly, in our dataset photosynthesis and metabolism genes were upregulated in resistant infected leaves (cluster T6). This upregulation of photosynthesis in response to *P. gregata* could be explained by two hypotheses. First, it is possible that during initial stages of pathogen infection photosynthesis and metabolism genes were downregulated, allowing the plant to instead use this energy for defense. One week after infection however, the resistant plants were able to control the *P. gregata* infection and return cell function back to normal. It is possible that if we sampled an earlier time point, we would notice photosynthesis and metabolism genes being downregulated in leaves. A second hypothesis is that photosynthesis rates must be increased after pathogen infection in order to provide the plant with enough energy to meet the increasing energy demands (Bolton, 2009).

Taken together, one week after *P. gregata* infection, soybean plants appear to switch off DNA replication and cell growth genes yet switch on photosynthesis and metabolism genes. Energy is critical during the execution of plant defense response due to the hundreds of genes from multiple defense pathways.

**Iron Homeostasis**

Surprisingly, many genes identified as being DE in response to *P. gregata* infection in our dataset have also been identified in response to iron deficiency chlorosis in soybean. Given that iron deficiency chlorosis and *P. gregata* infection in susceptible genotypes result in interveinal chlorosis of the leaves, stunting and reduced yield, we were interesting in making further
comparisons. Prior studies from our group have demonstrated the soybean lines tolerant to iron deficiency chlorosis regulate the expression of large suites of genes related to plant immunity and defense, DNA replication/gene silencing, photosynthesis and iron uptake/homeostasis (Atwood et al., 2014; Moran Lauter et al., 2014; O'Rourke et al., 2009). We observed the same classes of genes responding to *P. gregata* infection.

Iron plays an important role in plant-pathogen interactions. Both iron homeostasis and defense responses use common hormone signaling pathways such as ethylene, jasmonic acid, and salicylic acid (Aznar et al., 2015). As mentioned earlier, GO terms associated with these three hormones were identified as significant in clusters T1 and T3, the strongest differential expression occurred in leaves with little expression in stems or roots. Further, many transcription factors associated with defense also respond to abiotic stress. The balance between iron and defense signaling is complex. Plant roots secrete phenolic compounds under iron deficiency (Romheld and Marschner, 1986). These antimicrobial compounds both improve iron nutrition and protect the plant against pathogens. Therefore, iron deficient plants accumulate more phenolic compounds resulting in stronger resistance to pathogens compared to plants grown in sufficient conditions (Aznar et al., 2015). However, iron is also needed at specific sites to trigger reactive oxygen species (ROS) and hinder pathogen progression. ROS act as antimicrobial compounds, involved in the oxidative burst associated with the hypersensitive response.

Therefore, we examined our DE gene lists for homologs of known iron signaling and transport genes from Arabidopsis. In response to *P. gregata* treatment, we observed differential expression of fifteen soybean homologs of *AtFRO2, AtTIC110, AtHAS, AtMAT2,*
AtYSL1, AtFER1, AtFRO6 and AtbHLH105. All but one of the genes was induced in response to *P. gregata* infection in the resistant genotype. When we examined genotype and genotype by treatment differences between the *P. gregata* resistant and susceptible lines, we observed differential expression of 58 homologs of Arabidopsis iron responsive genes, including AtFRO2, AtFRO6, AtNRAMP3, AtNRAMP6, AtNAS1, AtNAS2, AtNAS4, AtYSL1, AtYSL3, AtYSL7, AtAHA2, AtAHA5, AtAHA11, AtbHLH105, AtbHLH29, AtbHLH38, AtFRD3, AtMAT2, AtMAT4, AtBTS, AtEIN3, AtPYE, AtOPT3, AtFER1, AtIREG2 and AtMTP8. All but fifteen of the genes were expressed at higher levels in the resistant genotype.

Interestingly AtTCP20 has recently been shown to have roles associated with the cell cycle, immunity and defense and nutrient acquisition. AtTCP20 can bind to the promoters of cyclin CYCB1;1 and several ribosomal proteins, to regulate growth and cell division (Li et al., 2005). TCP20, in combination with TCP7, TCP15, or TCP21, regulates expression of iron responsive bHLH38, bHLH39, bHLH100 and bHLH101 genes (Andriankaja et al., 2014). TCP20 is involved in regulating root growth to enhance nitrogen acquisition (Guan et al., 2014). In addition TCP20 plays a role in effector-triggered immunity. While we did not identify AtTCP20 homologs in our data set, many of the clusters differentially expressed in response to *P. gregata* infection were overrepresented with AtTCP20 binding sites. Earlier expression studies could shed light on TCP involvement in defense and iron deficiency responses in soybean.

**Toxin Metabolism**

In our analysis, single linkage clustering analysis allowed us to identify trends not apparent through hierarchical clustering analysis. Group 99 contained 31 DE genes, all orthologous to glutathione S-transferase (GST). GSTs are known to play an important role in
detoxification and redox buffering (Edwards et al., 2000); GSTs detoxify toxins by conjugating toxic substrates to form an s-glutathionylated reaction product, which is then transported into the vacuole for processing (Dixon et al., 2002).

In addition, we identified seven UDP-glucose transferases that were also differentially expressed in the Rbs3 region. In Arabidopsis, AtUGT73B5 and AtUGT73B3 are involved in the hypersensitive response (Simon et al., 2014), while AtUGT73C5 conjugates and detoxifies the fungal toxin deoxynivalenol mycotoxins produced by Fusarium species (Poppenberger et al., 2003). Gray and Chamberlain (1975) proposed that a toxin is involved in BSR pathogenesis causing leaf necrosis and chlorosis in P. gregata type A, and that one possible mechanism of BSR resistance is an insensitivity to toxins produced by P. gregata. Kobayashi and Ui (1980) later reported that gregatins, a family of compounds produced by P. gregata, were found to be toxic to adzuki bean, and that gregatins may also have an important role in pathogenesis in soybean. To our knowledge, the relationship between gregatins and BSR foliar symptom development has not been investigated further. A toxin causing foliar necrosis and chlorosis is not a foreign concept in soybean. The FvTox1 toxin produced by Fusarium viguliforme, the pathogen causing sudden death syndrome (SDS) in soybean, is a major pathogenicity factor involved in SDS foliar symptom development (Brar et al., 2011). Only SDS susceptible soybean lines are highly sensitive to FvTox1 proteins, resulting in loss of chlorophyll as well as necrosis symptoms on leaves (Brar et al., 2011). It is possible that foliar symptoms also develop on BSR susceptible soybean lines due to a toxin, and that GSTs aid in the detoxification of this toxin in BSR resistant soybean lines.
In conclusion, we report the first expression analyses of *P. gregata* infection of soybean. We find that infection results in the differential expression of genes relating to defense, cell cycle and iron homeostasis. In addition, we found that candidate genes in the *Rbs3* locus are associated with R gene mediated defense and toxin metabolism. These findings present new avenues for future soybean/*P. gregata* research that could have broad implications for other diseases and abiotic stressors affecting soybean and other crops. Further, our work has developed novel SNP sequences that can be used for marker assisted selection, fine mapping and charactering novel BSR resistance sources.

**Acknowledgements**

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**References**


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Tables and Figures

Table 1: BSR severity means of plants five weeks post infection. Eight plants were rated for foliar severity, stem severity, and recovery of P. gregata.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Treatment</th>
<th>Foliar Severity†</th>
<th>Stem Severity‡</th>
<th>Recovery of P. gregata§</th>
</tr>
</thead>
<tbody>
<tr>
<td>PI 437970 (Rbs3)</td>
<td>Resistant-Infected</td>
<td>5.5 a</td>
<td>45.1 b</td>
<td>2.1 a, b</td>
</tr>
<tr>
<td>PI 437970 (Rbs3)</td>
<td>Resistant-Mock</td>
<td>5.8 a</td>
<td>0 c</td>
<td>0 b</td>
</tr>
<tr>
<td>Corsoy 79</td>
<td>Susceptible-Infected</td>
<td>3.9 b</td>
<td>55.8 a</td>
<td>3.3 a</td>
</tr>
<tr>
<td>Corsoy 79</td>
<td>Susceptible-Mock</td>
<td>5.6 a</td>
<td>0 c</td>
<td>0 b</td>
</tr>
</tbody>
</table>

†Foliar severity was measured on a scale of 1-7, with 1 representing most severe symptoms.
‡Stem severity was determined by measuring the amount of internal stem browning divided by total plant height, and multiplied by 100 to obtain a percentage.
§Recovery of P. gregata was measured on a scale of 1-5 with 1 representing recovery of the fungus from the inoculation point only and 5 representing recovery of the fungus from the entire plant stem.
¶Means followed by the same letter in a column are not significantly different (P < 0.05; LSD).
Table 2: Number of significant (FDR < 0.001) differentially expressed (DE) genes responding to *P. gregata* treatment, genotype, genotype by treatment interaction, and genotype-specific response to *P. gregata* infection in leaf, stem, and root tissue.

<table>
<thead>
<tr>
<th>Treatment Effect</th>
<th>All Genotypes</th>
<th>Resistant Rbs3</th>
<th>Susceptible</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaves</td>
<td>160+, 135-</td>
<td>976+, 1516-</td>
<td>16+, 73-</td>
</tr>
<tr>
<td>Stems</td>
<td>647+, 266-</td>
<td>462+, 68-</td>
<td>297+, 127-</td>
</tr>
<tr>
<td>Roots</td>
<td>1+, 1-</td>
<td>4+, 12-</td>
<td>55+,108-</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Genotype Effect</th>
<th>DE Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Rbs3/Sus)</td>
<td></td>
</tr>
<tr>
<td>Leaves</td>
<td>1901+, 2230-</td>
</tr>
<tr>
<td>Stems</td>
<td>1017+,1573-</td>
</tr>
<tr>
<td>Roots</td>
<td>1228+, 866-</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Genotype x Treatment</th>
<th>DE Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Rbs3[Inf/Mock]/Sus[Inf/Mock])</td>
<td></td>
</tr>
<tr>
<td>Leaves</td>
<td>974</td>
</tr>
<tr>
<td>Stems</td>
<td>37</td>
</tr>
<tr>
<td>Roots</td>
<td>44</td>
</tr>
</tbody>
</table>
Figure 1: Hierarchical clustering of *P. gregata* treatment response. The treatment hierarchical clustering heat map was created with 3,788 significant (FDR < 0.001) unique DE genes responding to treatment, resistant response to *P. gregata* treatment, and susceptible response to *P. gregata* treatment. Hclust was used to cluster genes with similar expression patterns based on Z-score. The Z-score was calculated by comparing expression of the gene in a sample (in log2 counts per million, cpm) to the mean of all samples for the gene, divided by the standard deviation of the gene across all samples. The columns differentiate the three tissue types and four treatments within each tissue (resistant-mock (RM), resistant-infected (RI), susceptible-mock (SM), and susceptible-infected (SI)). The rows group genes by similar expression pattern, resulting in seven expression clusters delineated by colored bars on the Y-axis. Colors indicate Z-score values. A Z-score less than zero indicates lower expression than the mean, and a Z-score greater than zero indicates greater expression than the mean. DE genes responding to *P. gregata* treatment are listed in Additional File 1.
**Figure 2**: Gene ontology associated with *P. gregata* treatment response. Significantly (P-value <0.05) overrepresented biological process gene ontology (GO) terms within the DE genes responding to treatment were identified within each of the seven treatment clusters. A Fisher’s Exact Test (Fisher et al., 1960) and Bonferroni correction (Bonferroni, 1935), compared GO terms of genes within a cluster to GO terms for all genes in the genome. To reduce the number of significant GO terms and to eliminate redundancy, any GO terms whose genes completely overlapped were mapped to the largest significantly overrepresented GO term. The number in parentheses indicates the cluster the GO term was significant in. GO terms associated with the *P. gregata* treatment response are listed in Additional File 3.
Figure 3: Transcription factor expression patterns of the resistant and susceptible responses to
*P. gregata* treatment.
Significantly (P < 0.05) differentially expressed transcription factors were identified in each of
the seven treatment clusters from Figure 1. Expression of DE transcription factors was observed
across genotypes and tissues in both the resistant (A) and susceptible (B) response to *P.
gregata*. Absolute fold change is plotted on the x-axis, transcription factor families are plotted
on the y-axis. For visualization purposes, transcription factors with an absolute fold change
greater than 25 were plotted as 25. For a full list of transcription factors see Additional File 4.
The genotype hierarchical clustering heat map was created with 6,938 significant (FDR < 0.001) unique DE genes responding to genotype. Hclust was used to cluster genes with similar expression patterns based on Z-score. The columns differentiate the three tissue types and four treatments within each tissue (resistant-mock (RM), resistant-infected (RI), susceptible-mock (SM), and susceptible-infected (SI)). The rows group genes by similar expression pattern, resulting in eight expression clusters delineated by colored bars on the Y-axis. Colors indicate Z-score values. A Z-score less than zero indicates lower expression than the mean, and a Z-score greater than zero indicates greater expression than the mean. DE genes responding to genotype are listed in Additional File 6.
Figure 5: Hierarchical clustering of treatment x genotype response in leaves. The hierarchical clustering heat map was created with 974 significant (FDR < 0.001) DE genes responding to the treatment x genotype interaction in leaves. Hclust was used to cluster genes with similar expression patterns based on Z-score. The columns differentiate the four treatments within each tissue (resistant-infected, resistant-mock, susceptible-infected, and susceptible-mock). The rows group genes by similar expression pattern, resulting in two expression clusters delineated by colored bars on the Y-axis. Colors indicate Z-score values. A Z-score less than zero indicates lower expression than the mean, and a Z-score greater than zero indicates greater expression than the mean. DE genes for the genotype by treatment interaction for all tissues are listed in Additional File 8. The genotype by treatment response heat map for stem and root tissue can be found in Additional File 10. For visualization purposes, the heat maps are not to scale.
Figure 6: Identification of *Rbs3* candidate genes.

Differential gene expression of the 37 genes in the *Rbs3* region on chromosome 16 was analyzed (Chr16:33,333,000-33,819,000). Twenty one genes of thirty seven were significantly differentially expressed between the resistant and susceptible genotypes. Gene expression is presented as log2 based fold change differences between the susceptible and resistance genotypes. Additional information can be found in Additional File 1.
Figure 7: PI 437833 (Rbs2) response to *P. gregata* infection following VIGS. Images A-D illustrate rub-inoculation with one of four VIGS constructs. If a candidate gene contributes to resistance, silencing its expression should result in a susceptible phenotype, even though plants are genetically resistant. A-C. Resistant lines rub-inoculated with RLPA (A), RLPB (B), or RLPC (C). D. Resistant line rub-inoculated with empty vector (BPMV vector lacking an insert). All plants were infected with *P. gregata* two days after BPMV rub inoculation. Brown discoloration in the vascular tissue represents a susceptible reaction (A-C); healthy tissue represents a resistant reaction (D).
Additional Files

Additional File 1: Genes significantly differentially expressed in response to *P. gregata* treatment.

Additional File 2: Log fold change hierarchical clustering of *P. gregata* treatment response. The treatment hierarchical clustering heat map was created with 3,788 significant (FDR < 0.001) unique DE genes responding to treatment, resistant response to *P. gregata* treatment, and susceptible response to *P. gregata* treatment. The columns differentiate the treatments (leaves resistant (LR), leaves susceptible (LS), stems resistant (SR), stems susceptible (SS), roots resistant (RR), and roots susceptible (RS)). The rows group genes by similar expression pattern, resulting in seven expression clusters. Colors indicate gene expression values based on log fold change mapped to a color gradient from low (blue) to high (yellow) expression.

Additional File 3: Gene ontology (GO) terms significantly overrepresented within DE genes in response to *P. gregata* treatment in each cluster.

Additional File 4: Significantly differentially expressed transcription factors within each tissue and treatment cluster.

Additional File 5: Significantly overrepresented transcription factor binding sites within each cluster.

Additional File 6: Genes significantly differentially expressed in response to genotype.

Additional File 7: Gene ontology (GO) terms significantly overrepresented within DE genes in response to genotype.

Additional File 8: Genes significantly differentially expressed in response to treatment x genotype interaction.

Additional File 9: Gene ontology (GO) terms significantly overrepresented within DE genes in response to treatment x genotype interaction in each cluster.

Additional File 10: Hierarchical clustering of treatment x genotype response in stems and roots. The hierarchical clustering heat map was created with significant (FDR < 0.001) DE genes responding to the treatment x genotype interaction in stems (A) and roots (B). Hclust was used to cluster genes with similar expression patterns based on Z-score. The Z-score was calculated by comparing expression of the gene in a sample (in log2 counts per million, cpm) to the mean of all samples for the gene, divided by the standard deviation of the gene across all samples. The columns differentiate the four treatments within each tissue (resistant-infected, resistant-mock, susceptible-infected, and susceptible-mock). The rows group genes by similar expression pattern, resulting in two expression clusters for each tissue delineated by colored bars on the Y-
axis. Colors indicate Z-score values. A Z-score less than zero indicates lower expression than the mean, and a Z-score greater than zero indicates greater expression than the mean. DE genes for the genotype by treatment interaction for all tissues are listed in Additional File 8. For visualization purposes, the heat maps are not to scale.

**Additional File 11**: Gene families identified among differentially expressed genes

**Additional File 12**: SNP identification

**Additional File 13**: VIGS constructs
CHAPTER FIVE: GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

Pests and pathogens can drastically reduce soybean yield. In 2014, 13,636,691 bushels of soybean were lost to the disease brown stem rot (Koenning and Wrather, 2010). The results presented in this dissertation advance BSR resistance research and knowledge by combining traditional genetic studies, microscopic plant-pathogen interaction analyses, and genomic technologies.

The allelism test (Chapter 2) was conducted to identify and characterize novel sources of BSR resistance genes. Through this work, two plant introductions, PI 594638B and PI 594650A were identified as potentially containing a novel resistance allele or gene conferring resistance to *P. gregata*. A third plant introduction PI 594858B, was identified as either containing a novel source of resistance or to be expressing different mechanisms of resistance for BSR foliar and stem symptoms. The novel gene(s) for BSR resistance identified in this work have the potential to serve as donor genes or alternate sources of resistance in breeding programs, thus increasing the stability and durability of soybean host resistance to *P. gregata*. Given soybean’s narrow genetic base (Gizlice et al., 1994), reports of BSR symptoms developing on soybean lines containing the three known *Rbs* genes (Bachman et al., 1997; Hanson et al., 1988; Nelson et al., 1989) is alarming. Identifying and deploying novel resistance genes is crucial in order to continue protecting soybean yield against BSR. Future work will involve fine mapping the location of the novel resistance gene(s).

In Chapter 3, a new approach was utilized to investigate the soybean and *P. gregata* interaction, relating the presence and quantity of hyphae inside the soybean vascular tissue to
external foliar symptoms. This microscope work revealed that the three known BSR resistance genes may differ in their resistance mechanism, as was also indicated by the allelism study. We also concluded that there may be other pathogenicity factors associated with \textit{P. gregata}, such as a toxin that play a role in foliar symptom development. This unique microscopic approach allowed characterization of the physiological responses to BSR, not possible with traditional plant breeding or genetic studies. Future studies will investigate the role of a toxin in foliar symptom development and resistance to this toxin conferred by the \textit{Rbs} resistance genes.

In chapter 4, molecular responses to BSR were characterized in resistant and susceptible genotypes using RNA-Seq. This work demonstrated that \textit{P. gregata} infection results in the differential expression of genes relating to defense, cell cycle and iron homeostasis. This complex signaling network requires the differential expression of thousands of genes in leaves, stems, and roots. One week post infection, the strongest defense response was observed in resistant leaves. Candidate genes in the \textit{Rbs3} locus associated with \textit{R} gene mediated defense and toxin metabolism were also identified. Novel SNPs in the \textit{Rbs3} region were also identified that could be used marker assisted selection, fine mapping, and charactering novel BSR resistance sources.

This work provided powerful advancements to BSR research. We have utilized both traditional and non-traditional methods to answer biological questions regarding pathogen resistance. The research presented has leveraged knowledge from both traditional breeding and molecular genetics fields of study, presenting new avenues for future soybean/\textit{P. gregata} research. This work could have broad implications for other diseases and abiotic stressors, and the techniques presented can be applied to all areas of breeding research.
References


