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Mapping and identification of Candidate Arabidopsis *PSS5* and *PSS19* genes that confer nonhost resistance against the soybean pathogens, *Phytophthora sojae* and *Fusarium virguliforme*

by

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The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this thesis. The Graduate College will ensure this thesis is globally accessible and will not permit alterations after a degree is conferred.

Iowa State University

Ames, Iowa

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ABSTRACT

Phytophthora sojae is a hemi-biotrophic oomycete pathogen that causes root and stem rot disease in soybeans. Nonhost resistance (NHR) is broad-spectrum and provides resistance against all non-adapted pathogen species. Limited information is known about the molecular mechanism of NHR. To better understand the molecular mechanism of NHR, I have mapped and identified candidates for two new Arabidopsis nonhost resistancje genes, *P. sojae susceptible 5* (*PSS5*) and *P. sojae susceptible 19* (*PSS19*), by studying two putative mutants *pss5* and *pss19,* respectively, susceptible to the destructive soybean pathogen, *P. sojae.* Both mutants are also susceptible to *Fusarium virguliforme* that causes sudden death syndrome in soybean. By conducting bulked segregant analysis, the *PSS5* and *PSS19* were mapped to the south arm of chromosome 4 and north arm of chromosome 1, respectively. Comparison of the sequences of the bulked DNA samples from sixteen and twelve susceptible $F_{2:3}$ families homozygous for the *pss5* and *pss19* alleles, respectively, with the reference Col-0 genome sequence revealed four candidate genes for *PSS5* and six for *PSS19.* Analyses of the Salk T-DNA insertion mutants for these candidate genes identified the *AT4G10470* gene for *PSS5,* and *AT1G31930* for *PSS19* as strong candidate genes. Complementation analysis of the *pss5* mutant is being conducted to determine if *AT4G10470* is the *PSS5* gene and *AT1G31930* is *PSS19.* Once these genes are identified, it will be feasible to investigate if anyone of them can confer resistance against the soybean pathogens *P. sojae* and/or *F. virguliforme* in transgenic soybean plants.

CHAPTER 1. INTRODUCTION

Soybean overview

The soybean (*Glycine max* L. [Merr]) is one of the most economically important crops in the U.S. It is the second most produced crop (after corn) in Iowa [1]. Soybeans provide a large source of dietary protein, which is essential for human consumption and animal feed. Unfortunately, various pathogens attack soybean leading to yield suppression valued to over $3 billion annually [2].

Plant resistance overview

In nature, plants suffer from numerous biotic stresses including diseases caused by viruses, bacteria, oomycetes, fungi, nematodes, etc. In response to pathogen attack, plants use their immune system to defend against plant pathogens [3]. Effector-triggered immunity (ETI), also known as *R*-gene-mediated resistance or cultivar or race-specific resistance, is induced by single resistance (*R*) genes [4]. Generally, host cells recognize the pathogen(s) effector proteins through R proteins and induce the defense responses [4]. Effector proteins inducing ETI are encoded by avirulence (*Avr*) genes; and Flor [5-6] hypothesized that for each gene conditioning resistance in the host there is a corresponding gene for pathogenicity in the pathogen. This hypothesis later known as the gene-for-gene hypothesis. *R* genes mediate resistance by activating signal pathways regulated by ethylene, nitric oxide (NO), salicylic acid (SA) and jasmonic acid (JA) [7]. The hypersensitive response (HR) is first visible symptoms of a resistant host response induced by R proteins following recognition of corresponding effector proteins and is characterized by a rapid host cell death to limit the pathogen growth to the infection site. The HR is a form of programmed cell death (PCD) that evolved as a defense mechanism against pathogens [8].

However, the race-specific resistance is only effective in protecting plants against a small number of pathogen strains or isolates. Nonhost resistance (NHR), another form of resistance mechanism, is durable and broad-spectrum and provides full immunity to almost all pathogenic microorganisms [9-10].

The main difference between race-specific resistance and NHR is the type of pathogens, whether it's an adaptive or a non-adaptive pathogen. If a pathogen can infect and cause disease in a plant species, then the pathogen is termed adaptive pathogen to that plant species, and the species is considered as the host. If the pathogen fails to infect and cause disease in a plant species, then the pathogen is nonadaptive and the plant species is termed as nonhost [11-12]. Race-specific resistance or ETI is induced against adaptive pathogens; whereas, NHR is against nonadaptive pathogens.

In addition to ETI and NHR, there is also a third type resistance mechanism in plants known as basal defense mechanism which has subsequently been termed as PAMP-triggered immunity (PTI) [13]. It is activated during the initial phases of pathogen detection and is the first layer of the defense [14]. It is initiated with the perception of microbial, or pathogen-associated molecular patterns (MAMPs or PAMPs) [14-15]. Recent studies have shown that there may be some overlaps between basal defense and NHR [16].

Nonhost resistance overview

NHR is complex and multiple mechanisms for NHR are starting to emerge. Arabidopsis *NONHOST RESISTANCE 1* (*NHO1*) gene encoding a glycerol kinase, is the first known nonhost resistance gene [17-18]. The gene *NHO1* is induced by non-adapted bacterium *Pseudomonas syringae pv*. *phaseolicola*, and is required for expression of ETI and resistance to the fungal pathogen**,** *Botrytis cinerea* [17-18].

There are two molecular layers of NHR [19]. The first layer of NHR mainly suppresses the penetration process by the non-adapted pathogens [20]. Three *PENETRATION* genes, *PEN1, PEN2* and *PEN3,* have been shown to provide penetration resistance [20]. The *PEN1* gene encodes a syntaxin protein localized to the plasma membrane [21-22]. Following infection, the PEN1 protein becomes associated with golgi-complex and transport toxic free radicals to the infection sites to suppress penetration by a non-adaptive fungal barley pathogen in Arabidopsis [19, 23-24]. In addition, the gene plays a key role in the timely assembly of papillae at the pathogen infection sites [24]. *PEN2* gene is involved in restricting the penetration by two ascomycete powdery mildew fungi [25]. *PEN2* encodes a glycosyl hydrolase localized to peroxisomes and hydrolyzes the indole glucosinolates to highly toxic metabolites [19]. *PEN3* gene encodes a putative ATP-binding cassette (ABC) transporter localized to plasma membrane [26]. The ABC transporters in plants are well characterized, and ABC members have been implicated in the transportation for antifungal compounds, auxin, lipids, pigments and chlorophyll precursors [27]. It has been demonstrated that PEN3 collaborates with PEN2 for generating and transporting toxic compounds into the pathogen infection sites [28].

Once a pathogen overcomes the first layer of the defense, there is a second layer of NHR at the post-haustorial level, which is termed post-invasion resistance against the fungal pathogens [19]. This includes ROS, which activates various downstream signal pathways. For example, signal pathway mediated by the plant hormone salicylic acid (SA) plays a role in NHR of Arabidopsis against the nonhost pathogen *Xanthomonas citri* subsp. *citri* [29]. NHR against *Blumeria graminis* f. sp. *tritici* (*Bgt*) is mediated by sequentially deployed defense responses, which quantitatively contribute to pathogen resistance [30-31].

Other plant genes involved in the second layer of NHR include *ENHANCED DISEASE SUSCEPTIBILITY 1* (*EDS1*), *PHYTOALEXIN DEFICIENT 4 (PAD4*) and *SENESCENCE ASSOCIATED GENE 101* (*SAG101*) [19, 32-33]. Individual mutations in these genes have an effect on post-invasion defense in Arabidopsis against nonhost pathogen *B. graminis* f. sp. *hordei* [16]. *EDS1* and *PAD4* are also required for expression of resistance mediated by several Arabidopsis R proteins belong to the TIR-NB-LRR class [34-35]. The *EDS1-PAD4-SAG101* signaling complex is required for interfering the post invasion pathogen growth, the second layer of NHR [19]. This complex is well-known also to function in both PTI and ETI [19].

Arabidopsis genes *EDS1* and *NDR1* encode essential components for cultivar-specific resistance or ETI [34-35]. EDS1, PAD4 and SAG101 are also common regulators involved in ETI [36]. It has been shown that EDS1 interacts with both PAD4 and SAG101 leading to the EDS1-PAD4 and EDS1-SAG101 complexes localized to the cytosol. The SAG101-EDS1-PAD4 complexes has been localized to the nucleus. The EDS1-PAD4-SAG101 signaling complex is shown to function in *R* gene–triggered immunity [36].

Some effectors have been shown to be recognized by host and nonhost plant species. PcAvr3a1 of *Phytophthora capsici,* a homolog of Avr3a of *Phytophthora infestans,* is fully recognized by an R protein of nonhost *Nicotiana* species, which indicates the parallel mechanisms between race-specific resistance and NHR [37-39]. It has been reported that several genes and proteins play roles in both ETI and NHR. For example, *NHO1* and *GLYCOLATE OXIDASE (GOX)* are required for ETI and NHR in Arabidopsis [40]. *EDS1* also contributes to cultivar, nonhost and basal resistance [19].

Recently a forward genetic screen has been applied in Bhattacharyya lab and identified

several Arabidopsis *PSS* genes that confer NHR to two soybean pathogens: (i) the oomycete pathogen, *P. sojae*, and (ii) the fungal pathogen, *Fusarium virguliforme*. So far six *PSS* genes have been identified; *PSS1, PSS6, PSS20, PSS21, PSS25* and *PSS30* genes. *PSS1* encodes a glycine rich protein (*GRP1*) [41], *PSS6* encodes vesicle associated membrane protein 724 (VAMP 724), *PSS21* encodes an ABC1-like protein, *PSS25* encodes a member of the BEL family of homeodomain protein (BLH2), and *PSS30* a folate transporter (AtFOLT1) (The Bhattacharyya Lab, unpublished).

Phytophthora sojae overview

Phytophthora sojae, formerly known as *Phytophthora megasperma* f. sp. g*lycinea* and *P. megasperma* var. *sojae* before that, is an oomycete and a soil-borne pathogen [42]. It is considered as one of the most destructive pathogens of soybean [43]. The pathogen can kill seedlings before emergence from the soil, called as pre-emergence damping off; or seedlings may die right after emergence, which is called post-emergence damping off [42].

The pathogen causes root and stem rot in soybean. It can attack the soybean plants at any stage of growth. The disease is more likely to occur under cool and rainy weather [42]. Dull dark brown lesions can develop and extend upward on the stem from the soil line for older plants, occasionally to the tenth node. The lower taproot turns to dark brown and abnormally soft, and the entire root system can be rotted. Infected plants attacked by *P. sojae*, usually turn yellow, wilt and die in a short period [42]. *P. sojae* can be found mainly in drained and compacted heavy clay soils, especially in low areas with standing water for a couple of days after rainfall, or in areas with reduced tillage [42].

Arabidopsis thaliana

Arabidopsis thaliana is the plant model chosen for this study. Most *Phytophthora*

species including *P. sojae* cannot infect Arabidopsis [44]. The available genomic information and resources for the Arabidopsis system offer valuable prospects for dissecting its complex interaction with *Phytophthora* pathogens. Rarely, any growth through the stomata and the feeding structure haustoria are formed in the wild type Arabidopsis Columbia-0 (Col-0) plants [44].

The *PEN1* gene is essential for the timely assembly of papillae and callose deposition at the infection sites [21]. In the *pen1-1* mutant lacking the PEN1 function, single cells are penetrated by *P. sojae* [41]. The major objective of this study is to identify and investigate two Arabidopsis genes that contribute to the NHR mechanism.

Bulked segrgant analysis

Bulked segregant analysis (BSA) has been widely applied as a rapid molecular mapping method for genes that segregate as single Mendelian genes [45]. For this study, BSA was applied to segregating the $F_{2:3}$ populations of Arabidopsis. Three kinds of markers, single sequence length polymorphism (SSLP) markers, cleaved amplified polymorphic sequences (CAPS) markers and sequence-based polymorphism (SBP) markers, showing polymorphism between Arabidopsis Col-0 and Nd-0 ecotypes were applied for molecular mapping of two *PSS* genes [46]. Overall, the BSA has been successfully applied in the Bhattacharyya lab to map and identify several Arabidopsis *PSS* genes [e.g., 41, 47].

CHAPTER 2. INTRODUCTION MAPPING AND IDENTIFICATION OF CANDIDATES FOR TWO ARABIDOPSIS THALIANA NONHOST RESISTANCE GENES, *PHYTOPHTHORA SOJAE SUSCEPTIBLE* 5 (*PSS5*) AND 19 (*PSS19*)

Abstract

Phytophthora sojae is a hemi-biotrophic oomycete pathogen that causes root and stem rot disease in soybeans, which results in suppression of soybean yield valued to approximately $300 millions annually. Nonhost resistance (NHR) is broad-spectrum and provides resistance against all non-adapted pathogen species. A little is known about the molecular mechanism of NHR. To better understand the molecular mechanism of NHR, I have mapped and identified candidates for two new Arabidopsis nonhost resistancje genes, *P.* s*ojae susceptible 5* (*PSS5*) and *P.* s*ojae susceptible 19* (*PSS19*), by studying two putative mutants *pss5* and *pss19,* respectively, susceptible to the destructive soybean pathogen, *P. sojae.* Both mutants are also susceptible to *Fusarium virguliforme* that causes sudden death syndrome in soybean. By conducting bulked segregant analysis (BSA), the *PSS5* and *PSS19* were mapped to the south arm of chromosome 4 and north arm of chromosome 1, respectively. Comparison of the sequences of the bulked DNA samples from 16 and 12 susceptible $F_{2:3}$ families homozygous for the *pss5* and *pss19* alleles, respectively, with the reference Col-0 genome sequence revealed four candidate genes for *PSS5* and six for *PSS19*. Analyses of the Salk T-DNA insertion mutants for these candidate genes identified the *AT4G10470* gene for *PSS5* and *AT1G31930* for *PSS19* as strong candidate genes. Complementation analysis of the *pss5* mutant is being conducted to determine if *AT4G10470* is the *PSS5* gene and *AT1G31930* is *PSS19.* Once these genes are identified, it will be feasible to investigate if anyone of them can confer resistance against the soybean pathogens *P. sojae* and/or *F. virguliforme* in transgenic soybean plants.

Introduction

Soybean (*Glycine max* L. [Merr]) is one of the most economically important crops in the U.S. Soybeans is a major source of dietary protein and oil. Unfortunately, several pathogens attack soybean and cause serious annual yield suppression valued over $3 billion [2]. *P. sojae* was formerly known as *Phytophthora megasperma* f. sp. *glycinea* and prior to that as *P. megasperma* var. *sojae*. It is an oomycete and a soil-borne pathogen [42]. It is one of the most destructive pathogens of soybeans. The annual soybean yield suppression due to *P. sojae* have been valued to over $300 million [2].

Nonhost resistance (NHR) is a common form of plant defense mechanism exhibited by all members of a plant species against all non-adapted pathogens [10-11]. However, most nonhost plant resistance mechanisms are yet to be uncovered. Arabidopsis *Nonhost 1* (*NHO1*), encoding a glycerol kinase, is the first identified gene required for NHR against a non-adapted bacterial pathogen, *Pseudomonas syringae* pv. *phaseolicola* [17-18]. *NHO1* also plays a key role in the expression of gene-specific resistance against the bacterial pathogen, *Pseudomonas syringae* pv. *tomato*. It also confers immunity against the fungal pathogen, *Botrytis cinerea* [17-18].

There are two layers of NHR that protect Arabidopsis against pathogens [19]. The first layer of NHR, prevents the invasion by non-adapted pathogens [20]. Three Arabidopsis genes, *PEN1, PEN2* and *PEN3*, are involved in the first layer NHR or penetration resistance [20]. *PEN1* localized to the plasma membrane is a binary SNARE protein, a soluble N-ethylmaleimide–sensitive receptor syntaxin protein [21-22]. SNARE proteins are mainly involved in vesicle fusion and secretion of toxic free radicals to infection sites [23-24]. In the *pen1-1* mutant, papillae formation is delayed suggesting that *PEN1* plays a key role in papillae

formation at the fungal infection sites [24]. *PEN2* is involved in inhibiting the penetration by two ascomycete powdery mildew fungi [25]. The *PEN2* gene encodes a glycosyl hydrolase localized to peroxisomes and it hydrolyzes the indole glucosinolates to highly toxic metabolites [19]. *PEN3* encodes a putative ATP-binding cassette (ABC) protein transporter localized to the plasma membrane [26]**.** The ABC members have been implicated in the transportation of compounds including auxin, lipids, pigments and chlorophyll precursors and antifungal compounds [27]. It has been demonstrated that PEN3 collaborates with PEN2 to transport toxic chemicals elaborated by PEN2 into infection sites, and both proteins are required for extracellular accumulation of the glucan polymer, callose [28].

Once a pathogen overcomes the first layer of the NHR, they face a second layer of NHR, called post-invasion resistance [19]. At this layer, signal perception and signaling contribute to activation of post-haustorial resistance. The reactive oxygen species (ROS) activates downstream defense pathways; for example, salicylic acid (SA), a plant hormone signals the activation of plant defense mechanisms against the non-adaptive pathogen *Xanthomonas citri* subsp. *citri* [28-30]. *ENHANCED DISEASE SUSCEPTIBILITY 1* (*EDS1*), an important factor in the expression of plant immunity, is also a key component of NHR and actin cytoskeletal function [31-32].

Plant genes involved in basal defense include the *EDS1*, *PHYTOALEXIN DEFICIENT 4* (*PAD4*) and *SENESCENCE ASSOCIATED GENE 101* (*SAG101*), and they contribute to post-invasion defense [19, 31-32]. Specifically, individual mutations in these genes have an effect on the post-invasion defense in Arabidopsis against nonhost pathogen *B. graminis* f. sp. *hordei*. *EDS1-PAD4-SAG101* signaling complex is required for blocking the post-invasion pathogen growth [19]. This complex is known to function in ‘basal and *R* gene–triggered

immunity' [19]. *EDS1* interacts with both *PAD4* and *SAG101* leading to the *EDS1-PAD4* complex detected in both cytoplasm and nucleus and the *EDS1*-SAG101 complex in nucleus. The *EDS1-PAD4-SAG101* signaling complex is localized to nuclei and shown to function in basal and *R* gene–triggered immunity [36].

Arabidopsis thaliana was used as the model plant for this study. Arabidopsis is known to carry nonhost resistance genes against the oomycete pathogen *P. sojae* that causes root and stem rot in soybean [44]. The long-term research goal of the Bhattacharyya lab has been to identify and investigate most *PSS* genes that contribute to the NHR against two soybean pathogens, *P. sojae* and *F. virguliforme*. Thirty putative *P. sojae* susceptible (*pss*) mutants, *pss1* through *pss30*, were generated from screening of over 3,500 ethyl methane sulfonate (EMS)-induced $M_{2:3}$ families in Bhattacharyya lab [41, 47]. *PSS1* encodes a glycine-rich protein [47]. Additional five *PSS* genes, *PSS6, PSS20, PSS21*, *PSS25* and *PSS30* genes, have been identified (The Bhattacharyya Lab, unpublished). In this study, we have mapped and identified a strong candidate for each of the Arabidopsis NHR genes, *PSS5* and *PSS19*.

Results

PSS5 and *PSS19* genes segregate as Mendelian genes

Putative *pss5* and *pss19* mutants were crossed to Nd-0 and $F_{2:3}$ families were generated. Over 25 progenies of each $F_{2:3}$ family were evaluated for responses to *P. sojae* infection. Phenotypes of 70 $F_{2:3}$ families suggested that the segregation of the alleles at the *PSS5* locus fits to the 1:2:1 genotypic ratio for a Mendelian single gene model (Table 1). Similarly, investigation of the 58 $F_{2:3}$ families from a cross between *pss19* and Nd-0 revealed a 1:2:1 genotypic ratio for segregation of the alleles at the *PSS19* locus suggesting a single Mendelian

gene model for *PSS19* (Table 2).

The mapping of *PSS5*

By conducting the BSA, the *PSS5* gene was putatively mapped to the south arm of chromosome 4 (Figure 1) [45-46]. The CAPS marker 1H1L-1.6 showed the Col-0-specific allele among the $F_{2:3}$ families suggesting that this marker most likely closely linked to the *pss5* allele (Figure 1). The SBP marker, SBP4_6.51 showed recombination with the *PSS5* locus in several $F_{2:3}$ families indicating that *PSS5* is located south of this marker (Figure 1) [46]. Similarly, the SSLP marker CIW7 showed one recombination event and NGA1139 showed two with the *PSS5* locus among the $F_{2:3}$ families suggesting that the *PSS5* gene is located most likely north of these two markers (Figure 2). The *PSS5* gene was mapped to an 8.65-Mb genomic region between SBP4_6.51 and NGA1139 markers on the south arm of chromosome four (Figure 2).

Identification of Candidate *PSS5* Genes

To identify the candidate *PSS19* genes, we conducted Illumina sequencing for bulked DNA from 12 $F_{2:3}$ families homozygous for *pss5*. Sequence of the bulked DNA was compared to the reference Col-0 genome and also to the sequence of the *pen1-1* genome using the Bowtie program [48-49]. The *pss5* mutant was generated in the *pen1-1* mutant background and *pen1-1* was developed in Col-0. Therefore, common mutations between *pss5* and *pen1-1* were ignored. Four of the nine nonsynonymous mutations specific to the *pss5* mutant were considered for further study. Four genes, each carrying one of these four *pss5* specific mutations were considered as candidate *PSS5* genes (Table 3).

To identify the candidate *PSS5* gene from these four candidates, fourteen T-DNA insertion mutants for these four candidate *PSS5* genes were tested for responses to *P.*

sojae infection (Figure 3). From the 14 lines tested, two T-DNA mutant lines, SALK_108293 and CS052376 carrying T-DNA insertion mutations in the *AT4G10470* gene showed susceptibility to *P. sojae;* and were therefore, selected for further study (Figure 3). RT-PCR revealed reduced *AT4G10470* transcript levels in these two *P. sojae* susceptible mutants (Figure 4). T-DNA was inserted in the promoter region of the gene in both of these mutants resulting in reduced transcripts levels and susceptibility to *P. sojae.*

Complementation analysis to confirm that *AT4G10470* is the *PSS5* gene

To confirm that *AT4G10470* is the *PSS5* gene, complementation analysis is being conducted in the *pss5* EMS-induced mutant and the mutant SALK_108293 line with T-DNA insertion in the *AT4G10470* gene. The gene was cloned into the binary plasmid pISU-Agron5 vector as fusion genes with Promoter-2, Promoter-3 and Arabidopsis Ubiquitin-10 promoter, separately, and resulting plasmid constructs were transformed into the mutants. Promoters 2 and 3 are soybean promoters and are root and leaf specific promoters. Progenies of the basta resistant lines are being resprayed with basta to obtain transgenic lines for complementation analyses of the mutant phenotypes with the *AT4G10470* gene.

Mapping of the *PSS19* gene

Using the same BSA method used in mapping *PSS5*, the *PSS19* gene was putatively mapped to the centromeric region of chromosome 1 (Figure 5). The SSLP marker NGA63 showed recombination events with the *PSS19* locus among the $F_{2:3}$ families; but, no recombination of *PSS19* with OXFSSLP470359, LUGSSLO809 and CIW12 was also observed (Figure 6). Recombination of *PSS19* with SBP1_18.63 marker located in the south arm of chromosome 1 was observed (Figure 7). *PSS19* was mapped to a 13.38 Mb genomic region between markers, NGA63 and SBP1_18.63, most likely on the north arm of

chromosome 1 (Figure 5).

Identification of Candidate *PSS19* Genes

To identify the candidate *PSS19* genes, sequence of the *PSS19* region was obtained by sequencing the bulked DNA, pooled from DNA samples of twelve $F_{2:3}$ susceptible families homozygous for the *pss19* allele. Comparison of the sequence of the *PSS19* region was with that of the Col-0 and *pen1-1* genome sequences revealed 15 point mutations [48-49]. Six of these mutations were nonsynonymous and unique to the *pss19* mutant and were located in six candidate *PSS19* genes (Table 4).

To further identify the candidate *PSS19* gene, twenty-two T-DNA insertion Salk mutants for six candidate *PSS19* genes were tested for responses to *P. sojae* infection. Three T-DNA mutant lines, SALK_107656, SALK_030162 and SALK_141914, with T-DNA insertion in the exons of *AT1G31930* showed susceptibility to *P. sojae* infection (Figure 8).

Discussion

Nonhost resistance is the most common form of plant disease resistance, but its molecular mechanisms are remaining mostly unknown. Previously, the Bhattacharyya lab has discovered thirty putative mutants (*Phytophthora sojae* susceptible) from Arabidopsis that were penetrated by *P. sojae* to numerous cells. Fourteen out of the 30 mutants also showed susceptibility to *F. virgulforme*. So far six *PSS* genes have been identified: *PSS1* encodes a glycine rich protein, *GRP1* [41]; *PSS6* encodes a vesicle associated membrane protein 724 (*VAMP 724*); *PSS21* encodes an ABC1-like protein, *PSS25* encodes a member of the BEL family of homeodomain protein (*BLH2*) and *PSS30* encodes a folate transporter (*AtFOLT1*) (Table 5).

The *pss* mutants were generated in the *pen1-1* mutant, which is penetrated by *P. sojae* to single cells [41]. In this study, we have shown that both *PSS5* and *PSS19* were inherited like single Mendelian genes. Although, *pen1-1* is penetrated by *P. sojae* and *pss5* and *pss19* were generated in *pen1-1*, no epistatic effect of PEN1 on either PSS5 or PSS19 was observed. This was also supported by the susceptible responses of the T-DNA insertion mutants for the two *PSS* genes (Figures 3 and 8) that carry the wild-type *PEN1* gene.

In this study, we mapped and identified candidate genes for two of the 14 *pss* mutations, *pss5* and *pss19* that are susceptible to two soybean pathogens, *P. sojae* and *F. virguliforme.* Bulked segregant analysis (BSA) was applied on bulked DNA samples from 16 and 12 susceptible $F_{2:3}$ families homozygous for *pss5* and *pss19* alleles, respectively, to putatively map the *PSS5* and *PSS19* loci. The *PSS5* gene was mapped to an 8.65-Mb genomic region of the north-arm of chromosome 4, and the *PSS19* was mapped to the centromeric region of chromosome 1, in a 13.38-Mb genomic region. The bulked DNA samples were sequenced to identify the candidate *PSS5* and *PSS19* genes. Then the SALK T-DNA insertion mutants for the candidate genes were inoculated with *P. sojae* zoospores to identify the candidate *PSS5* or *PSS19* genes. At least two T-DNA insertion homozygous mutants for each candidate gene were selected to investigate for the possible loss of immunity against *P. sojae* (Figure 3 and 8).

Susceptibility of two independent T-DNA insertion mutants for *AT4G10470* suggests that *AT4G10470* is most likely the *PSS5* gene (Figure 3). The gene encodes a novel protein(s) with no known function. Therefore, it is unclear how this gene may regulate Arabidopsis nonhost resistance against the two soybean pathogens.

P. sojae susceptibility of three independent T-DNA insertion mutants for the *AT1G31930* gene strongly indicates that *AT1G31930* is the *PSS19* gene. *AT1G31930* encodes

a heterotrimeric guanine nucleotide-binding (G) protein, XLG3 (Extra-large G protein 3) that shows significant similarity to the G protein alpha subunit in its C terminal region [50-51]. The protein is involved in the regulation of root morphology and growth. Heterotrimeric guanine nucleotide-binding (G) proteins composed of three subunits, Gα, Gβ, and Gγ, and they function as molecular switches in signal transduction [50]. Three extra-large Gα (XLG1, XLG2, and XLG3) [52-54], one canonical Gα (GPA1) [55], one Gβ (AGB1) [56] and three Gγ (AGG1, AGG2, and AGG3) [57-59] subunits have been discovered in Arabidopsis. XLG1, XLG2, and XLG3 are localized to nuclei [60]. Unfortunately, their functions and modes of action remain largely unknown. XLG2 has been seen as one component of a G-protein complex, which differs from the prototypical heterotrimeric G-protein [60]. XLG2 may have 'distinct functions in modulating defense responses' [60]. Meanwhile, mutation of XLG3 impaired root waving and skewing, suggested its contribution to plant roots repairing [61-62]. However, there has been no study establishing that the XLG3 gene contributes to plant immunity. Therefore, further characterization would help us to establish a novel NHR mechanism governed by *PSS19*. We hypothesize that most likely *PSS19* may play signaling role in the expression of NHR against the soybean pathogens, *P. sojae* and *F. virguliforme*.

Materials and Methods

Arabidopsis detached leaf inoculation

Seeds of Arabidopsis Col-0, Nd-0, *pss5*, *pss19*, *pen1-1* and T-DNA insertion mutant lines were sown on LC1 soil-less mixture (Sun Grow Horticulture) and grown in a growth chamber, under a constant 16 h light (6 AM to 10 PM) and 8 h dark period cycle at 21°C [41]. The light intensity was 120-150 µmol/m^2/s, and the relative humidity was around 60% [41,

63]. Following sowing, the flats were covered with transparent domes to maintain a high humidity environment for 72 h. Ten days after sowing, the seedlings were transplanted (two seedlings per well of a 48-well flat) into a new LC1 soil mixture, and were covered with domes for three days. Seven days after transplantation, a fertilizer mixture of 15:15:15 :: N:P:K (1% concentration v/v) in water was applied to the transplanted seedlings [41]. They were watered every fifth day.

The *P. sojae* NW5A and PR6 isolates were maintained on V8 agar plates weekly. The plates were soaked and washed a day before inoculation to obtain zoospores [64]. From the 21-day-old Arabidopsis seedlings, the 4^{th}, 5^{th} and 6^{th} leaves from the apex were detached and placed on moist Whatman filter papers in Petri dishes [41]. Every leaf was inoculated with 25 µl of *P. sojae* zoospore suspensions ($3x10^5$/ml) [41]. The Petri dishes were covered, sealed and incubated under constant light (60µE/m^2/s) and at 25ºC [41]. The inoculated leaves in dishes were scored at 24, 48 and 72 h post-inoculation (hpi) as resistant or susceptible phenotype.

Bulked Segregant Analysis (BSA) and PCR

Young and fresh Arabidopsis leaves were selected from each plant for DNA extraction. The CTAB method was applied for Arabidopsis genomic DNA extraction [65]. Equal amount (10 µg) of DNA from 16 and 12 susceptible $F_{2:3}$ families homozygous for the *pss5* and *pss19* alleles, respectively, were mixed to obtain two bulked DNA samples. For each bulked DNA sample, the final concentration of DNA was around 25 ng/µl. The PCR reaction mixtures for SSLP markers contained 1 µl DNA template, 0.25 µM forward and reverse primer (Integrated DNA Technologies, Inc., Coralville, Iowa), 2 µM dNTPs (Thermo Fisher Scientific, Waltham, MA), 2 mM $MgCl_2$ **(**Bioline, Taunton, MA), and 0.5 U Taq polymerase (Denville Scientific, Inc., Metuchen, NJ) in 20 µl total volume [41]. PCR was conducted at 94ºC for 3 min

(denaturation) and then 35 cycles for amplification, with 94°C for 30 secs, 55°C for 30 secs and 72°C for 30 secs. Finally, the DNA mixture was incubated for the final extension at 72°C for 10 min [41]. PCR for the SBP and CAPS markers developed for SSLP-thin genomic regions was conducted as follows. PCR was conducted at 94°C for 3 min (denaturing), and then five cycles of 94°C for 30 sec followed by decreasing annealing temperatures from 55°C to 50°C (reduce 1°C/cycle) and 72°C for 1 min and then 35 cycles of 94°C for 30 secs, 50°C for 30 secs, and 72°C for 1 min and a single final extension step at 72°C for 10 min [41]. PCR was conducted in a MyCycler™ Thermal Cycler (BIO-RAD, Inc., Hercules, CA). The amplified products were digested with restriction enzymes and resolved on ~1-2% agarose gels by running at stable 10 V/cm. The PCR products amplified by SSLP markers were resolved on 4% agarose gels containing ethidium bromide by running at stable current (15 V/cm) [66]. The PCR products were visualized by illuminating under the UV light [41]. Primers used in the PCR for molecular markers linked to *PSS5* and *PSS19* are presented in Tables 6 and 7, respectively.

SNP detection by Bowtie analysis

The Illumina paired-end reads obtained from the bulked DNA from 16 and 12 susceptible $F_{2:3}$ families homozygous for either *pss5* or *pss19* allele, respectively, were mapped onto the Col-0 reference genome and mutations were identified using the Bowtie2 tool [48-49]. The Illumina paired-end reads for *pen1-1* were also mapped to Col-0 reference genome and mutations were identified. The short reads with at least 95% similarity to the reference genome were mapped. While mapping, a quality cutoff of 40 was used to ensure an error rate of less than 1 in 10,000 bp. The output from Bowtie2 in SAM format was imported to Samtools with the view command to produce the equivalent BAM file [49]. This file was sorted by the

SORT command. The sorted BAM file was piled up on the reference genome with the mpileup command in Samtools to produce the Variant Calling Format (vcf) file, to obtain the list of the SNPs with their quality scores, position, reference allele, alternate allele and all other necessary details in an Excel sheet. SNPs of *pss* mutants were compared with that of *pen1-1* in the Excel sheet to eliminate any common mutations originating from the *pen1-1* background.

RNA isolation and RT-PCR for *PSS5*

After inoculation and scoring, young leaves from each plant of Col-0 and two T-DNA insertion mutants were used to extract total RNAs [67]. Then DNase I (Invitrogen, Inc., Carlsbad, CA) was applied to each RNA sample to eliminate any contaminating genomic DNA [41, 68]. RT-PCR was conducted using the following the program: 75°C for 10 min, 42°C for 60 min and 75°C for 15 min [69]. RT- PCR was conducted using primers listed in Table 8. The PCR program included: 94°C for 3 min and 35 cycles of 95°C for 30 sec, 60°C or 55°C for 1 min and 72°C for 1 min, and the final extension cycle at 72°C for 10 min [41, 69]. Transcripts of *ACTIN* were amplified in the RT-PCR reactions as an internal control. Both RT-PCR and regular PCR were conducted in a MyCycler™ Thermal Cycler (BIO-RAD, Inc., Hercules, CA). The cDNAs were prepared using the manufacturer's protocol (Invitrogen, Inc., Carlsbad, CA) [69].

***PSS5* gene cloning, plant transformation and complementation analysis**

To confirm that *AT4G10470* is the *PSS5* gene, complementation analysis is being conducted in the *pss5* EMS-induced mutant and the SALK_108293 mutant line with T-DNA insertion in the *AT4G10470* gene. The full genomic length *PSS5* coding sequence was PCR amplified from the wild type Col-0 genomic DNA sample using gene-specific primers (Table 9). The gene was cloned into the pISU-Agron5 vector as fusion genes with Promoter-2,

Promoter-3 and Arabidopsis Ubiquitin-10 promoter, separately; and the resulting plasmid constructs were transformed into the *E. coli* DH10B cells. Promoters 2 and 3 are soybean promoters and are root and leaf specific promoters. The constructs were initially verified by conducting PCR (Table 9) and then by sequencing. The three constructs containing the *PSS5* gene fused individually to each of the three promoters were then transformed into the *Agrobacterium tumefaciens* EHA101 strain using the freeze-thaw method [47, 70] for transformation of the *pss5* and T-DNA insertion-induced *P. sojae*-susceptible SALK mutant line, SALK_108293. The *pss5* and T-DNA insertion SALK_108293 mutant line were transformed with the *A. tumefaciens* EHA101 isolate carrying the candidate *PSS5* gene by conducting floral dip inoculation method [47, 71]. The T_1 and T_2 progenies were screened for Basta^{TM} resistance by spraying with Liberty (80 µg/mL) herbicide [47]. Herbicide glufosinate-ammonium (Basta ^{TM}) was sprayed twice into transformed Arabidopsis seedlings, and herbicide resistant plants were selected to obtain progeny seeds. Progenies of the basta resistant lines will be resprayed with basta one more time before testing the lines for complementation of the mutant phenotypes with the *AT4G10470* gene.

Tables

Table 1. Segregation of *PSS5* alleles among the $F_{2:3}$ families.

Genotype	**Observed plants**	**Expected plants**
Homozygous resistant (*PSS5PSS5*)	19	17.5
Heterozygous (*PSS5pss5*)	35	35
Homozygous susceptible (*pss5pss5*)	16	17.5
Total	70	70
χ^2 value	0.26	
***p* value**	0.878	

Table 2. The segregation of *PSS19* alleles among the $F_{2:3}$ families.

Genotype	**Observed plants**	**Expected plants**
Homozygous resistant (*PSS19PSS19*)	17	14.5
Heterozygous (*PSS19pss19*)	29	29
Homozygous susceptible (*pss19pss19*)	12	14.5
Total	58	58
χ^2 value	0.86	
***p* value**	0.651	

Table 3. Four candidate *PSS5* genes carrying non-synonymous mutations were identified from the *PSS5* region. The candidate gene in bold font is the one, T-DNA insertion mutants of which showed susceptibility to *P. sojae* (Figure 3).

Point mutation locus	**Original**	**Mutant**	**Candidate Gene**	**Encoded protein**
6125170	G	A	*AT4G09690*	CYSTEINE/HISTIDINE-RICH C1 DOMAIN FAMILY PROTEIN
6476493	**A**	**G**	***AT4G10470***	**HYOPTHELICAL PROTEIN**
7274727	T	A	*AT4G12170*	THIOREDOXIN SUPERFAMILY PROTEIN
7980599	C	T	*AT4G13750*	EMBRYO DEFECTIVE 2597

Table 4. Six candidate *PSS19* genes carrying non-synonymous mutations were identified from the *PSS19* region. The candidate gene in bold font is the one, T-DNA insertion mutants of which showed susceptibility to *P. sojae* (Figure 8).

Point mutation locus	Original	Mutant	Candidate Gene	Encoded protein
5382436	G	A	*AT1G15650* *AT1G15660*	N/A CENTROMERE PROTEIN C (CENP-C)
5937896	C	T	*AT1G17340*	SUPPRESSOR OF ACTSAC5, SUPPRESSOR OF ACTIN 5
6057333	C	T	*AT1G17610*	CHILLING SENSITIVE 1
6833656	C	T	*AT1G19780*	CYCLIC NUCLEOTIDE GATED CATCNGC8, CNGC8, CYCLIC
11467314	**G**	**A**	***AT1G31930***	**EXTRA-LARGE GTP-BINDING PROTEIN 3, XLG3**
13539841	C	A	*AT1G36160*	ACC1, ACETYL-COA CARBOXYLASE 1, AT-ACC1, EMB22, EMBRYO DEFECTIVE 22, GK, GLOSSYHEAD 1, GSD1, GURKE, PAS3, PASTICCINO 3, SENSITIVE TO FREEZING 3, SFR3

Table 5. Summary of thirty putative Arabidopsis *pss* mutants characterized in the Bhattacharyya lab.

pss mutant	Phenotype to *F. virguliforme*	Mapping locus	Gene function & Protein
1	S	Chromosome 3	Glycine Rich Protein (*GRP1*)
2	R/S		
3	R/S		
4	R		
5	S	Chromosome 4	**Hypothetical protein**
6	S	Chromosome 4	Vesicle-Associated Membrane Protein (*VAMP724*)
7	R		
8	R		
9	R		
10	S		
11	R		
12	R		
13	R		
14	R		
15	S		
16	R/S		
17	R		
18	S		
19	S	Chromosome 1	**EXTRA-LARGE GTP-BINDING PROTEIN 3 (*XLG3*)**
20	S	Chromosome 4	BEL1-Like Homedomain 2 (*BLH2*)
21	S	Chromosome 5	ABC1-Like Protein 1 (*AtOSA1*)
22	R		
23	R		
24	R		
25	S	Chromosome 4	BEL1-Like Homedomain 2 (*BLH2*)
26	R		
27	R		
28	R		
29	R		
30	S	Chromosome 5	Folate transporter (*AtFOLT1*)

Table 6. Molecular markers mapped to the *PSS5* region.

Name	Marker type	Primer	Enzyme
SBP4_6.51	SBP	F: GGACAAGACCTTGATTTGAAGTTG R: GAGGGCTCACATTGGGTTTAATG	*Tsp*509I
1H1L-1.6	CAPS	F: CTAGAGCTTGAAAGTTGATG R: TTGAGTCCTTCTTGTCTG	*RsaI*, *Tsp509I*
CIW7	SSLP	F: AATTTGGAGATTAGCTGGAAT R: CCATGTTGATGATAAGCACAA	
NGA1139	SSLP	F: TTTTTCCTTGTGTTGCATTCC R: TAGCCGGATGAGTTGGTACC	

Table 7. Molecular markers mapped to the *PSS19* region.

Name	Marker type	Primer	Enzyme
NGA63	SSLP	F: AACCAAGGCACAGAAGCG R: ACCCAAGTGATCGCCACC	
SBP1_5.25	SBP	F: CACAAACCCTTCACCTCCAT R: GCAGTTGCCTAAAGGCTGAG	*MspI*
M59	CAPS	F: GTGCATGATATTGATGTACGC R: GAATGACATGAACACTTACACC	*RsaI*, *Tsp509I*
OXFSSLP470359	SSLP	F: CGTAGCTTCGTAATTCGTATAGT R: ATTGGAAGCAAAATTCTTGTT	
SBP1_8.08	SBP	F: AATTTGGAGATTAGCTGGAAT R: CCATGTTGATGATAAGCACAA	*RsaI*
LUGSSLP809	SSLP	F: AATTTGGAGATTAGCTGGAAT R: CCATGTTGATGATAAGCACAA	
CIW12	SSLP	F: AATTTGGAGATTAGCTGGAAT R: CCATGTTGATGATAAGCACAA	
SBP1_18.63	SBP	F: AATTTGGAGATTAGCTGGAAT R: CCATGTTGATGATAAGCACAA	*RsaI*

Table 8. Primers used in the *PSS5* RT-PCR experiment. *PSS5-BstXI* is used for Ubiquitin-10 clone and *PSS5-AscI* is applied for cloning with Promoter-2 and Promoter-3.

Name	Primer
PSS5	F: GCGTCCTGATCCATGATGCA R: GAAGCACGGTGCAGCAGATC
AtActin	F: GGCGATGAAGCTCAATCCAAACG R: GGTCACGACCAGCAAGATCAAGACG

Table 9. Primers used in cloning the *PSS5* gene. *PSS5-BstXI* primers were used for fusing the gene with the Ubiquitin-10 promoter and *PSS5-AscI* primers for fusing the gene individually with Promoter-2 and Promoter-3.

Name	**Primer**
PSS5	F: GCTCGTCGTCTCTGCAAAATCC
	R: CACGCCTCGATTCAGCCG
PSS5-AscI	F: ATATGGCGCGCCGCTCGTCGTCTCTGCAAAATCCCTTC
	R: ATATGGCGCGCCCACCGCCTCGATTCAGCCGCTTG
PSS5-Bst XI	F: GCGCCCATATATCTGGGCTCGTCGTCTCTGCAAAATCCCTTC
	R: GCGCCCATATATCTGGCACCGCCTCGATTCAGCCGCTTG
Checking Orientation	F: GCTGCAAATGACTTTGGTGATAGACA
	R: CATCTGCATCATGGATCAGGACGCC

Figures

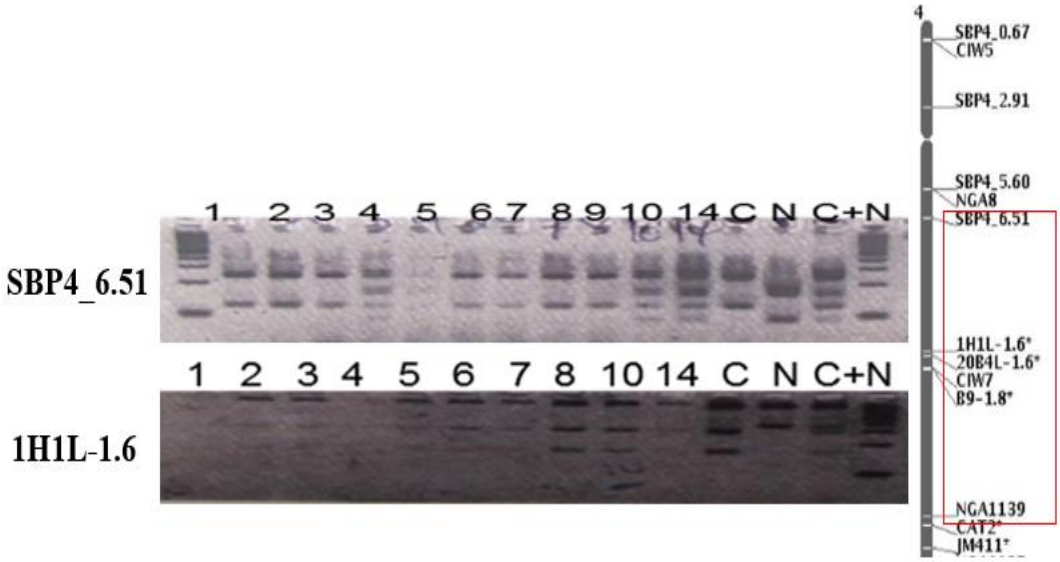

Figure 1. Amplification patterns of SBP4_6.51 and 1H1L-1.6 markers linked to *PSS5* among the susceptible $F_{2:3}$ families. B, bulked DNA of the susceptible families; C, Col-0; N, Nd-0; and C+N, mixed Col-0 and Nd-0 DNA samples in equal amounts. The *PSS5* region is boxed with a red line.

Figure 2. Amplification patterns of SSLP markers, CIW7 and NGA1139, linked to *PSS5* among the susceptible $F_{2:3}$ families. B, bulked DNA of the susceptible families; C, Col-0; N, Nd-0; and C+N, mixed Col-0 and Nd-0 DNA samples in equal amounts.

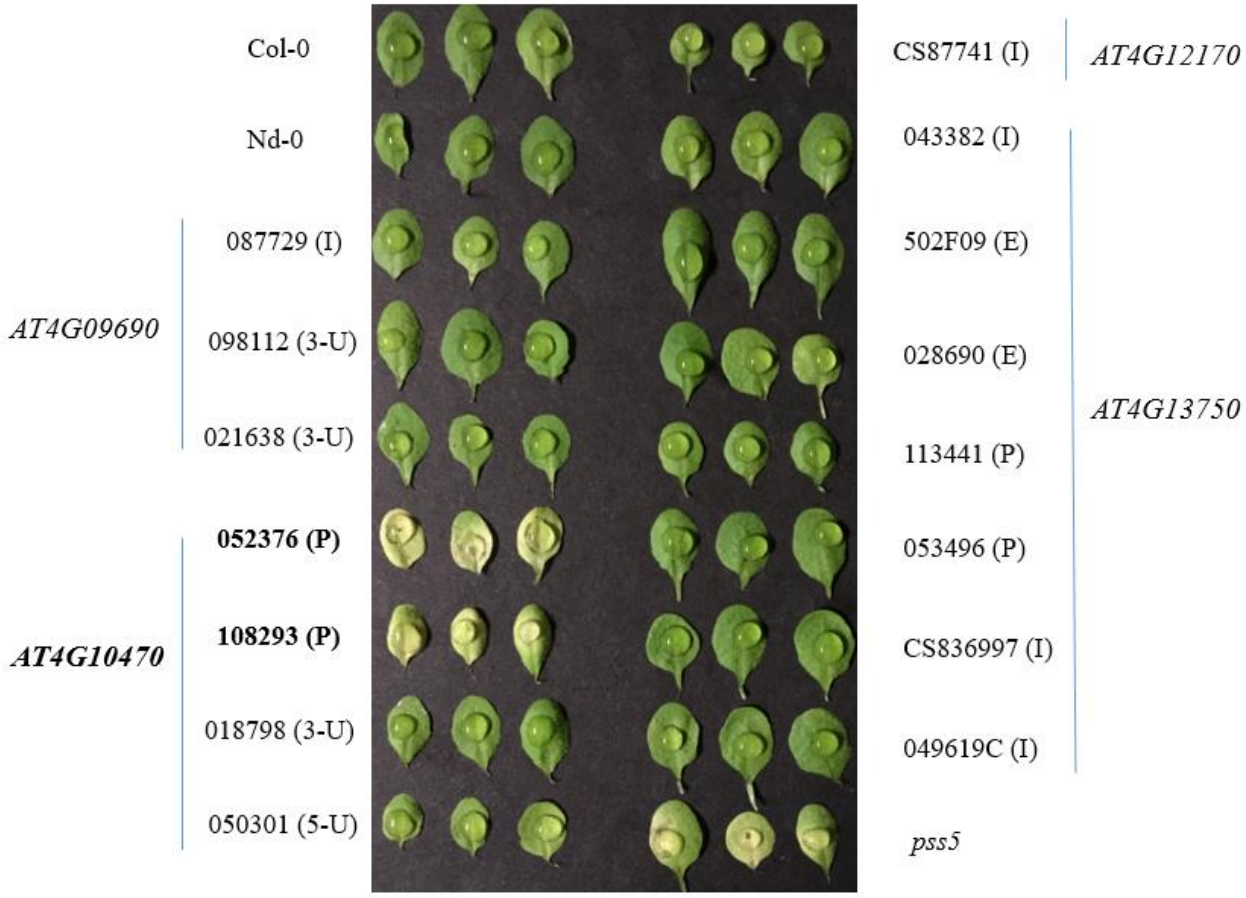

Figure 3. Responses of the Arabidopsis mutant lines carrying T-DNA insertions in candidate *PSS5* genes to *P. sojae*. Leaves of 21-day-old seedlings (three leaves per plant) were inoculated with 20 µl of *P. sojae* zoospore suspensions ($3x10^5$/ml). The Petri dishes were covered and sealed and incubated under constant light (50µE/m^2/s) at 25ºC. The inoculated leaves were scored at 48 and 72 h post-inoculation (hpi) for resistant and susceptible host responses. Mutants (CS052376 and SALK_108293) with two third or more susceptible plants contain T-DNA insertion in the promoter region of the candidate *PSS5* gene, *AT4G10470*.

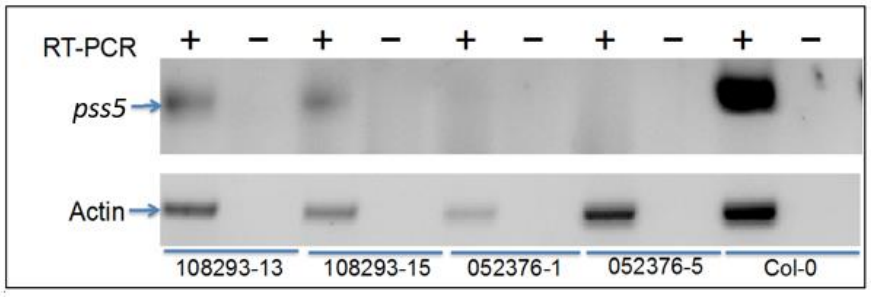

Figure 4. RT-PCR analysis of the T-DNA insertion lines. *AT4G10470* knock-down mutants showing susceptibility to *P. sojae* were investigated for expression of the *PSS5* candidate *AT4G10470* gene. Two mutants, SALK_108293 and CS052376, with T-DNA insertions in the promoter of *AT4G10470* gene showed susceptibility to *P. sojae* and reduced expression levels of transcripts for the *AT4G10470* gene. Constitutively expressed *Actin* gene was used as the internal control.

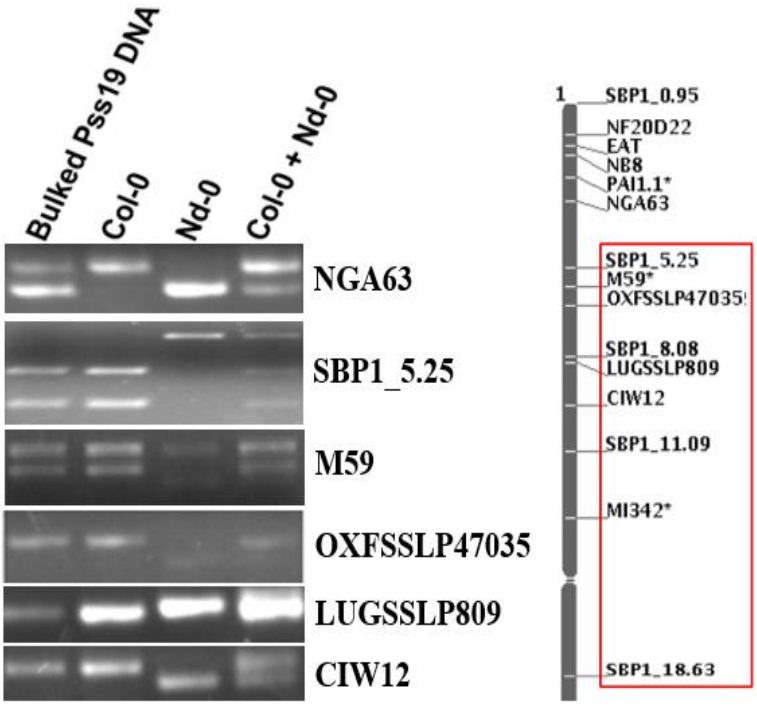

Figure 5. The bulked segregant analysis for the *PSS19* gene. DNA samples of 12 susceptible $F_{2:3}$ families were bulked and used to identify the *PSS19* linked molecular markers. The putative *PSS19* region is shown on the chromosome 1 using a box.

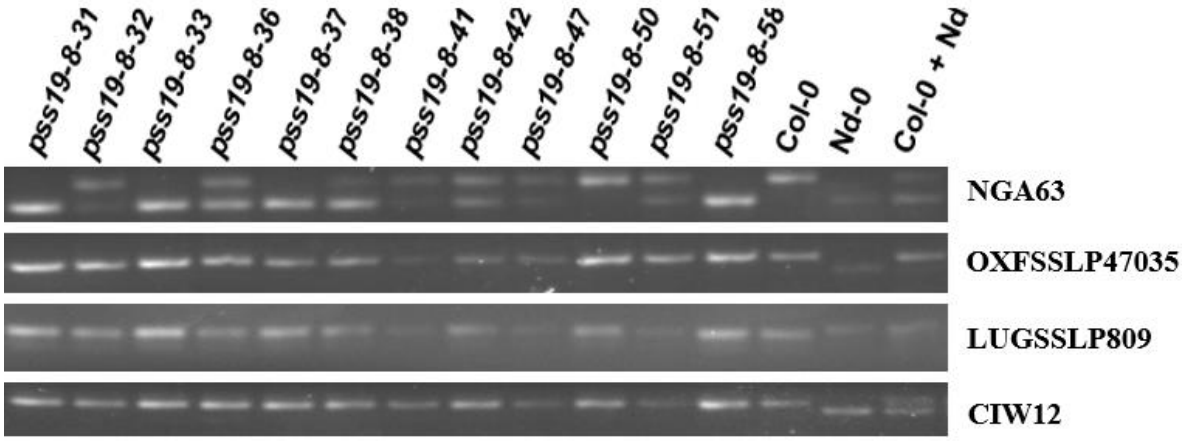

Figure 6 Amplification patterns of molecular markers (SSLP) linked to *PSS19* among the susceptible $F_{2:3}$ families. Controls are: Col-0; Nd-0; and Col-0 + Nd-0, DNA samples of Col-0 and Nd-0 mixed in equal amounts.

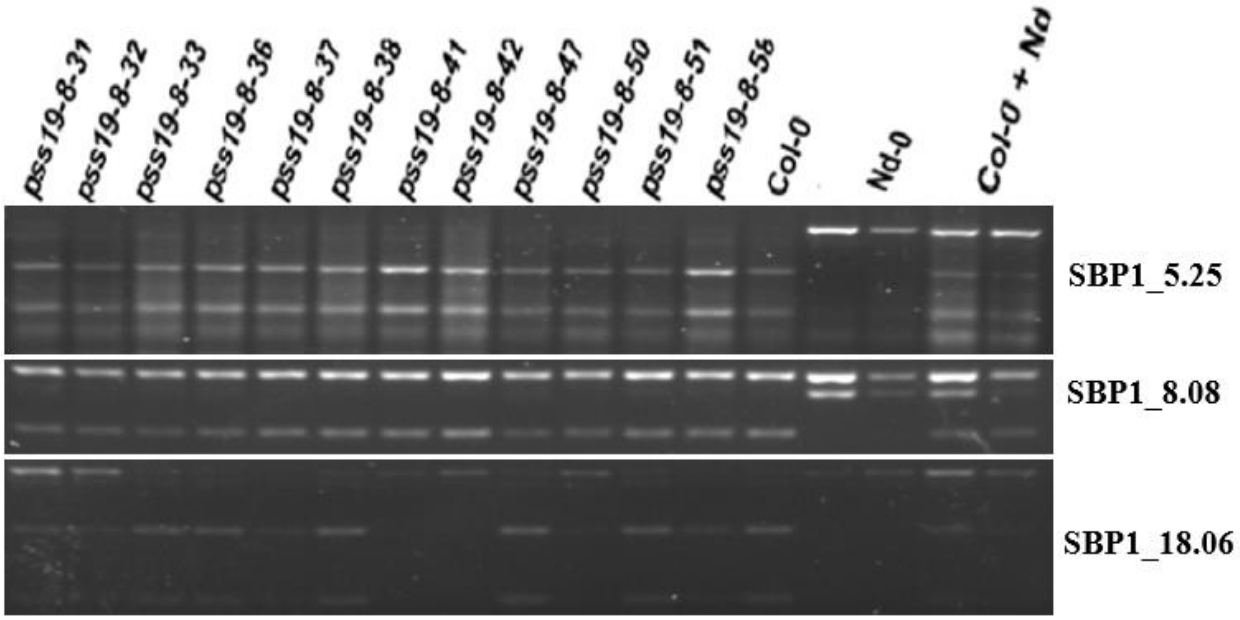

Figure 7. Amplification patterns of sequenced-based polymorphic (SBP) markers linked to *PSS19* among the susceptible $F_{2:3}$ families. Controls are: Col-0; Nd-0; and Col-0 + Nd-0, DNA samples of Col-0 and Nd-0 mixed in equal amounts

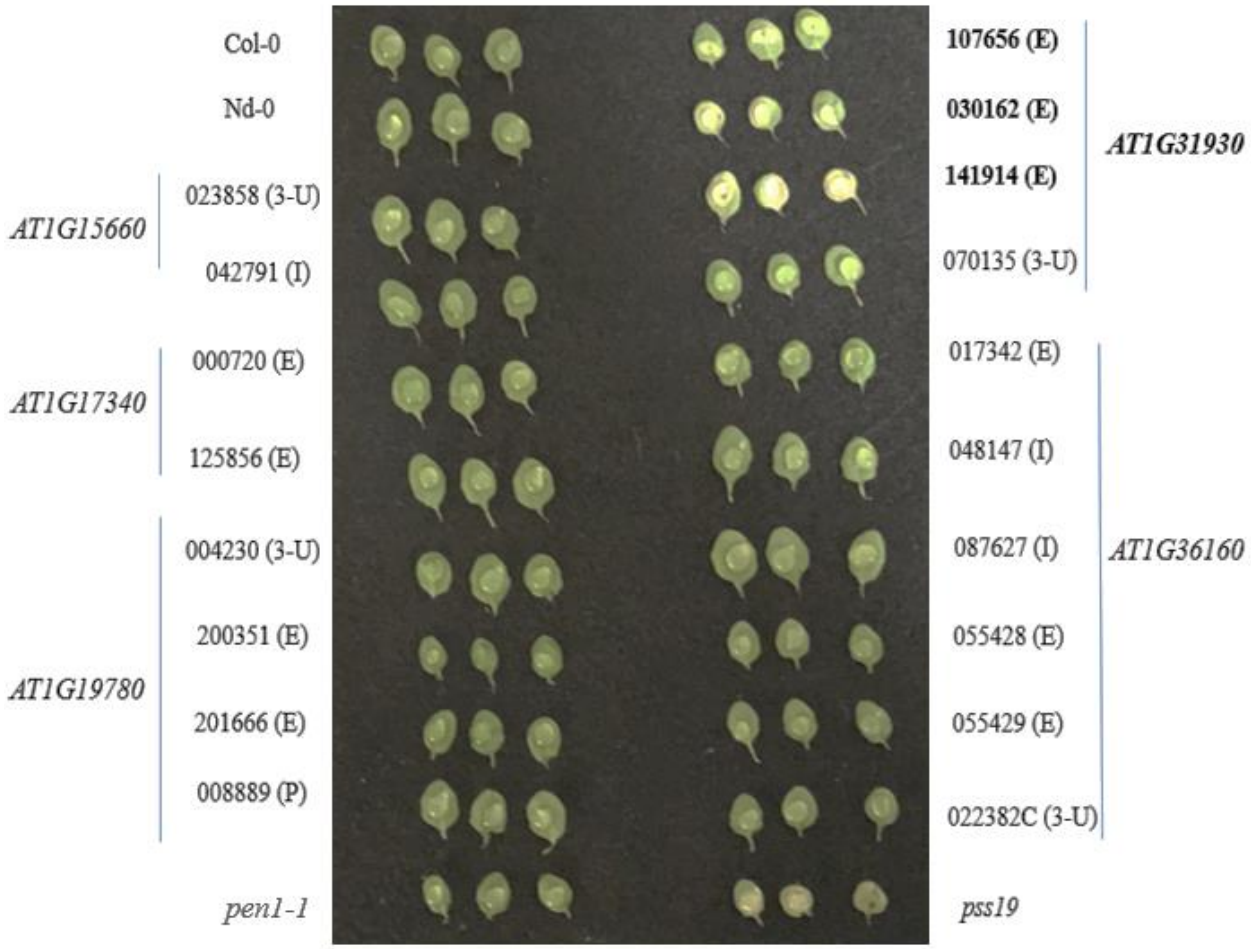

Figure 8. Responses of the Arabidopsis mutant lines carrying T-DNA insertions in candidate *PSS19* genes to *P. sojae*. Leaves of 21-day-old seedlings (three leaves per plant) were inoculated with 25 µl of *P. sojae* zoospore suspensions (spore count $3x10^5$/ml). The Petri dishes were covered and sealed and incubated under constant light (50µE/m^2/s) at 25ºC. The inoculated leaves were scored 48 and 72 h post-inoculation (hpi) for resistant and susceptible host responses. Mutants SALK_107656, SALK_030162 and SALK_141914 containing T-DNA insertions in exons of the *AT1G31930* gene showed susceptibility to *P. sojae*.

CHAPTER 3. CONCLUSIONS

Summary

In this study, I have identified a strong candidate gene for each of the two novel Arabidopsis NHR genes, *PSS5* and *PSS19*. The candidate for the *PSS5* gene encodes an unknown protein and *PSS19* encodes a heterotrimeric guanine nucleotide-binding (G) protein, XLG3 (Extra-large G protein 3). Both *PSS* genes confer nonhost resistance against the soybean oomycete pathogen, *P. sojae* and the fungal pathogen *F. virguliforme* in Arabidopsis.

Chapter two describes the identification of candidates for Arabidopsis *PSS5* and *PSS19* genes. The Bulked segregant analysis (BSA) [45] using three types of molecular markers, single sequence length polymorphism (SSLP) markers, CAPS and sequence based polymorphism (SBP) markers, the *PSS5* gene was mapped to an 8.65-Mb region on the south arm of chromosome 4, and *PSS19* to a 13.38-Mb region the centromeric region of chromosome 1. The map-based cloning of the Arabidopsis *PSS5* gene revealed four candidate *PSS5* genes each carrying one non-synonymous mutation. Following inoculation of the T-DNA insertion SALK mutant lines for each of the four candidate *PSS5* genes with *P. sojae* revealed two Arabidopsis SALK T-DNA insertion knock-down mutants that lack *Phytophthora* resistance. The two mutants carry T-DNA insertions in the promoter region of the *ATG4G10470* gene encoding an unknown protein and they showed down-regulation of the steady transcripts of the *ATG4G10470* gene. The gene was fused separately to three promoters – Promoter-2, Promoter-3 and Ubiquitin-10 in a binary plasmid vector. Promoters 2 and 3 are root- as well as leaf-specific promoters isolated from soybean. Ubiquitin-10 is a constitutively expressed Arabidopsis promoter. Complementation analysis of the *pss5* mutant and one Salk mutant with T-DNA insertion in *ATG4G10470* is in progress.

To identify the candidate *PSS19* genes, the sequence of the *PSS19* region was compared with that of the Col-0 and *pen1-1* genome sequences. We identified 15 point mutations. Six of these mutations were nonsynonymous and unique to the *pss19* mutant and were located in six candidate *PSS19* genes. Twenty-two T-DNA insertion Salk mutants for these six candidate *PSS19* genes were tested for responses to *P. sojae* infection. Three T-DNA mutant lines, SALK_107656, SALK_030162 and SALK_141914, with T-DNA insertion in the exons of *AT1G31930* showed susceptibility to *P. sojae* infection. Therefore, *AT1G31930* is a strong candidate for the *PSS19* gene. It encodes a heterotrimeric guanine nucleotide-binding (G) protein, XLG3 (Extra-large G protein 3) that shows significant similarity to the G protein alpha subunit in its C terminal region involved in the regulation of root morphology and growth.

Future plans

The study was focused on cloning two novel Arabidopsis genes *PSS5* and *PSS19*, which could provide NHR in soybean against its destructive pathogens. We have identified one strong candidate gene for each of these two Arabidopsis NHR genes. We are yet to perform the complementation analysis for both candidate genes to confirm that they are the *PSS* genes. For *PSS5* gene, *P. sojae* infection of the *ATG4G10470*-transformed Arabidopsis plants should determine if *ATG4G10470* can confer immunity against the nonhost pathogen; in other words, if it can complement the lost NHR function in the *pss5* mutant. For the *PSS19* gene, further investigation including cloning, transformation and complementation analyses are required to identify the gene. The strong candidate for the *PSS19* gene encodes an XLG3, a novel protein whose function in plant immunity has not yet been described.

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