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Inheritance, differential expression, and candidate gene analyses for Avr2 in Phytophthora sojae

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Abstract

Phytophthora sojae is an oomycete responsible for seed, root and stem rot of soybean plants. Managing this disease relies on growing soybean cultivars with race-specific resistance (*Rps*) genes that deliver complete host immunity in the presence of corresponding pathogen avirulence (Avr) effector proteins. The aims of this study were to characterize virulence towards *Rps2* among different *P. sojae* strains, track the inheritance of this trait, and attempt to identify an *Avr2* gene. Fifteen *P. sojae* strains were tested for virulence towards *Rps2* and crosses were performed between selected virulent and avirulent strains to follow the inheritance of virulence. Although parental strains were consistent in their virulence phenotype, many progeny were unstable. Of two candidate genes for *Avr2* tested, neither showed co-segregation with the *Rps2*-virulence trait. Overall, results indicate that virulence towards *Rps2* is inherited in a non-Mendelian fashion; the factors responsible could include epistasis, gene conversion, or other epigenetic phenomena.

Key words: *Phytophthora sojae*, avirulence, soybean, resistance, *Rps*, transcript, differential expression, polymorphism, *Avr2*, effector, inheritance, epigenetic, *Avh172*, genetic marker, candidate gene.

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List of Abbreviations

A	Avirulent
AAFC	Agriculture and Agri-Food Canada
ATP	Adenosine Triphosphate
Avr	Avirulence Gene
bp	Base Pair
BSA	Bovine Serum Albumin
BWA	Burrows-Wheeler Alignment
CAP	Cleaved Amplified Polymorphism
cDNA	Complementary Deoxyribonucleic Acid
CHP	Conserved Hypothetical Protein
dEER	Aspartate-Glutamate-Glutamate-Arginine
DESeq	Differentially Expressed Sequence
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylenediaminetetraacetae
ETI	Effector Triggered Immunity
F_1	First Generation
F_2	Second Generation
gDNA	Genomic Deoxyribonucleic Acid
h	Hour(s)
Kb	Kilo Base
L	Litre
LRR	Leucine Rich Repeat

M	Molar
N_2	Nitrogen
NGS	Next Generation Sequencing
Mb	Mega Base
mg	Milligram
min	minute
mL	Millilitre
MLG	Molecular Linkage Group
mM	Milli Molar
MQ	Milli-Q
NBS	Nucleotide Binding Sequence
^oC	Degrees Celsius
Padj	P Adjusted Value
PAMP	Pathogen Associated Molecular Pattern
PCR	Polymerase Chain Reaction
rcf	Relative Centrifugal Force
RE	Restriction enzyme
RFLP	Restriction Fragment Length Polymorphism
R-gene	Resistance Gene
RNA	Ribonucleic Acid
RNA-Seq	RNA sequenced
Rps	Resistance Gene to *Phytophthora sojae*
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
RXLR	Arginine-Any-Lysine-Arginine
SDS	Sodium Dodecyl Sulphate

SDW	Sterile Distilled Water
sec	seconds
SNP	Single Nucleotide Polymorphism
TAE	Tris-Acetate-EDTA
TBE	Tris-Borate-EDTA
V	Virulent
v/v	Volume/Volume
w/v	Weight/Volume
U	Unit
µg	Microgram
µL	Microliter
µM	Micrometer
Unk	Unknown (protein)
v	version

1 Introduction

1.1 Overview of *Phytophthora sojae*

1.1.1 Oomycete phylogeny and the evolution of *P. sojae*

Oomycetes (also known as 'water moulds') of the genus *Phytophthora* contain a large diversity of plant pathogens, many of which are devastating to agricultural settings and natural ecosystems. The genus name *Phytophthora* literally means "plant destroyer" in Greek, coined due to its destructive power on plant species. *Phytophthora sojae* (Kaufmann & Gerdemann, 1958) embodies its etymology by devastating soybean (*Glycine max* (L.) Merr) crops globally, causing seed, root and stem rot (Schmitthenner, 1999). Previously, *P. megasperma* var.*sojae* and *P. megasperma* f.sp. *glycinea* were also names used to describe the species. *Phytophthora sojae* was first documented in North America in the 1950s but its ancestry dates as far back as the Carboniferous period (Dorrance & Martin, 2000). Fossil records indicate that at least some oomycetes began as endophytes, growing endosymbiotically within plants (Tyler, 2007). Other studies assert that oomycetes began as marine parasites of seaweeds, diatoms and crustaceans based on phylogenetic analyses of their most basal lineages which account for only 5% of the species seen today. The majority of oomycetes (60%) are plant pathogens and the evolutionary history of modern taxa reveals that the shift towards plant parasitism evolved as a convergent trait in at least three different oomycete lineages (Kamoun et al., 2015)

Historically, *Phytophthora* and related genera were thought to reside within the kingdom Fungi based on their similarities in appearance, lifestyle and pathogenesis. A major similarity is their osmotrophic and filamentous lifestyle which is originally why taxonomists confused them with fungi. In addition, they also share presumably convergent traits like hyphal production and asexual dispersal spores which make them morphologically difficult to distinguish and classify. A major physical distinction, however, is the biflagellate (whiplash and tinsel) motile spores of the oomycetes. Modern molecular and biochemical analyses places the phylogenetic affiliation of oomycetes within the kingdom Stramenopila (Heterokonts) alongside diatoms and brown algae (Figure 1) (Thines & Kamoun, 2010; Tyler, 2007).

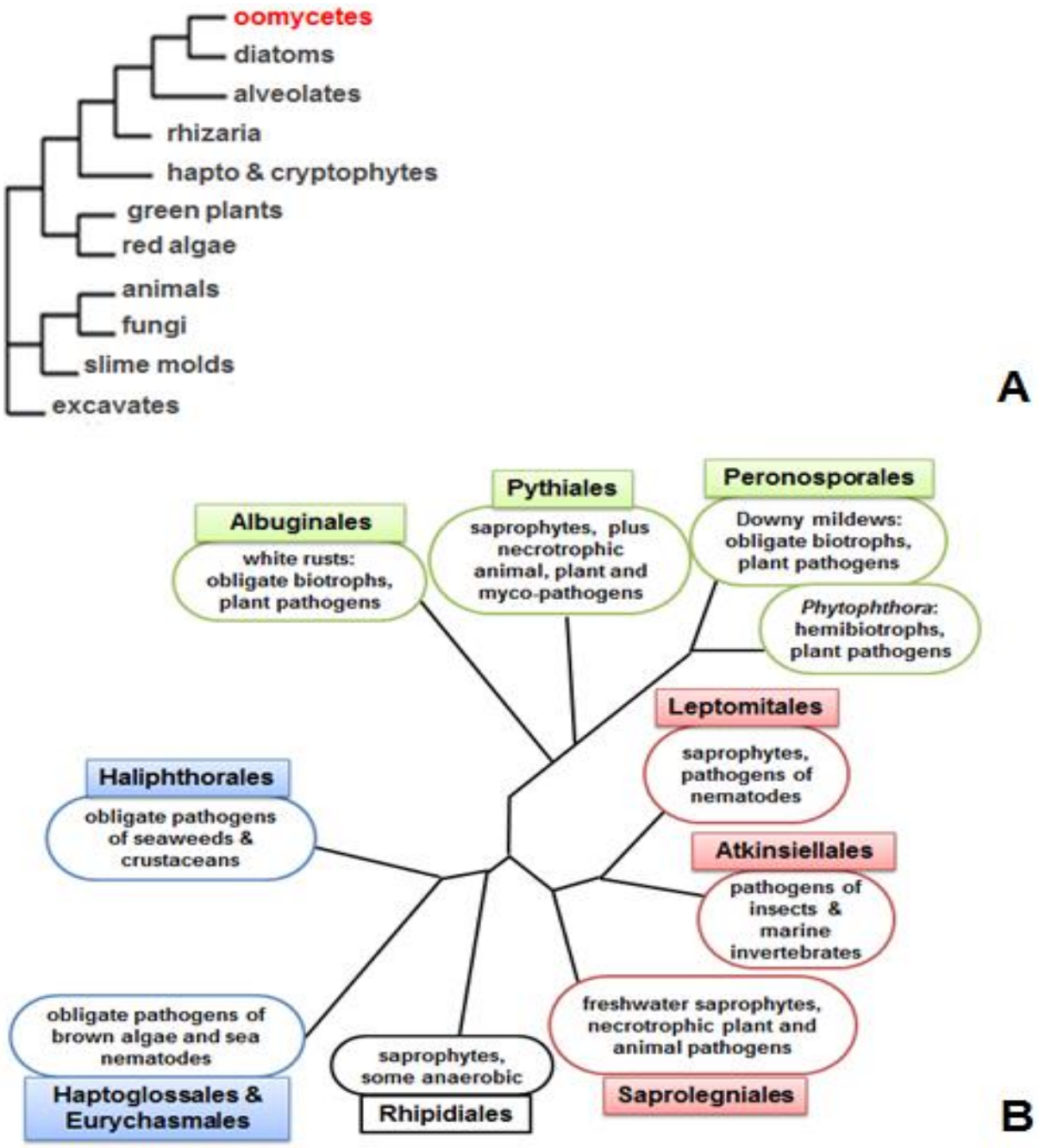

Figure 1: Phylogenetic relationships of oomycetes.

(A) A phylogram depicting a modern hypothetical evolutionary association of major eukaryotic kingdoms including the relative position of oomycetes. (B) Major orders and lifestyles of oomycetes based on ribosomal RNA profiles. The Peronosporalean group coloured green (~1,000 species, including *Phytophthora* species alongside downy mildews), the Saprolegnians (~500 species) coloured green, and the basal clades coloured blue are illustrated. Adapted from Judelson (2012).

Currently, one may find *Phytophthora* species in the Peronsosporales although the phylogenetic relationships among species within the genus are still being studied (Martin et al., 2014). In this group, biotrophy is thought to have evolved gradually. The development from opportunistic plant pathogens to the hemibiotrophic lifestyle (seen in many *Phytophthora* species today), and ultimately to obligate biotrophy is thought to have occurred in sequence. Unlike obligate biotrophs, *Phytophthora* species switch to a necrotrophic phase by deploying degradative enzymes and toxins to utilize dead host tissue for nutrition after surviving as biotrophs in the initial stages of infection (Tyler et al., 2006).

Due to the agro-economic importance of *Phytophthora* species in particular, there has been a rising interest in their genomics and molecular biology as well as obtaining phenotypic and genotypic data of species across the genus (Blair et al., 2008; Cooke et al., 2000). Description of species within the genus *Phytophthora* has steadily increased over the past 15 years providing at least 124 described plant pathogen species; the number expected to increase in the future due to the many provisional species named today. So far, three *Phytophthora* species have been fully sequenced and annotated including *P. sojae, P. ramorum* and *P. infestans* (Govers & Gijzen, 2006; Martens & Van de Peer, 2010) and whole genome sequence data are available for many others.

A combination of available DNA sequence data for multiple loci as well as the relatively low cost for producing such data and analyses have assisted researchers in differentiating Oomycete isolates and delineating species. Other notable genera and close relatives of *Phytophthora* within the family Pythiaceae include *Pythium* (plant and mammal parasites), *Peronospora* (plant parasite) and *Albugo* ('white rust' plant parasite) (Tyler & Gijzen, 2014). In a recent study of genus-wide *Phytophthora* phylogeny, Martin et al. (2014) analyzed mitochondrial and nuclear gene (separately and combined) datasets to observe relationships between species and produce a congruent tree topology for the genus. With a significantly high degree of support, the authors assign *P. sojae* and *P. cinnamoni var. robiniae, P. sojae* and *P. sp. niederhauserii, P. sojae* and *P. sp. niederhauserii* as sister species using mitochondrial, nuclear, and combined genes respectively.

1.1.2 Symptoms of infection

Phytophthora sojae is an agro-economically important plant pathogen responsible for stem and root rot in soybean. It can also infect members of the genus *Lupinus* but is only economically relevant to soybean (Erwin & Ribeiro, 1996; Kamoun et al., 2015; Tyler, 2007). *Phytophthora sojae* infects soybeans at any stage in growth or reproductive development (seed, seedling, or adult) and is usually promoted by wet or flooded soil conditions with poor drainage – hence its' classification as a "water mould" (Hamm et al., 1990). It is a soil-borne, diploid hemibiotroph that begins the infection process in the roots and eventually spreads to produce fatal cankers throughout the roots and up to the stem of the plant (Kamoun et al., 2015; Schmitthenner, 1999). Once the host tissue is penetrated and colonization begins, severe taproot and lateral root decay ensues along with girdling stem lesions causing the roots and stem to turn brown, in-between leaf veins to yellow, and chlorosis and necrosis to occur on basal leaves. Eventually, the entire plant suffers severe wilting and chlorotic leaves as they wither due to compromised roots and stems. These areas eventually turn orange-brown as the host dies – usually with the leaves still attached (Dorrance et al., 2007; Schmitthenner, 1999). Other symptoms include seed rot and pre-/post-emergence damping off of germinating seedlings when soil conditions favor moisture and warmth (25 to30°C). As the seedling emerges, light brown soft rot can be seen on the roots and hypocotyls and they die shortly thereafter (Tyler, 2007).

Soybean cultivars with a high level of partial resistance show a lesser degree of discoloration on the stem but may also display stunted growth, whereas moderately-resistant cultivars additionally display characteristic stem lesions that are brown, sunken and narrow, progressing up the stem before wilting. Mature cultivars that are highly tolerant display less obvious symptoms which generally include secondary root rot and discoloration of the taproot. Since the initial signs of infection are limited to slight chlorosis, wilting and stunting, it is easily mistaken for nitrogen deficiency, soil compaction or other root diseases. Nonetheless, mild symptoms of infection have the potential to reduce crop yields up to 40 percent (Schmitthenner, 1999). In laboratory settings conditioning optimal light, temperature and humidity, direct inoculation with *P. sojae* mycelia into the soybean stem causes disease symptoms to appear within three days (Figure 2).

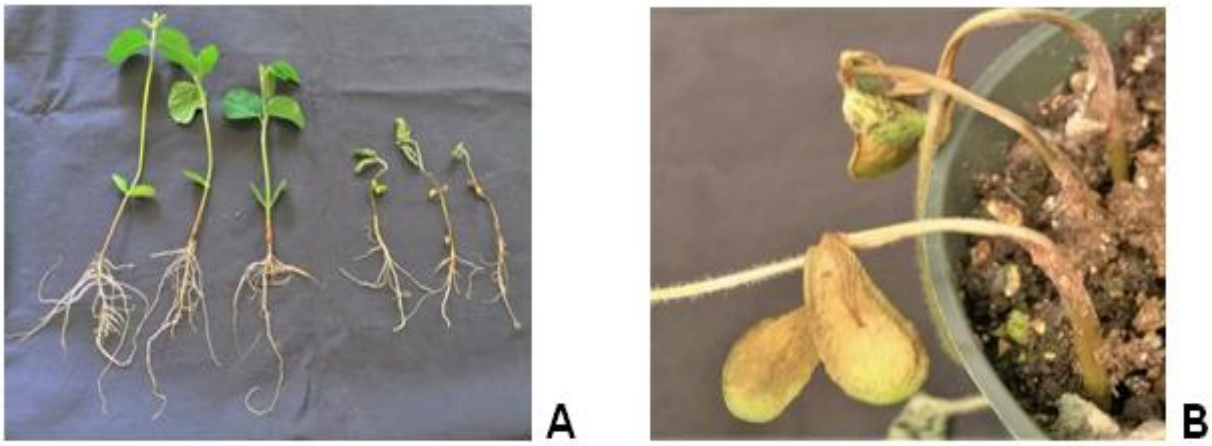

Figure 2: Symptoms of *P. sojae* infection on soybean

(A) Disease outcomes of laboratory inoculations on 3 resistant (left) and 3 susceptible soybean *Rps2* cultivars (right) at one week old. (B) A closer look at disease symptoms on a susceptible (*rps2*) soybean stems at one week old. Symptoms illustrated begin to show three days post-inoculation.

1.1.3 Lifecycle and morphology

Phytophthora sojae produces several types of spores after a period of vegetative growth, all of which play significant roles in its life and disease cycle (

Figure 3). In laboratory settings, *P. sojae* can be revived and cultured continuously based solely on hyphal growth however, in nature, the continuous production of spores is crucial to its survival. *Phytophthora sojae* cannot sustain itself as a saprophyte and must transition to a new host as its colonized plant begins to die (Judelson & Blanco, 2005)

In addition to oospores that are produced through sexual reproduction, there are two types of asexual spores in *P. sojae*: zoospores and chlamydospores (Judelson & Blanco, 2005; Tyler, 2007). The oospores and chlamydospores are resting spores and are useful for survival in between seasons (Schmitthenner, 1999). *P. sojae* is homothallic, containing both male

(antheridia) and female (oogonia) reproductive structures that are self-compatible. Oospores develop after an antheridium fertilizes an oogonium. Oospores (20 to 50 µm) are spherical, have thick inner and outer cell walls and can survive for long periods in the soil (Figure 4). During germination, the inner wall is absorbed and a germ tube is formed differentiating into either a sporangium or mycelium. Germination of oospores is non-synchronous and may not happen even if the external conditions are favorable. In the laboratory, the germination process can last from 2 to 30 days after separation from the mycelium (Bhat, 1993; Schmitthenner, 1999).

The zoospores are released from sporangia during wet conditions. Zoospores are wall-less and have two flagella to aid their dispersal in water. Once the sporangial papilla dissolves, zoospores are expelled from the sporangium via turgor pressure. Sporangia can produce 10 to 30 zoospores each. The zoospores are carried by water movements and can swim using their biflagellate motor consisting of a whiplash (push) and a tinsel (pull) flagella (Judelson & Blanco, 2005). *Phytophthora sojae* zoospores are attracted to isoflavonoid compounds exuded by soybean roots, and chemotactically travel through the water towards the host where they lose their flagella and encyst on the root surface (Morris et al., 1998; Tyler, 2007). Germination occurs immediately after encystment in which a germ tube extends on the root surface and swells to form an appressorium. With the use of turgor pressure and cell- wall degrading enzymes, the infection peg on the appressorium penetrates the epidermis allowing hyphae to travel through the opening and in between the living plant cells. These infection hyphae produce haustoria which penetrate the cell wall (but surrounded by the plasma membrane) of host cells to absorb nutrients (Judelson & Blanco, 2005; Kamoun et al., 2015; Latijnhouwers et al., 2003)

Phytophthora sojae mycelium optimally grows between 25- 27 °C and is coenocytic, aseptate and colorless, characteristic with its Oomycota lineage. However, it does not have characteristic culture morphology; growth patterns can vary widely between strains (Figure 5). Branching (90 degree angle) and curling of hyphae can occur during mycelial formation (Schmitthenner, 1999).

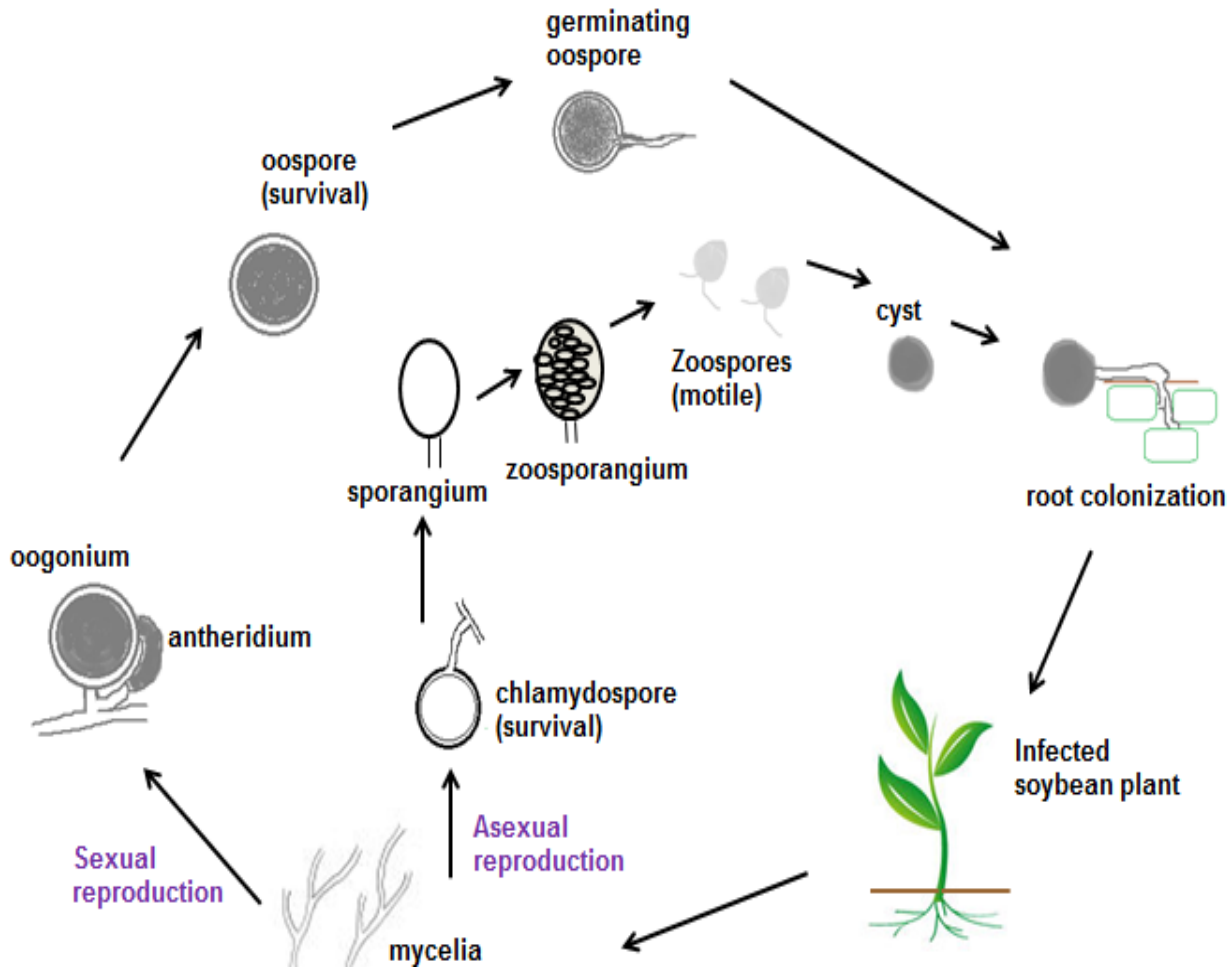

Figure 3: Lifecycle of *P. sojae*. Adapted from Tyler (2007).

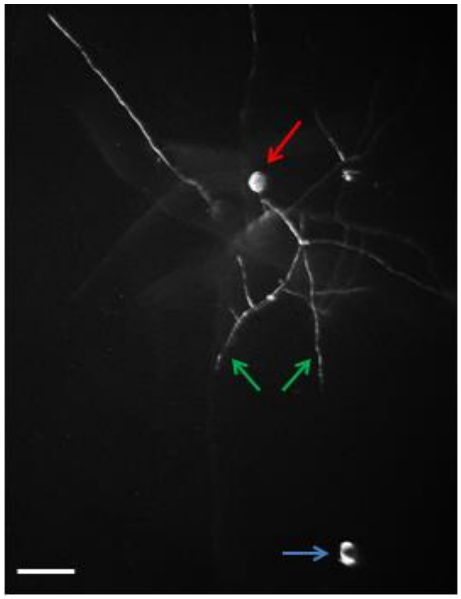

Figure 4: Germinated oospore under laboratory conditions.

Oospores obtained from a *P. sojae* race 2 x race 7 genetic cross with a maturation period of 30 days. After isolation from the crossing media, oospores were grown on 1.5 (w/v) water agar substituted with β-sitosterol and rifampicin (10 µg/µL) at 26°C. Image was taken after four days using a stereomicroscope (60X). Blue arrow = ungerminated oospore; green arrow = hyphae; red arrow = germinated oospore. Scale bar represents 100 µm.

Figure 5: Culture morphologies of *P. sojae* strains.

Left = race 7; right = race 2. Cultures were grown on regular V8 media (see Table 2) for seven days at 26°C.

1.2 Agricultural aspects of *P. sojae*

1.2.1 Economical impact

Soybean, originally from Asia, is the largest oilseed crop produced and consumed in the world (Schmitthenner, 1999). Although 50 countries now grow soybean, the United States remains the top producer (Schmitthenner, 1999; Wilcox, 2004). Canada exports nearly $2 billion worth of soybeans annually and is a significant contender in the global soybean market. On average, Canada produces five to six million metric tonnes of soybean per year and Ontario is the provincial leader producing over half the crop. Over the past 10 years, Canadian soybean production has increased by 98.71 percent (Soy Canada, 2015). The strong global market for soybeans increases the necessity and for sustainable production practices and for controlling the spread of disease.

Phytophthora sojae poses a major threat to soybean production in exporter countries around the world (Schmitthenner, 1999; Sugimoto et al., 2012). On a global scale, the soybean pathogen causes around $1 to 2 billion in crop losses and control measures per annum (Dong, Yu, et al., 2011; Tyler, 2007) and an estimated $200 million in North America alone (Erwin & Ribeiro, 1996; Lin et al., 2014). *Phytophthora* root-rot was first reported in Canada in 1954 and spread throughout Southwestern Ontario during the 1950s and 1960s to susceptible soybean cultivars (Hildebrand, 1959; Xue et al., 2015). Today, *P. sojae* costs Canadians $50 million in crop loss per year. Annual surveys in Canada and the US show that despite resistance measures, *P. sojae* remains widespread which suggest that the virulence profiles of prominent strains have adapted since their first discovery to defeat host resistance genes (Xue et al., 2015).

1.2.2 Control measures

Phytophthora sojae and other *Phytophthora* root rots are a challenge to agricultural fields because they are difficult to control (Schmitthenner, 1985). So far, the use of race-specific resistant cultivars has been the most cost-efficient and successful approach to control the spread of *P. sojae,* namely due to the qualitative immune response activated by the host (Dorrance et al., 2007; Erwin & Ribeiro, 1996; Qutob, 2000; Xiao et al., 2002). Soybean resistance genes to *P. sojae* are referred to as *Rps* genes. To effectively utilize *Rps* gene-mediated resistance, it is

imperative soybean growers accurately know which *P. sojae* races are present in the field so that a cultivar with the corresponding resistance gene for the pathotype can be planted (Tyler, 2007; Xiao et al., 2002). Repeatedly using one specific cultivar also promotes the emergence of new *P. sojae* races which overcome currently resistant cultivars (Förster et al.,1994; McDowell & Woffenden, 2003; Xiao et al., 2002). Schmitthenner (1999) reports that new *P. sojae* strains appear within 8 to 10 years to defeat cultivars that are widely used, which is an especially dangerous outcome if that cultivar contains several *Rps* genes. Therefore, aside from cultivar breeding efforts to exploit this resistance-gene-mediated defence, other cultural and chemical approaches also need to be employed to tackle the disease. Increasing and improving soil drainage, rotating crops and reducing soil compaction are also effective ways to control the spread of the soil-borne, water- mobile (Erwin & Ribeiro, 1996; Schmitthenner, 1999). However, crop rotation may only have a minor effect on disease management as oospores overwinter and can survive without a host in the soil for years (Xiao et al., 2002). In addition, chemical applications are often used to reduce the spread of the pathogen. The systemic fungicide metalaxyl, which is specific for oomycetes, is used as either a seed treatment for disease tolerant cultivars (Apron) or an in-furrow soil spray (Ridomil). Note that metalaxyl treatment applied to the soil rather than the seed is a more durable form of protection and disease control because it is directly absorbed by the roots which is the entry point for disease spreading zoospores (Schmitthenner, 1999). Other fungicides including ETMT and pyroxyfur have also aided in *P. sojae* disease control, but the overall cost for maintaining and applying fungicide treatments to field crops is steep. In addition, fungicides like metalaxyl have been known to decrease in effectiveness due to tolerance developed by the root rot pathogen. Therefore, though breeding race-specific resistant cultivars is a cost-efficient and widely popular disease control practice, an integrated approach including chemical and physical measures may further aid in controlling the spread of *P. sojae* (Kaitany, 2001; Xiao et al., 2002).

1.3 Genetics of plant-host relationship

1.3.1 Avirulence (*Avr*) and resistance (*R*) gene characterization

Members of the *Phytopthora* genus have been considered some of the most destructive and devastating plant pathogens in the world, especially to dicot plants (Erwin & Ribeiro, 1996). Previously, little attention was given to oomycetes at the molecular level, despite their unique evolutionary history and high economic importance. However, with the development of advanced molecular analysis tools and a heightened interest in these species, researchers have put that trend to rest (Bos et al., 2003). Genome sequencing of *P. sojae*, a model species for the *Phytophthora* genus, predicts 26,584 genes encoded in a genome of 95 Mb (Tyler & Gijzen, 2014).

By its simplest definition, a plant pathogen avirulence (*Avr*) gene encodes for a protein that is targeted by a host plant containing the corresponding resistance gene, regardless of its function or pathogenic capabilities. For host plants and phytopathogenic bacteria or eukaryotes, the interaction between the host resistance (*R*) gene and pathogen *Avr* gene triggers a cascade of strong immuno-defense responses that arrests the spread the pathogen, called the hypersensitive response (de Wit, 1994; Laugé & De Wit, 1998). Avirulence gene products targeted by *R* genes are called effector proteins. There are large and diverse types of effectors produced by eukaryotic and bacterial plant pathogens but relatively few are known to interact with host *R* genes and thereby named *Avr* genes (Rietman et al., 2010). Many of these effector-encoding genes have the capability of becoming *Avr* genes if a matching *R* genes can be identified in the host plant (Luderer & Joosten, 2001).

In *P. sojae*, there are over 350 avirulence homolog genes (*Avh* genes) that encode predicted RXLR effector proteins. These effectors proteins possess a conserved N-terminal RXLR (Arg-anyaminoacid-Leu-Arg) and dEER (Asp-Glu-Glu-Arg) motif downstream from a signal peptide (Qutob et al., 2009; Vleeshouwers & Oliver, 2014; Q. Wang et al., 2011) (Figure 6). Previous *Avr* gene pursuits in *P. sojae* have utilized *Avh* genes as candidates, with some success. Although many plant *R* genes (termed *Rps* genes with respect to soybean resistance against *P. sojae*) have been cloned, few soybean *Rps* genes have been identified at the molecular level. Most known plant *R* genes encode for receptor proteins having nucleotide binding sites characterized by

leucine-rich repeats (NBS-LRR) that occur at an intracellular location, and soybean *Rps* genes are assumed to be similar (Chen et al., 2012; McDowell & Woffenden, 2003; Shan et al., 2004). This suggest that for avirulence to occur, phytopathogens must translocate Avr proteins into plant cells where they interact with cytoplasmic R-proteins (Bonas & Lahaye, 2002). The LRR domain, which in some cases is believed to account for R-protein specificity towards the *Avr* gene product, is the most variable region amongst resistance genes (DeYoung & Innes, 2006). Like *Avh* genes, hundreds genes encoding NB-LRR proteins are predicted to exist in a given plant species. The R-proteins are immune receptors and adaptable surveillance proteins that detect rapidly evolving pathogen effectors in a co-evolutionary tug-of- war (Van Der Biezen & Jones, 1998).

The RXLR effector proteins in oomycetes constitute a large and highly diverse super-family of secreted proteins (Jiang et al., 2008; Tyler et al., 2006). This RXLR motif has played a critical role in the bioinformatic identification of candidate *Avr* genes from *Phytophthora* species, *P. sojae* included (Anderson et al., 2015). In addition, it resembles a host-targeting signal in *Plasmodium* species that is required for entry into the host cytoplasm. Although the function of the RXLR-dEER motif is not known for certain, some studies suggest that it aids in the translocation of the effectors into the host cells (Dou et al., 2008; Grouffaud et al., 2010; Tyler et al., 2006). Conservation of this motif was first observed in whole-genome analyses in *P. sojae* and *P. ramorum* using *Avr1b-1* as query to interrogate predicted proteins. This re-iterative search resulted in the identification of a large number of predicted proteins that were named *Avh* genes. Aside from the signal peptide, RXLR and dEER motifs, more variable regions called K, W, L and Y motifs are also present in many effector proteins of this class and are thought to serve important functional roles (Jiang et al., 2008; Qutob et al., 2009). In the absence of a host immune receptor encoded by a corresponding *R*-gene, effector proteins serve virulence functions that contribute to pathogen fitness. These specialized proteins modulate the host physiology to support colonization. They include but are not limited to inhibitors of the plant immune response, toxins, proteases and other degradation enzymes (Gijzen et al., 2014; Judelson, 2012). Though their mode of entry into the cell is unknown, studies predict that the RXLR and/or C-terminal domain may contribute to lipid binding on the host cell membrane, aiding in effector translocation mentioned above (Dou et al., 2008; Grouffaud et al., 2010; Na et al., 2013; Sun et

al., 2013) The C-terminus has also been reported to be highly polymorphic in Avr proteins, a feature that enables strains to overcome resistance to particular *Rps* genes (Dou et al., 2008)

To date, there are 15 described soybean *Rps* genes that defend against *P. sojae* infections. The *Rps1* and *Rps3* loci possess multiple specificities that behave genetically as alleles. Many more *Rps* genes are predicted to exist in soybean germplasms but await further characterization (MacGregor et al., 2002; May et al., 2002; Sandhu et al., 2005; Tyler & Gijzen, 2014; Tyler, 2002; Whisson et al., 2004). Molecular markers that link each of the *P.sojae* resistance genes have been reported and some genes (*Rps1*, *Rps2*, *Rps3*, *Rps4*, *Rps5*, *Rps6*, *Rps7*, and *Rps8)* have association to molecular linkage groups (MLGs) (Demirbas et al., 2001; Sandhu et al., 2005). The *Rps2* region is part of a MLG called "J" and includes at least three tightly linked genes: *Rps2* responsible for resistance to *Phytophthora* infections, *Rmd-c* for resistance against soybean powdery mildew (*Erysiphe diffusa*) and a gene controlling nodulation (Graham et al., 2002; MacGregor et al., 2002; Sandhu et al., 2005).

Corresponding to soybean resistance genes, there are predicted to be 15 *Avr* genes in *P. sojae*. So far, 11 of the *P. sojae Avr* genes have been identified and named based on recognition by their *Rps* gene counterparts; these are *Avr1a, Avr1b, Avr1c Avr1d, Avr1k, Avr3a, Avr3b, Avr3c, Avr4, Avr5* and *Avr6.* Clustering and genetic linkage of *Avr* genes is seen in the cases of *Avr1b/1k, Avr4/6, Avr3a/5 and Avr1a/1c* (Dong et al., 2009; Dong, Yin, et al., 2011; Dong, Yu, et al., 2011; Dou et al., 2010; MacGregor et al., 2002; Na et al., 2014; Shan et al., 2004; Song et al., 2013).

Figure 6: A schematic of *P. sojae* effector proteins.
Each effector is composed of a signal peptide followed by downstream amino acid RXLR and dEER motifs. The carboxy-terminal effector domain is highly variable and may contain one or more W/L/K/Y motifs that may form a single or multiple subunit domains. Adapted from Raffaele & Kamoun (2012).

1.3.2 Inheritance of *Avr* genes

Genetic analysis of (a)virulence in *P. sojae* is challenging because this organism is homothallic (capable of self-fertilizing), diploid and experiences non-synchronous germination of its sexual oospores which can impede progeny isolation (Gijzen et al., 1996; MacGregor et al., 2002).The use of molecular markers to detect outcrossing between different genotypes has been beneficial in genetic studies of *P. sojae* virulence (Qutob et al., 2013; Whisson et al., 1995). The ability to detect F_1 hybrids derived from divergent parental strains and subsequently obtain F_2 progeny as a mapping population has enabled researchers to identify *P. sojae* avirulence genes on the basis of DNA marker recombination and co-segregation frequencies with avirulent phenotypes (Gijzen et al., 1996; May et al., 2002). Genetic crosses of *P. sojae* strains indicate that *Avr1a, 1b, 1k, 3a, 4, 5, 6* can act as single dominant alleles , suggesting that avirulence is a simple dominant trait and a should segregate as such in F_1 and F_2 populations in a 1:0 and 3:1 ratio of avirulent: virulent phenotypic, respectively, as per Mendelian law (Figure 7) (Gijzen et al., 1996; May et al., 2002; Tyler et al., 1995; Whisson et al., 1995). However, virulence phenotypes and DNA markers in progeny of *P. sojae* genetic crosses often do not follow Mendelian patterns which suggests that the genetic background of the various strains that are crossed can affect the segregation ratios (May et al., 2002; Qutob et al., 2013).

Cleaved amplified polymorphic sequence (CAPs) markers, an extension of the widely used restriction fragment length polymorphic (RFLP) genetic markers, are DNA markers that are useful to distinguish hybrid progeny in genetic crosses between *P. sojae* genotypes (Dong et al., 2009; MacGregor et al., 2002; Na et al., 2014; Na et al., 2013; Tyler et al., 1995). A co-dominant CAPs marker is the amplification of a short DNA sequence (via PCR) containing a restriction enzyme (RE) cut site that is either present or absent in genetically different strains. This type of molecular marker (co-dominant) can differentiate heterozygous and homozygous genetic patterns. The resulting fragment lengths can then be visualized and differentiated via gel electrophoresis. As opposed of RFLP analyses, this method is relatively quicker allowing for larger populations to be scored. It also does not involve the use of radioactivity or time-consuming DNA blotting procedures and thus allows large mapping populations to be scored quickly using only small quantities of DNA (Jarvis, 1994; Konieczny & Ausubel, 1993). In this study, CAPs markers are used to distinguish outcrossed hybrids from self-fertilized parental

strains and to determine if candidate gene markers co-segregate with an avirulent phenotype in F_1 and F_2 progeny.

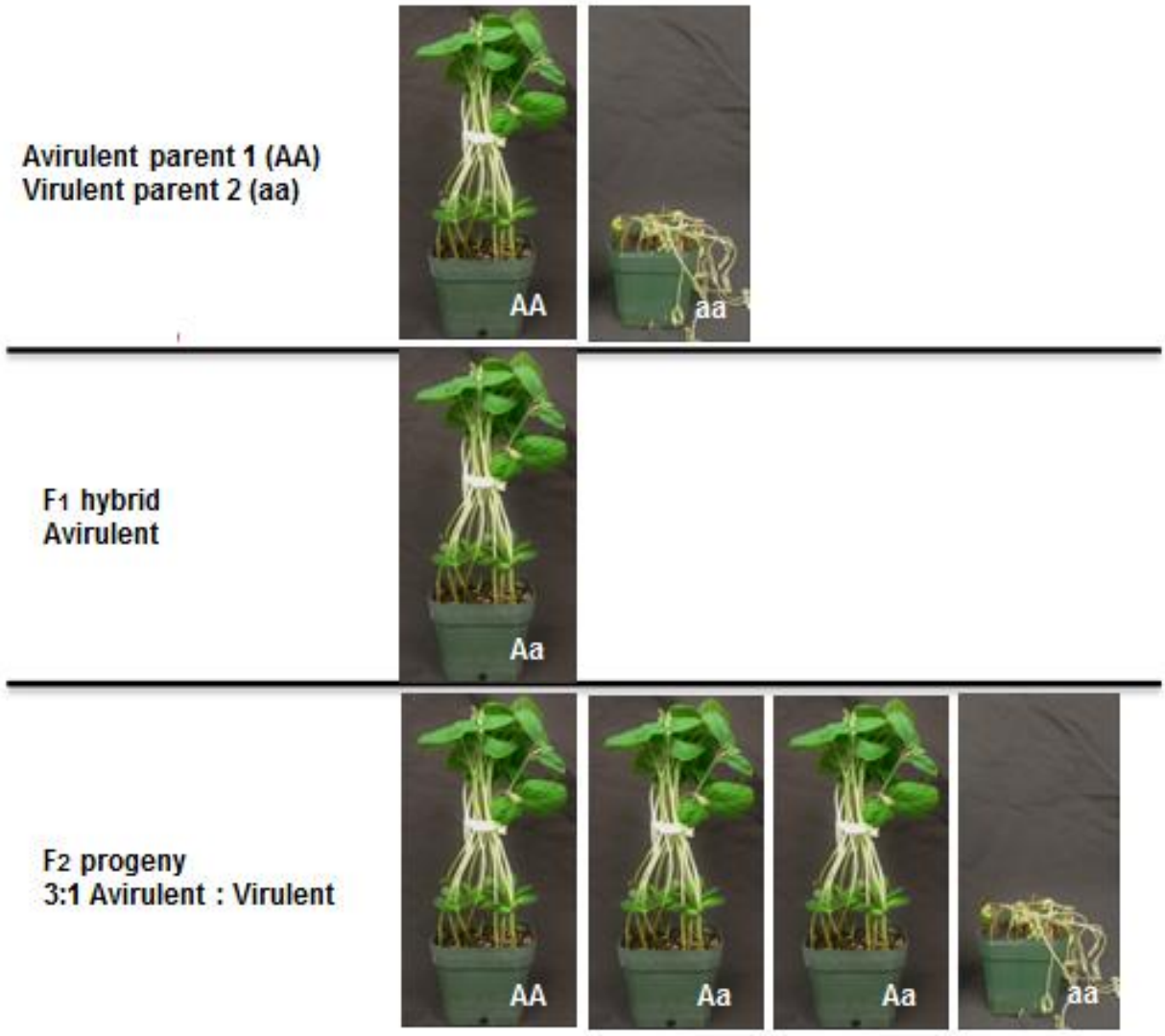

Figure 7: Expected Mendelian inheritance of an avirulence gene in a genetic cross. Outcrossing of avirulent (AA) and virulent (aa) homozygous *P. sojae* parent strains results in the *Avr* gene (A) segregating as a single dominant allele. The F_1 progeny are heterozygous and express the dominant avirulent allele. The F_2 progeny, acquired from self-fertilization of F_1 hybrids, display a 3:1 avirulent to virulent phenotypic ratio which corresponds to the segregation of the dominant trait. Photographs of virulent and avirulent *P. sojae* interactions are representative images of virulence outcomes; not actual images from a *P. sojae* outcross.

1.3.3 Gene-for-gene and co-evolutionary relationship between pathogen effectors and plant resistance genes

The interdependence between host-plant immunity and pathogen virulence is historically known as the gene-for-gene relationship. First described by H.H. Flor in 1956 while studying flax rust disease resistance, this model laid the foundation for understanding effector and receptor specificity in disease resistance and is widely applied to phytopathogenic studies today (Bos et al., 2003; Cui et al., 2015; de Wit, 1992; Stukenbrock & McDonald, 2009). The gene-for-gene model implies that for each resistance gene (*R*) in the host there is a corresponding avirulence gene (*Avr*) in the pathogen. The interaction of *R* and *Avr* gene products result in plant resistance to disease, through a process that is now known as effector triggered immunity (ETI). The ETI is usually accompanied by a hypersensitive response (HR) that helps to immediately stop pathogen growth (Bos et al., 2003; Cui et al., 2015; Flor, 1971; MacGregor et al., 2002). Activation of the host HR via effector recognition triggers a cascade of immune responses involving an influx of reactive oxygen species (ROS), ion fluxes across the plasma membrane (increase in calcium production), MAPK movements, transcriptional activation of defense gene and the production of secondary metabolites (Cui et al., 2015). In the classic gene-for-gene model, pathogen *Avr* and host *R* genes are dominant alleles where both must be present for resistance to occur (Flor, 1971).

Exactly how the pathogen effector is recognized by host immune receptors is variable and depends on the system under study (Bonas & Lahaye, 2002; Bos et al., 2003) A direct ligand-receptor recognition between the effector and receptor can occur. However, indirect relationships involving a co-receptor as the *Avr* target, with the R-protein immune receptor "guarding" a protein with a high-affinity binding site for effector, or binding to digestion products from *Avr* activity, have also been proposed (Bonas & Lahaye, 2002; Luderer & Joosten, 2001; van der Hoorn & Kamoun, 2008)

Gain-of-virulence changes to *Avr* genes dramatically increase pathogen fitness towards plants carrying the corresponding *R*-gene. Effectors contribute to virulence by inhibiting plant defenses but can inadvertently 'trip the wire' activating ETI when they are recognized by an immune receptors. Specific RXLR effectors can therefore evolve to evade detection by R proteins while still maintaining virulence functions (Gijzen et al., 2014; Raffaele & Kamoun, 2012). There is

co-evolution between phytopathogens and their plant hosts as both parties can elicit strong attack and defense mechanisms that are vital to each species survival. As an *Avr* gene evolves to circumvent detection, the complementary host *R* gene may evolve to track the pathogen effector and regain immunity. The majority of pathogen effectors show high levels of polymorphisms in their target-binding domains which reflects the diversity and distribution of corresponding host genes (Stukenbrock & McDonald, 2009; Vleeshouwers & Oliver, 2014)

Gain-of-virulence changes can result from conventional genetic mutations in the *Avr* gene, but other mechanisms can also be responsible. For example, Vleeshouwers & Oliver (2014) review the literature and show that gene silencing of *Avr* genes in the absence of conventional genetic mutations is a common method to escape host immunity, as it is reported in over half of the known *Avr* genes in *P. sojae*. It has been proposed that reversible epigenetic regulators may control gene silencing and the expression of *Avr* effectors (Gijzen et al., 2014; Kasuga & Gijzen, 2013; Whisson et al., 2014). Additional gain-of-virulence tactics such as *Avr* gene deletion amino acids changes, and frame shifts are also used by *P. sojae* to evade host detection (Cui et al., 2012; Song et al., 2013; Yin et al., 2013).

1.4 Thesis Statement and Objectives

The activation of plant immunity by pathogen Avr factors is a current and active research topic in plant pathology, especially in relation to diagnostics and crop protection. Molecular studies on the virulence determinants of *P. sojae*, however, are incomplete. Past studies on *P. sojae* phytopathology has uncovered the existence of Avr proteins (or *Avr* genes) that interact with soybean *Rps* gene products to deliver plant immunity. This study aims to identify the *Avr2* gene in *P. sojae*, which is predicted to encode an effector that triggers immunity on soybean plants with the *Rps2* gene.

Hypothesis: In *P. sojae*, avirulence to soybean resistance gene *Rps2* is determined by the corresponding gene *Avr2* which can be genetically mapped and identified at the DNA sequence level.

Objectives

The main objective of this study is to identify the *Avr2* gene in *P. sojae*. To complete this goal, the following sub-objectives are applied:

I. Identify *P. sojae* strains that differ in virulence towards soybean lines with and without the *Rps2* resistance gene, using phenotyping assays. Strains that are virulent towards control plants (*rps*) but avirulent to test plants (*Rps2*) are predicted to possess and express the *Avr2* gene, whereas strains that are virulent towards both types of plants are predicted to differ at the *Avr2* locus in sequence or expression.

II. Use *Rps2*-avirulent and -virulent *P. sojae* strains in a genetic cross to determine the inheritance pattern of *Avr2* in F_1 and F_2 generations. Segregation of the *Avr2* gene is determined using phenotype assays of each population.

III. Select candidate genes and test them for co-segregation with *Avr2*. Genetic markers will be designed for each candidate and co-segregation occurs when the DNA markers and virulence phenotypes match in progeny from the cross of *P. sojae* strains that differ in virulence to *Rps2*. Candidate genes are selected based on differential expression and sequence polymorphisms between *P. sojae* strains that are avirulent or virulent towards *Rps2*. Two different approaches to identify candidates are:
    a. *Avh* gene catalog and the use of DNA sequence polymorphism analysis
    b. RNA transcript catalog and deep sequencing data to find differentially expressed genes

2 Materials and Methods

2.1 *Phytophthora sojae* strains and plant materials

Phytophthora sojae parental isolates (races 1 to 25) used in this study were obtained from Dr. Mark Gijzen's collection at Agriculture and Agri-Food Canada (AAFC) in London, ON (Table 1**)**. A collection of F_2 isolates (race 2 x race 7 cross) from a previous study (Qutob et al., 2009) along with parental strains were originally preserved in liquid nitrogen (N_2) as mycelial disks (5 mm diameter) infused with 10% glycerol in 2.0 mL cryogenic tubes. For liquid N_2 culture revival, mycelial disks were thawed at room temperature for 30 min , transferred to 'regular' plates V8 consisting of 2.5% (v/v) V8 vegetable juice (Table 2) and incubated at 26°C degrees in the dark for 5 days. All other isolates were routinely maintained in the same conditions on regular V8 agar. To store cultures for longer than 3 months, mycelial disks (5mm diameter) were removed from the edge of each culture and transferred to 'long term storage' plates (Table 2) stored at 16 °C in darkness

Soybean (Glycine max) cultivars Williams (*rps*) and Harosoy (*rps*) with their corresponding *Rps2* isolines L76-1988 and L70-6494 respectively were obtained from AAFC in London, ON and used to evaluate the virulence of *P. sojae* isolates (parental strains, F_1s and F_2s). Seedlings were grown in 10 cm pots containing soil-less mix (Pro-Mix 'BX', Premier Horticulture Ltd, Riviere-du-Loup, Canada) under controlled growth chamber conditions (16 h photoperiod with 25°C day and 16°C night temperatures) and watered daily before inoculation for all virulence assays.

Table 1: List of *Phytophthora sojae* strains used in this study.

Name	Strain	Origin[1]	Investigator[2]
race 1	48FPA18	Ohio	F. Schmitthenner
race 2	P6497	Mississippi	B.M. Tyler
race 3	25MEX4	Ohio	F. Schmitthenner
race 6	ACR6	London, ON	T. Anderson
race 7	P7064	Canada	B.M. Tyler
race 8	ACR8	Unknown	T. Anderson
race 9	ACR9	Harrow, ON	T. Anderson
race 10	ACR10	Stoneville, MS	T. Anderson
race 12	ACR12	Stoneville, MS	T. Anderson
race 16	ACR16	Stoneville, MS	T. Anderson
race 17	P7074	Stoneville, MS	T. Anderson
race 19	P7076	Stoneville, MS	B.M. Tyler
race 20	ACR20	Unknown	T. Anderson
race 21	ACR21	Lafayette, IN	T. Anderson
race 25	ACR25	Lafayette, IN	T. Anderson

[1] Original site of isolation.

[2] Investigator who provided the original culture to AAFC.

2.2 Virulence assays and scoring phenotypes

To determine the virulence of *P. sojae* cultures towards *Rps2*,strains (races 1 to 25), F_1 and F_2 progeny were phenotypically scored using the hypocotyl inoculation test as described in Dorrance et al. (2008). Each virulence assay was independently repeated at least twice using two soybean isolines per isolate per repeat: Williams (*rps*) and Harosoy (*rps*) as positive controls for virulence and L70-1988 (*Rps2*) and L70-6494 (*Rps2*) as test plants. Seeds (15 to 20 seeds per 10 cm pot) were grown in a controlled growth chamber in 16 h/ 25°C light (day) period and 16°C night temperatures for six days prior to inoculation. Inoculum for each isolate consisted of a mycelial slurry derived from seven-day old strains grown on 0.9% (v/v) V8 juice agar. Mycelial

agar strips were sliced with a scalpel and placed into a 10 mL syringe attached to an 18- gauge needle. The agar culture was then forced though the syringe three times to obtain a smooth, consistent inoculum texture (slurry). Inoculation consisted of making a slit with the needle ~1 cm long into the hypocotyl of the seedlings, deep enough to pierce the epidermis. The inoculum (0.4 to 0.5 mL per seedling) was then squeezed into the wound using the syringe. Inoculated plants were covered with plastic bags to maintain humidity and kept in a dark growth chamber for two days (Figure 8). An additional three days was allowed for disease symptoms to develop before isolates were scored for virulence. Susceptible plants die or develop clear symptoms of infection three to five days after inoculation whereas resistant plants, exhibiting HR, form a scar around the wound but otherwise grow and remain healthy. Isolate phenotypes were scored as either avirulent (< 25% seedlings killed), virulent (> 75% seedlings killed) or intermediate (26 to 75% seedlings killed) (Dorrance et al., 2008). A one-tailed Fisher's exact test was also used to test for a significant association of virulence towards *Rps2* plants with respect to the level of virulence on the control plants.

Figure 8: A schematic of hypocotyl inoculation assay used for virulence testing.
(A) A mycelial slurry consisting of homogenized culture and media is injected into the hypocotyl of soybean stems. (B) Inoculated seedlings are covered to retain moisture and incubated in the dark for 3 days. (C) Virulence outcomes (avirulent, virulent or intermediate) are scored based on seedling survival.

2.3 Genetic crossing of *P. sojae* strains

Outcrosses of *P. sojae* parental strains race 10 x race 17 and race 2 x race 7 were performed and F_1 hybrids were obtained for the latter cross. F_2 progeny (race 2 x race 7) were subsequently derived from self-fertilized F_1 hybrids. Parental strains and F_1s were grown on regular V8 plates (2.5% (v/v) V8 juice) for five days at 26°C in the dark to acquire fresh growth. Mycelial disks (5mm diameter) were cut from the growing edges and transferred to β-sitosterol supplemented 'crossing plates' (Table 2) where they were incubated at 26°C in the dark for seven days or until mycelia grew to the edge of the plates. For crossing pairs of parental strains, mycelial agar strips cut from both races were placed in a 10 mL syringe, forced through an 18-gauage needle and repeated until the cultures were well-mixed. Two 1 mL deposits were placed 2 cm apart in the center of a new crossing plate and repeated for a total of five plates/replicate per cross. The cross plates were then air-sealed with plastic wrap (Saran), covered with aluminum foil to maintain complete darkness, and incubated at 26°C for at least 31 days to produce mature oospores. Self-fertilization of F_1 hybrids was performed in the same manner; except for the homogenization step (two 5 mm diameter mycelial disks were directly placed across from each other in the new crossing medium).

2.4 Isolation and extraction of oospores

Mature oospores obtained from genetic crosses of *P. sojae* parental strains and F_1 hybrids (section 2.3) were isolated from the crossing media and maintained separately for future DNA and RNA analyses. The following steps to isolate oospores are also described in Qutob et al. (2009).

(Steps I-VII were conducted at a laminar flow bench)

I. Two plates containing mature oospore culture were sliced into agar strips (~5 cm x 2 cm), placed into a sterile Waring blender containing 100 mL of sterile 4°C Milli-Q (MQ) water, and macerated for 120 s (with a 60 s break after 60 s to prevent the mixture from overheating

II. Using a vacuum filtration, the culture was then sieved twice through a sterile 75 μm nylon membrane to remove mycelia and agar chunks and collected in 50 mL conical tubes.

III. To kill hyphae still present in the suspension, the conical tubes were frozen for 24 h at -20°C and then thawed for 10 min at 45°C.

IV. The samples were re-filtered twice using a sterile 75 μm nylon membrane and collected in new 50 mL conical tubes which were centrifuged at 1,700 rcf for 15 min to pellet the oospores (oospore pellet looks like a light brown cluster at the base of the tube).

V. β-glucuronidase was added to the suspension to a final concentration of 2000 U/mL and mixed by lightly shaking the tubes. The suspension was incubated at 37°C for 16 h.

VI. The oospore mixture was washed three times by centrifugation at 1,700 rcf for 10 min. After removing agar and mycelial fragments with a sterile Pasteur pipette after each spin round, the isolated oospore pellet was re-suspended in 5 to 8 mL of sterile MQ water.

VII. Antibiotics kanamycin (50 μg/mL) and ampicillin (100 μg/mL) were added to the suspension and approximately 500 oospores were aseptically spread on β-sitosterol (10 μg/mL) and rifampicin (10 μg/mL) treated 'water plates' (Table 2).

VIII. The water plates were incubated in the dark at 26°C for at least 3 days to allow for oospore germination. Oospores do not germinate synchronously so the plates were checked daily using a stereomicroscope (60X).

IX. Germinating oospores were immediately picked from the agar using a sterile diamond-head transfer needle and placed separately on 9 cm 26% (v/v) V8 agar plates (supplemented with rifampicin (10 μg/mL) for growth at 25°C for 7 days in the dark.

The oospore cultures collected from both parental crosses (race 10 x race 17 and race 2 x race 7) were used for mycelial DNA extraction and genetic analyses to distinguish hybrid progeny. Subsequent F_1 and F_2 progeny from the race 2 x race 7 cross, were maintained on regular plates, transferred to long term storage plates and cryogenically preserved in liquid N_2 as described in (Dorrance et al., 2008).

Table 2: Preparation and usage of *P. sojae* culture media in this study

Media name[1]	Ingredients[2]	Application
26% (v/v) V8 juice agar 'Regular'	V8 juice: 156 mL Agar: 8.4 g CaCO3: 1.6g SDW: 440 mL	Routine growth, revival and maintenance
2.5% (v/v) V8 juice agar 'Crossing'	V8 juice: 15 mL Agar: 8.4 g, SDW: 585 mL β-sistosterol – 10 μg/mL	Genetic crosses (oospore incubation)
0.9% (w/v) V8 juice agar 'Virulence assay'	V8 juice: 156 mL Agar: 5.4 g $CaCO_3$: 1.6 g SDW: 440 mL	Phenotyping *P. sojae* strains
1.5% (w/v) water agar 'Water agar'	Agar: 9 g SDW: 440 mL β-sistosterol: 0.006 g Rifampicin: 10 μg/mL (after autoclaving)	Isolation of oospores (oospore germination)
1.4% (w/v) water agar 'Long-term storage'	Agar: 8.4 g SDW: 584 mL V8 juice: 16 mL	Culture storage > 3 months

[1] Media referred to as common name (in parentheses) throughout thesis.

[2] Total volume = 600 mL. Ingredients were thoroughly mixed, autoclaved, aseptically poured into sterile petri-dishes (90 x 16 mm) and stored at room temperature. SDW = sterile distilled water. V8 juice = Original V8® vegetable juice.

2.5 Genomic DNA isolation

Genomic DNA (gDNA) was extracted from parental strains (race 1 to 25) and oospores collected from crosses, including F_1 and F_2 progeny from race 2 x race 7, for genetic marker analyses. Oospores and parental strains were grown on regular plates for 5 days in the dark at 26°C prior to DNA extraction. Under a laminar flow hood, mycelial fragments (1 mL equivalent) were scraped from the surface of culture plates using sterile 200 µL pipette tips to isolate DNA using the UltraClean Microbial DNA isolation kit (Mo Bio Laboratories, Solana Beach, Calif.) as per manufacturer's directions. The final volume of DNA (50 µL) was eluted in EDTA-free Tris buffer (10 mM) and stored at -20°C until further use. DNA was quantified using a small volume spectrophotometer (NanoDrop, Thermo Scientific, USA).

2.6 Genetic markers for hybrid determination and co-segregation tests

Cleaved amplified polymorphic sequences (CAPs) corresponding to co-dominant genomic DNA (gDNA) markers were designed and used to distinguish F_1 hybrids from self-fertilized progeny in crosses (race 2 x race 7; race 10 x race17) and to test candidate genes for co-segregation with *Rps2* virulence phenotypes in parental strains, F_2, and F_1 populations. For hybrid determination, each CAPs marker consisted of an amplified region that contains a restriction cut site that is polymorphic between the avirulent and virulent parental strains. After digestion, amplified DNA product from the parental allele lacking the restriction enzyme site retains its original amplicon size (one larger band) while the other allele is digested (two smaller bands). Parental strains are pre-tested to ensure they are homozygous for the CAPs markers. Hybrid progeny are heterozygous and would thus display three bands representing both alleles, whereas self-fertilized progeny possess either one or two bands. Similarly, for candidate gene co-segregation tests, a region of the candidate gene is amplified containing a polymorphic restriction cut site present in either the avirulent or virulent parent strains

To determine the presence of single nucleotide polymorphisms (SNPs) that are potential cleavage sites for restriction enzymes between avirulent and virulent phenotypes, gDNA sequences for parental strains and candidate genes were uploaded to a specialized CAPs marker developer software called SNP2CAPS (Thiel et al., 2004). A list of the CAPs markers, their

applications and expected band sizes is displayed in Table 3. To determine hybrids in race 10 x race 17 cross, oospore DNA was extracted, amplified and digested using two different co-dominant CAPs markers named *Avr3a* and *Scaf_69* to establish confidence. For race 2 x race 7, *Avr3a* and *Avh320* CAPs markers were used.

Table 3: Restriction digest patterns of CAPs markers used for genetic analyses.

Marker	Restriction Enzyme	Marker size (bp)[1]		Application[2]
		race 2	***race 7***	
Avr3a	*AluI*	163, 130	293	HD
Avh320	*ClaI*	268, 132	400	HD
Avh172	*BaeGI*	291, 95	386	CGA
Avh8	*BstXI*	393	291, 102	CGA
Avh124	*AclI*	200, 154	354	CGA
Avh232	*AccI*	392, 268	660	CGA
Avh270	*BaeGI*	180	95, 85	CGA
		race 10	***race 17***	
Avr3a	*AluI*	163, 130	293	HD
Scaf_69	*PciI*	235, 135	360	HD

[1] Expected fragment lengths of amplified genomic DNA after restriction enzyme digest. Cleaved amplified polymorphic sequence (CAPs) marker determined by SNP2CAPs software (Thiel et al., 2004).

[2] HD = Hybrid determination; CGA = Candidate gene analysis.

2.7 Polymerase chain reaction (PCR) and restriction enzyme (RE) digestion

A three-step PCR was applied to all DNA samples used for hybrid determination and other genetic marker analyses. Initial denaturation temperature was set at 94°C for 2 min and the cycle started with a second denaturation temperature of 94°C for 40 s, an annealing temperature of 58°C for 40 s, and extension at 72°C for 40 seconds. At the end of the cycle, the extension temperature continued at 72°C for 7 min followed by a 4°C holding temperature. Thirty cycles were used for both regular and RT-PCR starting with a 15ng gDNA/cDNA template. The PCR

mixture was a total volume of 20 µL which consisted of 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 1.25 U Taq polymerase, 1X PCR buffer as directed (Invitrogen, Life Technologies), 0.5 µM each of forward and reverse oligonucleotide primers and MQ water. PCR gels consisted of 1.5 % (w/v) agarose dissolved in 1X TBE buffer with 0.05 % fluorescent nucleic acid dye GelRed (Biotium). Amplified DNA bands were quantified by gel electrophoresis and visualized by UV transillumination (BioRad Gel Doc). A list of the PCR/RT-PCR primers used in this study can be found in Table 4. Primers were designed using Primer3 software (v 0.4.0) and the presence of hairpins and self-dimers assessed using PrimerQuest Tools (Integrated DNA Technologies). All primers contained less than two hairpins and dimerization capabilities.

For CAPs marker utilization, amplified DNA products were digested with the pertaining restriction enzymes (Table 3). The RE mixtures were performed in a total volume of 25 µL: 12.5 µL of PCR product and 12.5 µL of enzyme/buffer mixture (Three units of relevant enzyme, recommended buffer and BSA obtained from New England Biolabs). Incubation time and temperature were dependent on the enzyme used as per manufacturer's directions. DNA bands were quantified and visualized is the same manner as PCR products except for the use of 2.5% (w/v) agarose gels instead.

Table 4: Oligonucleotide primers used in this study.

Primer Name[1]		5'-3' Sequence	Application[2]
Avr3a	F	GCTGCTTCCTTCCTGGTTGC	HD,C
	R	GCTGCTGCCTTTTGCTTCTC	
Avh320	F	AACGCTCTCGAAAGTGGC	HD,C
	R	AAAGAACTTCGACAG CC	
Scaf_69	F	GCGGATATCTAAGCGCACTC	HD, C
	R	TCATCTCAACGACCCCTCTT	
Avh172	F	AGCATCGAGGCTTCTACGAA	CGA,C,R
	R	TGCGCACTTGTCTGCGAGCAT	
Cand2	F	CTCCTCGGGTTCACCGT	CGA,R
	R	ACCGTATCCGAGCCCTT	
Actin	F	CGAAATTGTGCGCGACATCAAG	Co
	R	GGTACCGCCCGACAGCACGAT	
Avh8	F	TCTCTCCTTCGTCCTGCTC	CGA, C
	R	CGTGGGCGGTATTTCTCTTT	
Avh124	F	CATATTTCCTGCTCCTGCTC	CGA, C
	R	TCTCAGCTTATTCAAGCTCTTC	
Avh232	F	TATGCCAAACAGGCATCTC	CGA, C
	R	GTCGATAATGTCGTCCCTTG	
Avh270	F	CGAGCAAGAAGACGATAACC	CGA, C
	R	TTCCTCCAGTTCACCCTGTC	

[1] F = Forward primer; R= Reverse primer.

[2] HD = Hybrid determination; CGA = Candidate gene analysis; Co= Control for gene expression in RT-PCR analyses; C = amplification (PCR) of CAPs region; R = amplification of transcript region via RT-PCR.

2.8 RNA isolation for reverse transcriptase polymerase chain reaction (RT-PCR) and RNA-sequencing

To test candidate genes for co-segregation with virulence phenotypes at the transcriptional level, total RNA was isolated from select races, parental strains, F_1 and F_2 progeny (race 2 x race 7), reverse transcribed to create complementary DNA (cDNA) and amplified by RT-PCR using gene-specific primers. To acquire mycelia for RNA extraction, *Phytophthora sojae* isolates were first freshly cultured on 'regular' V8 plates. Under a laminar flow bench, mycelial plugs (5mm diameter) from the growth edges were transferred to sterile porous cellophane disks (Ultra Clear Cellophane, RPI Crop) placed directly over regular V8 media (Table 2) and incubated for 7 days at 26°C in darkness. The cellophane disks, now interwoven with mycelia, was sealed in sterile foil packets, placed in liquid N_2 for 5 min, and stored at -80°C if necessary. The snap-frozen disks were then ground into a fine powder using a mortar and pestle and suspended in liquid N_2 to prevent RNA degradation. Total RNA extraction was performed on the powdered mycelia in a fume-hood using a solution of phenol-guanidine isothiocyanate (TRIZOL, Life Technologies, Invitrogen) according to instructions provided by the manufacturer. The RNA pellet attained was dissolved in DEPC-treated water and stored at -80°C until further use. RNA samples were quantified using a NanoDrop spectrophotometer (Thermo Scientific, USA). To determine the integrity of samples, 1 µg of RNA combined with 1X RNA Gel- Loading Buffer (2:1 ratio) was separated on 1.5% (w/v) agarose gel using 1X TAE buffer. Each RNA isolation yielded four replicate tubes also stored at -80°C.

RNA samples used for next-generation sequencing were diluted to 300 ng/µL using DEPC treated water to achieve a final volume of 15 µL and shipped on dry ice.

To perform RT-PCR on *P. sojae* isolates (F_1 and F_2 progeny and strains), cDNA was synthesized from total RNA samples. Two µg of RNA was first treated with DNAse I (Amplification Grade, Invitrogen) to remove remaining DNA in the samples and then subjected to cDNA synthesis using Superscript III First Strand Synthesis System (Invitrogen) as directed by the manufacturer. The RT-PCR mixture, cycles and product visualization mirror that of regular PCR (Section 2.7) using cDNA as the template instead. Primers for candidate genes (*Avh172* and *Cand2*) aimed for RT-PCR (Table 4) were also designed in the same manner described in Section 2.7. Primers specific for *P. sojae* gene *Actin* were also applied to each cDNA isolate and amplified by RT-

PCR (*Actin* used as a positive control for transcriptional gene expression). In addition, candidate gene primers were also applied to gDNA (race 2) for RT-PCR to validate amplification success.

2.9 Single nucleotide polymorphism (SNP) analysis of *Avh* genes

The predicted set of *Avh* genes encodes small secreted proteins with RXLR-dEER motifs downstream from the signal peptide. The presence of these features characterizes *Avh* genes as candidate effector proteins. Known *Avr* genes that encode effector proteins often exhibit high levels of polymorphism to evade host cell detection.

Three *P. sojae* strains (race 7, race 17 and race 19) along with the reference genome (race 2), which represent a wide spectrum of genetic variation that occurs in the species, were compared to identify polymorphism in effector proteins (Wang et al., 2011) Race 2 and race 19 are avirulent towards *Rps2* and race 7 and race 17 are virulent. I expected that sequence variants in *Avh* genes between the *Rps2*-aviurlent and -virulent groups could be used to select *Avr2* candidates.

Candidate genes for *Avr2* were selected from 378 *Avh* genes that contain sequence polymorphisms between *Rps2*- avirulent (race 2 and race 19) and - virulent (race 7 and race 17) strains, resulting in both synonymous and non-synonymous changes. Sequence and polymorphic data for this comparison was provided by Wang et al. (2011). Co-dominant CAPs markers were designed for the selected *Avh* genes and tested for co-segregation or association with the virulence trait in F_2, F_1 and parental populations. Selected candidate *Avh* genes contained one or more high quality SNPs in the one or more of following comparisons: race 7 and race 2, race 7 and race 19, race 17 and race 2, race 17 and race 19.

2.10 Deep RNA sequencing (RNA-Seq) and differentially expressed sequence (DESeq) analysis

To explore the transcriptional differences between *P. sojae* cultures that differ in virulence towards *Rps2*, expression profiles of avirulent and virulent isolates were compared using RNA-Seq data. RNA-Seq is a recently developed method used to map and quantify transcriptomes

using deep-sequencing technologies. In short, total RNA is converted to a cDNA library containing fragments. The cDNA library is then high-throughput sequenced to obtain short reads (typically 30 to 400 nucleotides). The reads are then aligned to a reference transcript to produce a genome-scale map that describes the level of expression for each gene (Wang et al., 2009).

A total of 16 RNA samples were prepared for the RNA-Seq analysis, as follows. One set of eight samples from parental strains and a second set of eight samples from F_2 isolates. Each set included four *Rps2*-virulent and four *Rps2*-avirulent phenotypes. The RNA samples were sequenced using Illumina HiSeq 2000 technology at the McGill University and Genome Quebec Innovation Centre (Table 2.5). The reads were trimmed, filtered for quality and aligned to predicted transcripts from the *Phytophthora sojae* race 2 (reference strain P6497) v.3.0 genome assembly downloaded from JGI Genome Portal. Sequence reads were mapped using Burrows Wheeler Alignment (BWA v. 0.6.1) to generate a Sequence Alignment Map (SAM). From there, SAM-tools (v 0.1.18) was used to import count data into the DESeq package (DESeq v 1.22.0) offered in Bioconductor. The raw data was first normalized so that count values are brought to a common scale, making them comparable and easier to visualize.

The basic task of this package was to analyze count data from RNA-Seq and detect differentially expressed genes/sequences (DESeq) between two groups. Reports for each sample include the base mean number of reads assigned to a gene which projects the level of expression to calculate the log2Fold change (i.e. log2 of avirulent/virulent counts) and the statistical significance of this change (p-value). The p- value adjusted, however, is used to accurately establish significance as it controls the False Discovery Rate seen in multiple testing.

To ensure RNA samples accurately correlate with the phenotype group they are assigned in DESeq analyses, mycelial plugs taken from the same culture-plate/at the same time were used to extract RNA for sequencing and to perform virulence assays.

In addition to parental strains and F_2 isolates, transcriptional profiles of RNA-Seq data from 12 other races (7 avirulent, 5 virulent towards *Rps2*) cataloged in Mark Gijzen's laboratory (Agriculture and Agri-Food Canada, London, ON) were also used for DESeq analyses. These strains were also re-phenotyped to ensure accuracy in group assignment. Candidate genes from each group (parental, F_2 and races) were selected for marker co-segregation with *Avr2* if they

were highly expressed in the avirulent group compared to the virulent group and if they encoded for an avirulence protein. Each significantly differentially expressed gene was queried for sequence similarity using JGI Genome Browser and Blastp alignment search tools

3 Results

3.1 *Phytophthora sojae* parental strains differ in virulence towards soybean *Rps2* plants

In this initial analysis to determine any differences in virulence towards *Rps2* in *P. sojae*, a total of eight strains were tested for virulence against *Rps2* plants (L76-1988 and L70-6494) and their corresponding *rps* isolines (Williams and Haro (1-7). Nearly 100 *Rps2* and 60 *rps* seedlings were inoculated across two biological repeats of each cultivar and Fisher's Exact Test was used to determine the significance of virulence differences between the two treatments (challenge on *rps* versus *Rps2* plants). Of the six *P. sojae* strains that are avirulent towards *Rps2* but that retained virulence towards *rps*, race 10 and race 2 returned the lowest *p*-values for this test, as shown in Table 5. Two *P. sojae* strains (race 7 and race 17) could defeat *Rps2* and thus are virulent towards both types of plants. These four strains (races 10, 2, 17 and 7) were selected for further study.

Table 5: Virulence testing of *Phytophthora sojae* parental strains against *Rps2* plants

	Virulence[1]		
Strain	*Rps2*	*rps*	P- value[2]
Race 1	22/95 A	54/62 V	6.5E-16
Race 2	17/92 A	44/45 V	1.2E-20
Race 7	86/92 V	47/53 V	n.s.
Race 10	14/91 A	50/52 V	4.2E-23
Race 12	11/102 A	14/53 I	9.0E-03
Race 17	81/95 V	48/58 V	n.s.
Race 19	16/100 A	27/48 I	2.4E-07
Race 20	0/92 A	23/53 I	2.0E-12

[1] A= avirulent; Intermediate = I; V= virulent. The virulence of each strain was tested by inoculating seedlings containing resistance gene *Rps2* (L76-1988 and L70-6494) and no known resistance gene *rps* (Williams and Haro(1-7)). The number of dead seedlings/total inoculated seedlings is shown, summed from two biological replicates.

[2] Determined by Fisher Exact Test (one-tailed) used to compare the frequency of seedling survival between *Rps2* and *rps* (control) cultivars. The p-value (< 0.01) indicates isolate is avirulent towards *Rps2* cultivars and virulent towards control *rps*; n.s. = not significant

3.2 Identification of F_1 hybrids

In order to identify F_1 hybrid progeny, co-dominant CAPs markers polymorphic in the parent strains were used to screen random progeny isolated from outcrossing race 10 x race 17 and race 2 x race 7. Multiple oospore and DNA isolation trials were performed to isolate hybrid progeny (Table 6). For the race 10 x race 17 cross a total of 280 progeny were genetically analyzed for hybridization, and 142 progeny from the race 2 x race 7 cross. The vast majority of progeny from each cross resulted from self-fertilization of the avirulent parental strains (race 10 and race 2), according to the DNA marker analysis. Two CAPs markers for each cross were used to determine parentage of the progeny: *Avr3a* and *Scaf_69* (race 10 x race 17) and *Avr3a* and *Avh320* (race 2 x race 7) as shown in Table 3. No viable hybrids were recovered from the race 10 x race 17 cross, and only two progeny originated from race 17. However, in the race 2 x race 7 cross, six F_1 hybrids were recovered (Figure 9). Each F_1 hybrid (race 2 x race 7) was tested for virulence towards *Rps2* and self-fertilized to produce six separate F_2 populations containing 20 individuals each.

Table 6: Self-fertilization and hybridization progeny outcomes from outcrossing *P.sojae* parental strains.

Cross strains (Parent 1 x Parent 2) [1]	No. of oospore isolation trials[2]	No. of oospores obtained[3]	CAPs markers[4]	Marker Segregation[5]		
				Parent 1	Parent 2	F_1 hybrids
race 2 x race 7	7	142	*Avr3a, Avh320*	131	5	6
race 10 x race 17	10	280	*Avr3a, Scaf_69*	278	2	0

[1] Parental strains: *Rps2*-avirulent= race 2, race 10; *Rps2*-virulent= race 7, race 17.

[2] See methods for details on protocol.

[3] Total number of oospores isolated and tested for hybridization.

[4] Cleaved amplified polymorphic sequence (CAPs) markers that distinguish parental and hybrid DNA patterns using strain specific restriction enzymes. Marker details in Table 3 and Table 4.

[5] Outcome from the CAPs marker analysis. Parent1 and Parent 2 refer to self-fertilized progeny from either parent. The F_1 hybrids refer to outcrossed progeny between Parent 1 and Parent 2.

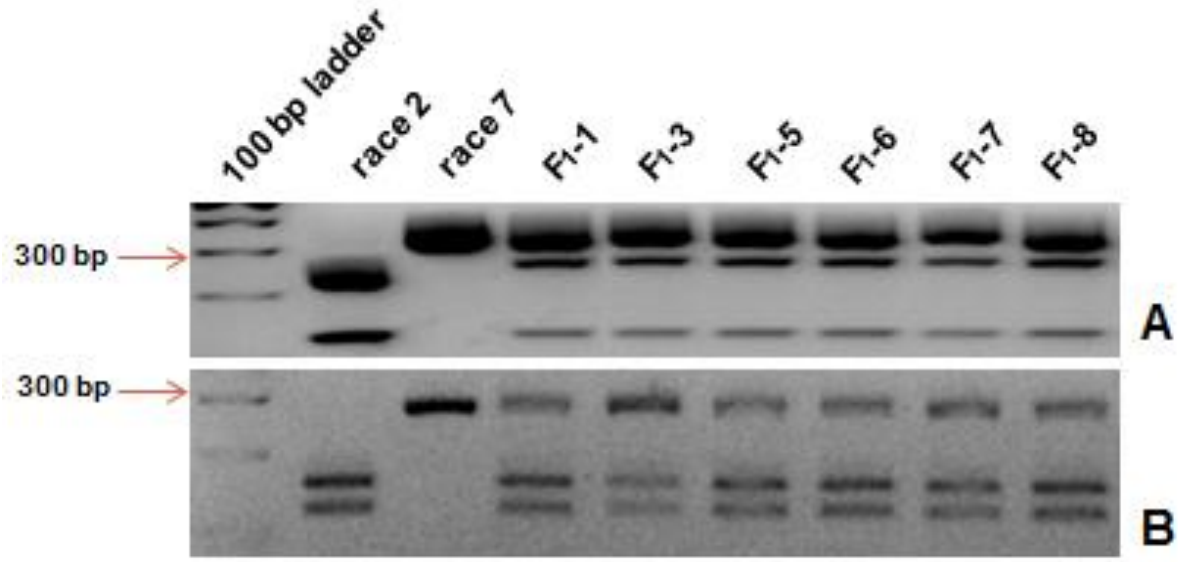

Figure 9: Hybrid determination (race 2 x race 7) using CAPs markers.
(A) *Avr3a* marker and (B) *Avh320* marker showing six F_1 progeny (race 2 x and race 7) containing both parental DNA digestion patterns. Marker size details in Table 3.

3.3 Abnormal inheritance of *Avr2* in F_1 and F_2 progeny

Homozygosity in *P. sojae* field isolates and the segregation of avirulence as a single dominant trait in outcrosses is a common pattern and a default expectation (Förster et al., 1994; Tyler et al., 1995). This is the outcome that I expected for inheritance of virulence towards *Rps2* in my F_1 and F_2 progeny.

The phenotypic expression of virulence towards plants carrying the *Rps2* resistance gene did not follow Mendelian segregation as expected, instead giving a 1:1 avirulent to virulent ratio in the six F_1 hybrids obtained from a cross of race 2 x race 7 (F_1-1,3,5,6,7,8), as shown in

Table 7. Virulent F_1 progeny include F_1- 1, F_1- 7 and F_1- 8 and avirulent F_1 progeny are F_1- 3, F_1- 5 and F_1- 6 (Figure 10). In subsequent virulence assays of F_2 progeny where F_1 parental strains were included as controls, previously avirulent isolates (F_1-3, 5 and 6) converted to virulent phenotypes or switched between avirulence and virulence in between trials in the case of F_1-6 (Table 11, Table 12, and Table 13). Virulent F_1 progeny (F_1-1, 7, 8), however, remained phenotypically consistent throughout.

The F_2 populations also displayed unexpected inheritance patterns with respect to their virulence towards *Rps2*. Many progeny in the F_2 populations created from F_1-3, F_1-5 and F_1-6 individuals exhibited inconsistent phenotypes when repeatedly tested for virulence towards *Rps2* as shown in Table 11, Table 12, and Table 13.Additional virulence tests were performed for isolates that returned inconsistent phenotypes in the first two trials. The instability of virulence towards *Rps2* appeared to be an unusual feature that was not resolved by further tests.

In F_2 populations derived from virulent individuals F_1-1, F_1-7 and F_1-8, almost all 60 progeny (20 in each population) were virulent towards *Rps2*, with two avirulent exceptions in the F_2-8 population (Table 8, Table 9 and Table 10). These groups underwent only two biological repeats of the virulence test due to consistent phenotype scores. A separate F_1 individual from a cross of race 2 x race 7, and corresponding F_2 population, from a previous study were retrieved from cryogenic storage and tested for virulence to *Rps2*. In this case, the F_1-74 isolate was virulent, as were 53 out of 65 F_2 progeny as shown in Table 14.

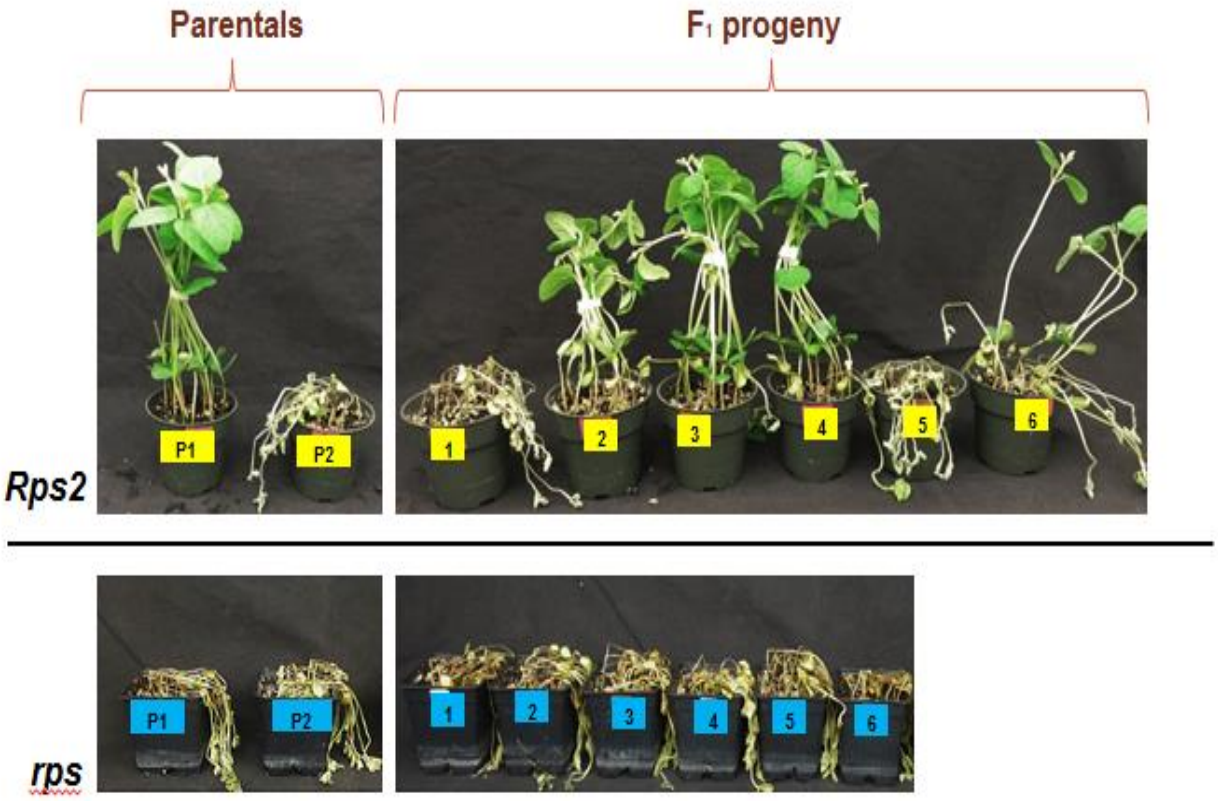

Figure 10: Photographs of plants after inoculation, illustrating phenotypes of F_1 progeny (race 2 x race 7) against *Rps2* and *rps* cultivars.

Parentals P1 and P2 refer to race 2 and race 7 respectively. Avirulent progeny (2, 3 and 4) represent F_1-3, F_1-5 and F_1-6 respectively. Virulent progeny (1, 5, and 6) represent F_1-1, F_1-7 and F_1-8 respectively. Details on inoculation and scoring method in Section 2.2.

Table 7: Virulence scores of F_1 hybrids obtained from crossing race 2 x race 7.

Isolate	Virulence[1]		P- value[2]
	Rps2	*rps*	
F_1-1	55/56 V	56/56 V	n.s.
F_1-3	16/53 I	54/54 V	2.0E-16
F_1-5	3/54 A	52/52 V	4.3E-27
F_1-6	9/54 A	48/51 V	5.2E-17
F_1-7	53/54 V	53/53 V	n.s.
F_1-8	43/49 V	50/50 V	n.s.

[1] A= avirulent; I= intermediate; V= virulent. The virulence of each strain was tested by inoculating seedlings containing resistance gene *Rps2* (L76-1988 and L70-6494) and no known resistance gene *rps* (Williams and Haro(1-7)). The number of dead seedlings/total inoculated seedlings is shown across two biological repeats.

[2] Determined by Fisher Exact Test (one-tailed) used to compare the frequency of seedling survival between *Rps2* and *rps* (control) cultivars. P-value (< 0.01) indicates isolate is avirulent towards *Rps2* cultivars and virulent towards control *rps*; n.s. = not significant.

Table 8: Virulence scores of F_2 progeny obtained from self-fertilization of F_1-1 hybrid (race 2 x race 7).

Isolate	Virulence[1]		P-value[2]
	Rps2	*rps*	
F_1-1	30/30 V	30/30 V	n.s.
F_2-1-1	30/30 V	30/30 V	n.s.
F_2-1-2	30/30 V	30/30 V	n.s.
F_2-1-3	30/30 V	30/30 V	n.s.
F_2-1-4	30/30 V	30/30 V	n.s.
F_2-1-5	30/30 V	30/30 V	n.s.
F_2-1-6	28/30 V	30/30 V	n.s.
F_2-1-7	29/30 V	30/30 V	n.s.
F_2-1-8	30/30 V	30/30 V	n.s.
F_2-1-9	30/30 V	30/30 V	n.s.
F_2-1-10	30/30 V	30/30 V	n.s.
F_2-1-11	30/30 V	30/30 V	n.s.
F_2-1-12	15/30 I	30/30 V	n.s.
F_2-1-13	30/30 V	30/30 V	n.s.
F_2-1-14	30/30 V	30/30 V	n.s.
F_2-1-15	30/30 V	30/30 V	n.s.
F_2-1-16	30/30 V	30/30 V	n.s.
F_2-1-17	30/30 V	30/30 V	n.s.
F_2-1-18	30/30 V	30/30 V	n.s.
F_2-1-19	30/30 V	30/30 V	n.s.
F_2-1-20	30/30 V	30/30 V	n.s.

[1] A= avirulent; V= virulent; I= intermediate. The virulence of each strain was tested by inoculating seedlings containing resistance gene *Rps2* (L76-1988 and L70-6494) and no known resistance gene *rps* (Williams and Haro(1-7)). The number of dead seedlings/total inoculated seedlings is shown across 2 biological repeats.

[2] Determined by Fisher Exact Test (one-tailed) used to compare the frequency of seedling survival between *Rps2* and *rps* (control) cultivars. P value (< 0.01) indicates isolate is avirulent towards *Rps2* cultivars and virulent towards control *rps*; n.s. = not significant.

Table 9: Virulence scores of F_2 progeny obtained from self-fertilization of F_1-7 hybrid (race 2 x race 7).

Isolate	Virulence[1]		P-value[2]
	Rps2	*rps*	
F_1-7	30/30 V	30/30 V	n.s.
F_2-7-1	30/30 V	30/30 V	n.s.
F_2-7-2	30/30 V	30/30 V	n.s.
F_2-7-3	30/30 V	30/30 V	n.s.
F_2-7-4	30/30 V	30/30 V	n.s.
F_2-7-5	30/30 V	30/30 V	n.s.
F_2-7-6	29/30 V	30/30 V	n.s.
F_2-7-7	30/30 V	30/30 V	n.s.
F_2-7-8	29/30 V	30/30 V	n.s.
F_2-7-9	30/30 V	30/30 V	n.s.
F_2-7-10	30/30 V	30/30 V	n.s.
F_2-7-11	30/30 V	30/30 V	n.s.
F_2-7-12	30/30 V	30/30 V	n.s.
F_2-7-13	30/30 V	30/30 V	n.s.
F_2-7-14	30/30 V	30/30 V	n.s.
F_2-7-15	30/30 V	30/30 V	n.s.
F_2-7-16	30/30 V	30/30 V	n.s.
F_2-7-17	30/30 V	30/30 V	n.s.
F_2-7-18	30/30 V	30/30 V	n.s.
F_2-7-19	30/30 V	30/30 V	n.s.
F_2-7-20	30/30 V	30/30 V	n.s.

[1] A= avirulent; V= virulent. The virulence of each strain was tested by inoculating seedlings containing resistance gene *Rps2* (L76-1988 and L70-6494) and no known resistance gene *rps* (Williams and Haro(1-7)). The number of dead seedlings/total inoculated seedlings is shown across 2 biological repeats.

[2] Determined by Fisher Exact Test (one-tailed) used to compare the frequency of seedling survival between *Rps2* and *rps* (control) cultivars. P-value (< 0.01) indicates isolate is avirulent towards *Rps2* cultivars and virulent towards control *rps*; n.s. = not significant.

Table 10: Virulence scores of F_2 progeny obtained from self-fertilization of F_1-8 hybrid (race 2 x race 7).

Isolate	Virulence[1]		P-value[2]
	Rps2	*rps*	
F_1-8	30/30 V	30/30 V	n.s.
F_2-8-1	25/30 V	30/30 V	n.s.
F_2-8-2	30/30 V	30/30 V	n.s.
F_2-8-3	28/30 V	30/30 V	n.s.
F_2-8-4	30/30 V	30/30 V	n.s.
F_2-8-5	30/30 V	30/30 V	n.s.
F_2-8-6	30/30 V	30/30 V	n.s.
F_2-8-7	19/30 I	30/30 V	n.s.
F_2-8-8	30/30 V	30/30 V	n.s.
F_2-8-9	29/30 V	30/30 V	n.s.
F_2-8-10	27/30 V	30/30 V	n.s.
F_2-8-11	12/30 I	30/30 V	9.4E-08
F_2-8-12	30/30 V	30/30 V	n.s.
F_2-8-13	30/30 V	30/30 V	n.s.
F_2-8-14	29/30 V	30/30 V	n.s.
F_2-8-15	30/30 V	30/30 V	n.s.
F_2-8-16	28/30 V	30/30 V	n.s.
F_2-8-17	25/30 V	30/30 V	n.s.
F_2-8-18	30/30 V	30/30 V	n.s.
F_2-8-19	21/30 I	30/30 V	n.s.
F_2-8-20	11/30 I	30/30 V	2.7E-08

[1] A= avirulent; V= virulent; I= intermediate. The virulence of each strain was tested by inoculating seedlings containing resistance gene *Rps2* (L76-1988 and L70-6494) and no known resistance gene *rps* (Williams and Haro(1-7). The number of dead seedlings/total inoculated seedlings is shown across 2 biological repeats.

[2] Determined by Fisher Exact Test (one-tailed) used to compare the frequency of seedling survival between *Rps2* and *rps* (control) cultivars. P-value (< 0.01) indicates isolate is avirulent towards *Rps2* cultivars and virulent towards control *rps*; n.s. = not significant.

Table 11: Virulence scores of F_2 progeny obtained from self-fertilization of F_1-3 hybrid (race 2 x race 7).

Isolate	Trial	Virulence[1]		P-value[2]
		Rps2	*rps*	
F_1-3	1	13/13 V	15/15 V	n.s.
	2	15/15 V	15/15 V	n.s.
	3	20/20 V	15/15 V	n.s.
F_2-3-1	1	13/13 V	15/15 V	3.5E-04
	2	15/15 V	15/15 V	1.1E-03
	3	18/20 V	15/15 V	n.s.
	4	5/20 A	15/15 V	4.8E-06
F_2-3-2	1	14/15 V	15/15 V	n.s.
	2	15/15 V	15/15 V	n.s.
F_2-3-3	1	15/15 V	15/15 V	n.s.
	2	15/15 V	15/15 V	n.s.
F_2-3-4	1	4/16 A	15/15 V	1.3E-05
	2	7/15 I	15/15 V	1.1E-03
	3	16/20 V	15/15 V	n.s.
	4	18/20 V	15/15 V	n.s.
F_2-3-5	1	9/15 I	15/15 V	8.4E-03
	2	7/15 I	15/15 V	1.1E-03
	3	17/20 V	15/15 V	n.s.
F_2-3-6	1	15/15 V	15/15 V	n.s.
	2	14/15 V	15/15 V	n.s.
F_2-3-7	1	8/16 I	15/15 V	9.6E-03
	2	5/15 A	15/15 V	1.0E-04
	3	14/20 V	15/15 V	n.s.
	4	2/20 A	15/15 V	4.2E-08
F_2-3-8	1	9/15 I	15/15 V	8.4E-03
	2	5/15 I	15/15 V	1.0E-04
	3	18/20 V	15/15 V	n.s.
F_2-3-9	1	6/15 I	15/15 V	3.5E-04
	2	15/15 V	15/15 V	n.s.
	3	19/20 V	15/15 V	n.s.
F_2-3-10	1	4/14 I	15/15 V	5.0E-05
	2	5/15 I	15/15 V	1.0E-04
F_2-3-11	1	3/15 A	15/15 V	5.3E-06
	2	1/15 A	15/15 V	1.0E-07
F_2-3-12	1	13/16 V	15/15 V	n.s.
	2	0/15 A	15/15 V	6.4E-09

Table 11 (continued): Virulence scores of F_2 progeny obtained from self-fertilization of F_1-3 hybrid (race 2 x race 7).

Isolate	Trial	Virulence[1]		P-value[2]
		Rps2	*rps*	
	3	20/20 V	15/15 V	n.s.
	4	7/20 I	15/15 V	5.3E-05
F_2-3-13	1	13/15 V	15/15 V	n.s.
	2	5/20 A	15/15 V	1.0E-04
	3	20/20 V	15/15 V	n.s.
	4	8/20 I	15/15 V	1.5E-04
F_2-3-14	1	14/15 V	15/15 V	n.s.
	2	12/15 V	15/15 V	n.s.
F_2-3-15	1	13/20 V	15/15 V	n.s.
	2	3/15 A	15/15 V	5.3E-06
	3	20/20 V	15/15 V	n.s.
F_2-3-16	1	14/15 V	15/15 V	n.s.
	2	9/15 I	15/15 V	8.4E-03
	3	20/20 V	15/15 V	n.s.
F_2-3-17	1	11/15 V	15/15 V	n.s.
	2	10/15 I	15/15 V	n.s.
	3	20/20 V	15/15 V	n.s.
F_2-3-18	1	14/15 V	15/15 V	n.s.
	2	12/15 V	15/15 V	n.s.
F_2-3-19	1	2/15 A	15/15 V	8.8E-07
	2	1/15 A	15/15 V	1.0E-07
F_2-3-20	1	12/15 V	15/15 V	n.s.
	2	2/15 A	15/15 V	8.8E-07
	3	16/20 V	15/15 V	n.s.
	4	14/20 I	15/15 V	n.s.

[1] A= avirulent; I = intermediate; V= virulent. The virulence of each strain was tested by inoculating seedlings containing resistance gene *Rps2* (L76-1988 and L70-6494) and no known resistance gene *rps* (Williams and Haro(1-7)). The number of dead seedlings/total inoculated seedlings is shown across at least two biological repeats. Additional trials (>2) were done on the basis of inconsistent scores in trials 1 and 2.

[2] Determined by Fisher Exact Test (one-tailed) used to compare the frequency of seedling survival between *Rps2* and *rps* (control) cultivars. P-value (< 0.01) indicates isolate is avirulent towards *Rps2* cultivars and virulent towards control *rps*; n.s. = not significant.

Table 12: Virulence scores of F_2 progeny obtained from self-fertilization of F_1-5 hybrid (race 2 x race 7).

Isolate	Trial	Virulence[1]		P-value[2]
		Rps2	*rps*	
F_1-5	1	2/15 A	15/15 V	3.6E-03
	2	3/15 A	15/15 V	5.3E-06
	3	6/20 I	15/15 V	1.7E-05
	4	7/20 I	15/15 V	5.3E-05
F_2-5-1	1	15/15 V	15/15 V	n.s.
	2	12/15 V	15/15 V	n.s.
F_2-5-2	1	10/12 V	15/15 V	n.s.
	2	7/15 I	15/15 V	1.1E-03
	3	5/20 A	15/15 V	4.8E-06
	4	2/20 A	15/15 V	4.2E-08
F_2-5-3	1	8/15 I	15/15 V	3.2E-03
	2	6/15 I	15/15 V	3.5E-04
	3	15/20 V	15/15 V	n.s.
	4	2/20 A	15/15 V	4.2E-08
F_2-5-4	1	6/16 I	15/15 V	1.8E-04
	2	6/15 I	15/15 V	3.5E-04
	3	5/20 A	15/15 V	4.8E-06
F_2-5-5	1	5/15 A	15/15 V	1.0E-04
	2	0/15 A	15/15 V	6.4E-09
F_2-5-6	1	10/15 V	15/15 V	n.s.
	2	4/15 I	15/15 V	2.5E-05
	3	6/20 I	15/15 V	1.7E-05
	4	0/20 A	15/15 V	3.1E-10
F_2-5-7	1	5/15 A	15/15 V	1.0E-04
	2	0/14 A	15/15 V	1.3E-08
F_2-5-8	1	0/15 A	15/15 V	6.4E-09
	2	0/15 A	15/15 V	6.4E-09
F_2-5-9	1	6/15 I	15/15 V	3.5E-04
	2	0/15 A	15/15 V	6.4E-09
	3	5/15 A	15/15 V	4.8E-06
F_2-5-10	1	0/15 A	15/15 V	6.4E-09
	2	2/15 A	15/15 V	8.8E-07
F_2-5-11	1	2/15 A	15/15 V	8.8E-07
	2	1/15 A	15/15 V	1.0E-07
F_2-5-12	1	5/11 A	15/15 V	2.0E-03
	2	1/15 A	15/15 V	1.0E-07

Table 12 (continued): Virulence scores of F_2 progeny obtained from self-fertilization of F_1-5 hybrid (race 2 x race 7).

Isolate	Trial	Virulence[1]		P-value[2]
		Rps2	*rps*	
	3	14/20 I	15/15 V	n.s.
	4	2/20 A	15/15 V	4.2E-08
F_2-5-13	1	0/15 A	15/15 V	6.4E-09
	2	0/15 A	15/15 V	6.4E-09
F_2-5-14	1	0/15 A	15/15 V	6.4E-09
	2	1/15 A	15/15 V	1.0E-07
F_2-5-15	1	0/15 A	15/15 V	6.4E-09
	2	0/15 A	15/15 V	6.4E-09
F_2-5-16	1	0/15 A	15/15 V	6.4E-09
	2	1/15 A	15/15 V	1.0E-07
F_2-5-17	1	2/15 A	15/15 V	8.8E-07
	2	0/15 A	15/15 V	6.4E-09
F_2-5-18	1	6/15 I	15/15 V	3.5E-04
	2	5/15 A	15/15 V	1.0E-04
	3	13/20 I	15/15 V	n.s.
	4	13/20 I	15/15 V	n.s.
F_2-5-19	1	1/15 A	15/15 V	1.0E-07
	2	0/15 A	15/15 V	6.4E-09
F_2-5-20	1	8/15 I	15/15 V	3.2E-03
	2	2/15 A	15/15 V	8.8E-07
	3	15/20 V	15/15 V	n.s.
	4	15/20 V	15/15 V	n.s.

[1] A= avirulent; I= Intermediate; V= virulent. The virulence of each strain was tested by inoculating seedlings containing resistance gene *Rps2* (L76-1988 and L70-6494) and no known resistance gene *rps* (Williams and Haro(1-7)). The number of dead seedlings/total inoculated seedlings is shown across at least two biological repeats. Additional trials (>2) were done on the basis of inconsistent scores in trials 1 and 2.

[2] Determined by Fisher Exact Test (one-tailed) used to compare the frequency of seedling survival between *Rps2* and *rps* (control) cultivars. P-value (< 0.01) indicates isolate is avirulent towards *Rps2* cultivars and virulent towards control *rps*; n.s. = not significant.

Table 13: Virulence scores of F_2 progeny obtained from self-fertilization of F_1-6 hybrid (race 2 x race 7).

Isolate	Trial	Virulence[1]		P-value[2]
		Rps2	*rps*	
F_1-6	1	2/15 A	15/15 V	8.8E-07
	2	12/15 V	15/15 V	n.s.
	3	9/20 I	15/15 V	4.0E-04
F_2-6-1	1	3/15 A	15/15 V	5.3E-06
	2	5/14 I	15/15 V	2.0E-04
	3	4/20 A	15/15 V	1.2E-06
F_2-6-2	1	7/15 A	15/15 V	1.1E-03
	2	15/0 V	15/15 V	n.s.
	3	6/20 I	15/15 V	1.7E-05
F_2-6-3	1	1/15 A	15/15 V	1.0E-07
	2	3/15 A	15/15 V	5.3E-06
F_2-6-4	1	2/15 A	15/15 V	8.8E-07
	2	4/15 I	15/15 V	2.5E-05
F_2-6-5	1	0/11 A	15/15 V	1.3E-07
	2	3/15 A	15/15 V	5.3E-06
F_2-6-6	1	4/15 I	15/15 V	2.5E-05
	2	11/15 I	15/15 V	n.s.
	3	0/20 A	15/15 V	3.1E-10
F_2-6-7	1	13/15 V	15/15 V	n.s.
	2	14/15 V	15/15 V	n.s.
F_2-6-8	1	9/15 I	15/15 V	8.4E-03
	2	8/15 I	15/15 V	3.2E-03
	3	16/20 V	15/15 V	n.s.
F_2-6-9	1	2/15 A	15/15 V	8.8E-07
	2	2/15 A	15/15 V	8.8E-07
F_2-6-10	1	14/15 V	15/15 V	n.s.
	2	9/15 I	15/15 V	8.4E-03
F_2-6-11	1	10/15 I	15/15 V	n.s.
	2	10/15 I	15/15 V	n.s.
F_2-6-12	1	6/13 I	15/15 V	1.4E-03
	2	11/15 1	15/15 V	n.s.
	3	14/20 I	15/15 V	n.s.
F_2-6-13	1	9/14 I	15/15 V	n.s.
	2	15/15 V	15/15 V	n.s.
	3	20/20 V	15/15 V	n.s.

Table 13 (continued): Virulence scores of F_2 progeny obtained from self-fertilization of F_1-6 hybrid (race 2 x race 7).

Isolate	Trial	Virulence[1]		P-value[2]
		Rps2	*rps*	
F_2-6-14	1	15/15 V	15/15 V	n.s.
	2	11/15 I	15/15 V	n.s.
F_2-6-15	1	11/15 I	15/15 V	n.s.
	2	12/15 V	15/15 V	n.s.
F_2-6-16	1	15/15 V	15/15 V	n.s.
	2	13/15 V	15/15 V	n.s.
F_2-6-17	1	9/15 I	15/15 V	8.4E-03
	2	1/15 A	15/15 V	1.0E-07
	3	3/20 A	15/15 V	2.5E-07
F_2-6-18	1	13/15 V	15/15 V	n.s.
	2	15/15 V	15/15 V	n.s.
F_2-6-19	1	11/15 I	15/15 V	n.s.
	2	7/15 I	15/15 V	1.1E-03
	3	15/20 V	15/15 V	n.s.
F_2-6-20	1	6/15 I	15/15 V	3.5E-04
	2	8/15 I	15/15 V	3.2E-03
	3	0/20 A	15/15 V	3.1E-10

[1] A= avirulent; V= virulent. The virulence of each strain was tested by inoculating seedlings containing resistance gene *Rps2* (L76-1988 and L70-6494) and no known resistance gene *rps* (Williams and Haro(1-7)). The number of dead seedlings/total inoculated seedlings is shown across at least two biological repeats. Additional trials (>2) were done on the basis of inconsistent scores in trials 1 and 2.

[2] Determined by Fisher Exact Test (one-tailed) used to compare the frequency of seedling survival between *Rps2* and *rps* (control) cultivars. P-value (< 0.01) indicates isolate is avirulent towards *Rps2* cultivars and virulent towards control *rps*; n.s. = not significant.

Table 14: Virulence scores of F_2 progeny obtained from self-fertilization of F_1-47 hybrid (race 2 x race 7). Isolates were revived from cryogenically stored cultures used in previous work done in Mark Gijzen's laboratory (Agriculture and Agri-Food Canada).

Isolate	Virulence[1]		P-value[2]
	Rps2	*rps*	
F_1-47-1	56/60 V	60/60 V	n.s.
F_2 progeny			
47-1-2	19/31 I	14/28 I	n.s.
47-1-3	15/31 I	2/27 A	3.2E-04
47-1-5	31/58 I	59/59 V	1.1E-10
47-1-6	30/33 V	28/28 V	n.s.
47-1-7	10/30 I	27/27 V	2.5E-08
47-1-8	58/58 V	57/57 V	n.s.
47-1-15	28/29 V	29/29 V	n.s.
47-1-16	0/31 A	3/30 A	n.s.
47-1-17	26/31 V	29/29 V	n.s.
47-1-18	18/29 I	23/25 V	9.0E-03
47-1-20	9/30 I	30/30 V	1.8E-09
47-1-22	31/31 V	26/28 V	n.s.
47-1-23	0/31 A	9/29 I	6.8E-04
47-1-25	13/32 I	31/31 V	5.7E-08
47-1-28	15/30 I	30/30 V	n.s.
47-1-29	31/31 V	28/30 V	n.s.
47-1-31	60/60 V	57/57 V	n.s.
47-1-32	15/61 A	29/30 V	1.1E-11
47-1-33	44/45 V	60/60 V	n.s.
47-1-35	43/69 V	60/60 V	n.s.
47-1-36	60/60 V	60/60 V	n.s.
47-1-37	60/60 V	59/59 V	n.s.
47-1-39	60/60 V	57/57 V	n.s.
47-1-40	60/60 V	59/59 V	n.s.

Table 14 (continued): Virulence scores of F_2 progeny obtained from self-fertilization F_1-47 hybrid (race 2 x race 7).

Isolate	Virulence[1]		P-value[2]
	Rps2	*rps*	
47-1-41	43/57 V	59/59 V	n.s.
47-1-42	9/60 A	60/60 V	5.9E-25
47-1-43	52/57 V	59/60 V	n.s.
47-1-44	43/57 V	60/60 V	n.s.
47-1-50	59/59 V	59/59 V	n.s.
47-1-51	60/60 V	58/58 V	n.s.
47-1-52	55/60 V	60/60 V	n.s.
47-1-57	60/60 V	60/60 V	n.s.
47-1-58	28/28 V	25/25 V	n.s.
47-1-64	25/31 V	30/31 V	n.s.
47-1-65	57/60 V	56/58 V	n.s.
47-1-70	30/31 V	26/26 V	n.s.
47-1-79	9/31 I	28/28 V	2.2E-09
47-1-81	28/29 V	28/28 V	n.s.
47-1-82	22/31 I	27/27 V	n.s.
47-1-84	4/30 A	12/27 I	8.2E-03
47-1-85	61/61 V	54/57 V	n.s.
47-1-86	57/57 V	56/56 V	n.s.
47-1-87	32/32 V	31/31 V	n.s.
47-1-85	61/61 V	54/57 V	n.s.
47-1-86	57/57 V	56/56 V	n.s.
47-1-88	58/60 V	42/42 V	n.s.
47-1-93	0/32 A	30/31 V	3.6E-17
47-1-97	59/59 V	59/59 V	n.s.
47-1-99	60/60 V	60/60 V	n.s.
47-1-TM53	61/61 V	60/61 V	n.s.
47-1-TM54	44/60 I	57/57 V	n.s.

Table 14 (continued): Virulence scores of F_2 progeny obtained from self-fertilization F_1-47 hybrid (race 2 x race 7).

Isolate	Virulence[1]		P-value[2]
	Rps2	*rps*	
47-1-TM131	54/59 V	59/59 V	n.s.
47-1-TM132	60/60 V	53/53 V	n.s.
47-1-TM134	49/58 V	60/60 V	n.s.
47-1-TM136	50/60 V	56/59 V	n.s.
47-1-TM137	50/59 V	58/60 V	n.s.
47-1-TM138	48/55 V	60/60 V	n.s.
47-1-TM139	56/58 V	60/60 V	n.s.
47-1-TM140	48/58 V	60/60 V	n.s.
47-1-TM141	50/55 V	53/58 V	n.s.
47-1-TM145	52/55 V	56/56 V	n.s.
47-1-TM146	44/59 V	60/60 V	n.s.
47-1-TM155	60/60 V	54/54 V	n.s.

[1] A= avirulent; I = indeterminate; V= virulent. The virulence of each strain was tested by inoculating seedlings containing resistance gene *Rps2* (L76-1988 and L70-6494) and no known resistance gene *rps* (Williams and Haro(1-7)). The number of dead seedlings/total inoculated seedlings is shown across 2 biological repeats.

[2] Determined by Fisher Exact Test (one-tailed) used to compare the frequency of seedling survival between *Rps2* and *rps* (control) cultivars. P-value (< 0.01) indicates isolate is avirulent towards *Rps2* cultivars and virulent towards control *rps*; n.s. = not significant.

3.4 *Avh* genes selected as candidates for *Avr2*

Gene sequences for 378 *Avh* genes in race 7, race 17 and race 19 were compared against the reference *P. sojae* genome (race 2) to identify single nucleotide polymorphisms (SNPs) that would cause amino changes. Polymorphic data was obtained and analyzed from Wang et al. (2011).

A total of 29 candidate genes for *Avr2* were selected based on two criteria: first, the presence of a high quality polymorphism in the virulent strains (race 7 and race 17) compared the avirulent strains (*P. sojae* reference strain race 2 and race 19), and second, the ability to produce a viable genetic marker for segregation tests was necessary (Table 15). Of this group, 19 *Avh* genes displayed nonsynonymous mutations (i.e. leading to amino acid changes) and all experienced synonymous mutations (nucleotide substitutions or SNPs not resulting in amino acid changes). Out of the three *Avh* genes reported for insertion/deletion changes, two (*Avh172* and *Avh163*) exhibited very high levels of polymorphisms with respect to race 2, including non-synonymous mutations.

Four of the candidate *Avh* genes were chosen randomly and tested for co-segregation with the *Rps2*-virulence trait in selected F_1 and F_2 individuals that displayed consistent phenotypes. None of candidate *Avh* gene markers co-segregated with the *Rps2*-virulence trait (Figure 11).

Table 15: A list of candidate *Avh* genes based on polymorphic comparisons among *P. sojae* strains that differ in virulence towards *Rps2*.

Candidate gene[1]	No. of Polymorphisms[2]			Type[3]
	Race 7 (Virulent)	Race 17 (Virulent)	Race 19 (Avirulent)	
Avh3c	13	19	0	S
Avh8	1	1	0	S, N
Avh18a	2	1	0	S, N
Avh29	20	20	0	S, N
Avh30	2	1	0	S, N
Avh78	4	4	0	S, N
Avh84	2	2	0	S
Avh97	10	-	0	S, N
Avh105	1	8	0	S, N
Avh124	4	3	0	S, N
Avh137	1	1	0	S
Avh148	4	1	0	S, N, I
Avh160	17	5	0	S, N
Avh163	75	46	0	S, N, I
Avh164	0	1	0	S
Avh168	25	-	0	S, N
Avh170	2	2	0	S
Avh172	53	69	0	S, N, I
Avh206	1	1	0	S
Avh208	7	17	0	S
Avh232	1	1	0	S, N
Avh234	4	1	0	S, N
Avh256	1	2	0	S, N
Avh264	50	49	0	S, N
Avh270	2	2	0	S
Avh347	0	1	0	S, N
Avh437	24	24	0	S, N
Avh452	1	1	0	S
Avh162	1	1	0	S

[1] Avirulence homolog (*Avh*) gene sequences and polymorphic data obtained from Wang et al. (2011).

[2] Total number of unique statistically significant sequence polymorphisms for each strain in a 4-way alignment compared to *P. sojae* reference strain race 2.

[3] Type of polymorphisms observed compared to race 2: S = synonymous substitutions, N= non-synonymous substitutions resulting in amino acid changes, I = insertions/deletions.

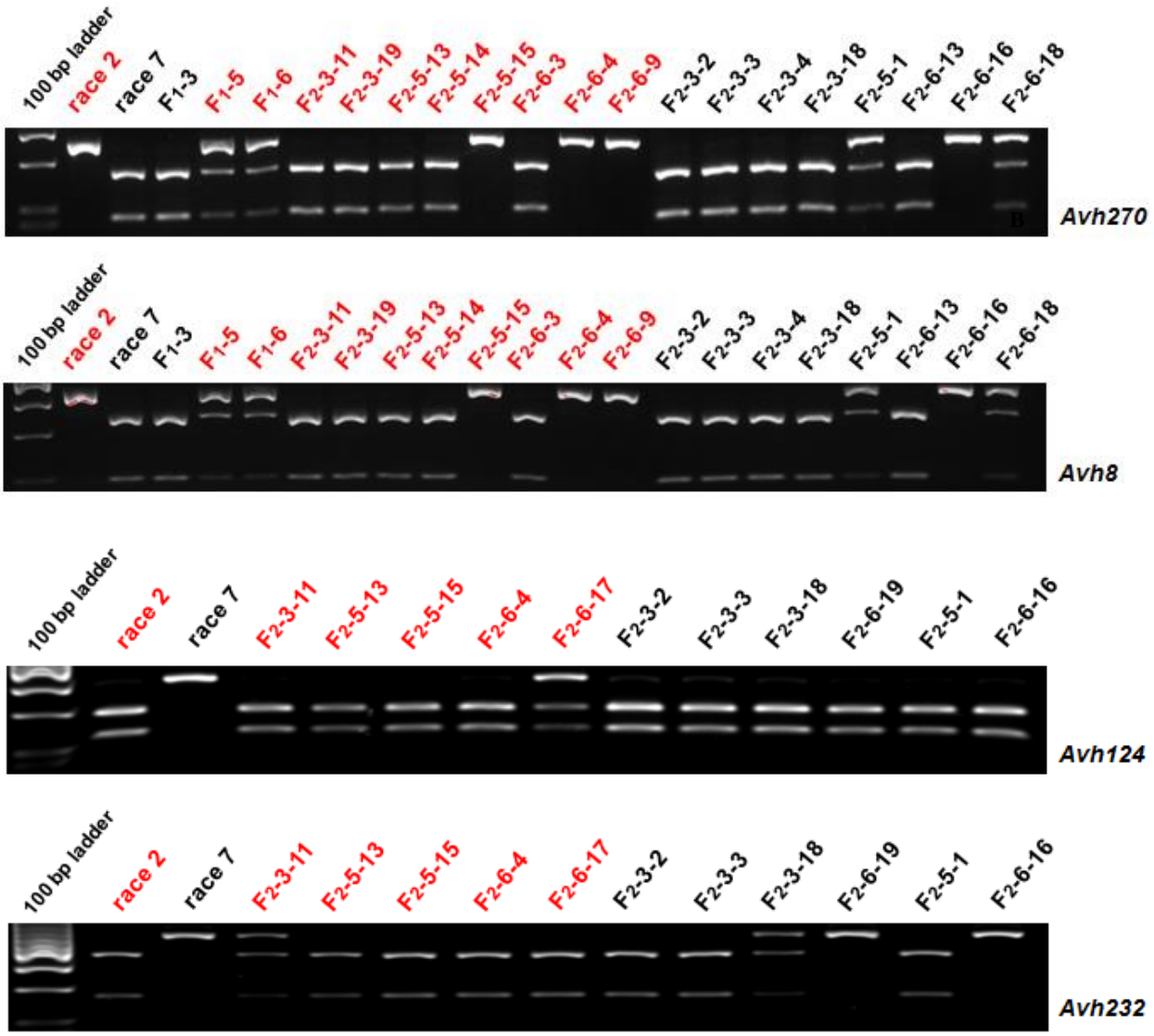

Figure 11: Marker co-segregation analyses of candidate genes derived from *Avh* polymorphic comparisons.

CAPs marker segregation for *Avh* genes (*Avh270*, *Avh8*, *Avh124*, *Avh132*) scored in parental strains (race 2, race 7), hybrids (F_1-3, F_1-5, F_1-6) and select F_2 progeny with consistent phenotypes is shown. Avirulent individuals are colored red; virulent individuals are colored black (note: F_1-5 and F_1-6 later switched to virulent phenotypes). Marker size details in Table 3.

3.5 Candidate *Avr2* genes selected from differential expression analysis

RNA-Sequence (RNA-Seq) data was used to perform differentially expressed sequence (DESeq) analyses of parental strains, select F_2 (race 2 x race 7) progeny and other *P. sojae* races from the laboratory core collection. Virulence scores and mapping data for all isolates used in this analysis are displayed in Table 16. The analysis was performed to identify transcripts that differed in expression based upon virulence to *Rps2* plants. For each differentially expressed gene, the transcript identification number (ID) obtained from *Phytophthora sojae* v 3.0 assembly is shown. The transcriptome of this assembly consists of 26,497 predicted genes. Also shown are the avirulent and virulent base mean counts, which sum all the count data for each member in both groups, and the adjusted p-value (P_{adj}) to correct false discovery rates seen in multiple testing. All significantly differentially expressed transcripts were queried for RXLR matches using Blast tools (JGI and NCBI) with the best protein matches and expect values (Table 17). Genes with higher expression levels in the avirulent group members compared to virulent members were of special interest as *Avr2* candidates.

In the first analysis, 12 *P. sojae* races in the laboratory collection (races 1, 2, 7, 8, 9, 10, 12, 17, 19, 20, 21, 25) were re-tested for virulence towards *Rps2* plants and placed into avirulent and virulent groups. Seven strains were avirulent (races 1, 2, 10, 12, 19, 20 and 25) and five strains (races 7, 8, 9, 17, 21) were virulent towards *Rps2* according to statistical tests. Base mean counts were compared as an average of individuals in both groups to assess expression level. A total of 14 differentially expressed genes were identified between avirulent and virulent strains (Table 17). From that list, queried genes were identified as unknown proteins with the exception of one gene (ID: 356110) which matched exactly to *Avh172*, making it a prime candidate for *Avr2*

In addition, the count data for this gene shows expression levels approximately 30 times higher in the aviurlent group compared to the virulent group. The *Avh172* gene has transcript length of 838 bp and a coding sequence of length of 684 bp which includes the associated protein domains typical of *Avr* effectors: a signal peptide followed by an RXLR and dEER motifs and the presence of W/L/Y motifs (Figure 12). The previous *Avh* candidate gene selection included *Avh172* as it was found to be highly polymorphic between *P. sojae* stains that differ in virulence to *Rps2* (Section 3.4, Table 15)

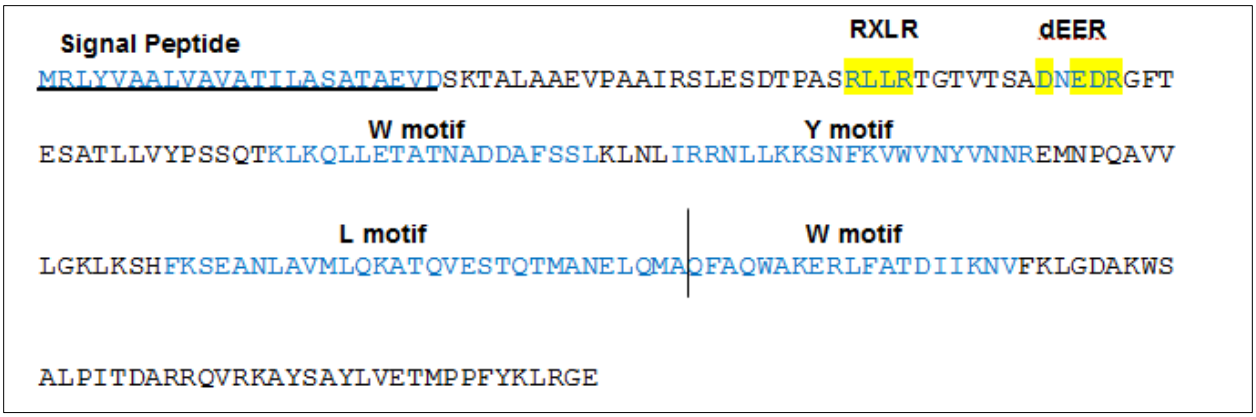

Figure 12: Effector domains of *Avh*172.
Protein sequence (227 amino acids) and domain data obtained from NCBI Blast (GenBank ID: AEK80823.1)

For the second analysis, two independent biological replicates each of parental races 2, 7, 17, and 20 (eight samples in total) were re-tested for virulence towards *Rps2* cultivars and deep-sequenced to obtain cDNA libraries for DESeq analysis. Races 2 and 20 were avirulent and race 17 and 7 were virulent towards *Rps2*, as expected from previous virulence assays. A total of 26 genes were identified as significantly different in expression but none mapped to any predicted *Avh* gene. However, some proteins were identified as part of other gene super-families (actin cross-linking, polymerases, metallo- dependent phosphatases, pyruvate phosphates kinases, and transporter proteins) which contribute to disease development in other eukaryotic and *Phytophthora* species (Aravind & Koonin, 1998; Marshall et al., 2001; Murphy & Young, 2015; Tenllado et al., 2004). One gene (ID: 356543), identified as *P. sojae* avirulence protein *Avr1b-1*, was previously cloned and characterized using race 2 and race 7 as parental strains (Shan et al., 2004), which accounts for its presence in this differential expression test using the same parental strains.

A total of eight phenotypically reliable F_2 individuals (race 2 x race 7) were re-tested for virulence against *Rps2* and deep-sequenced for a third DESeq analysis. As many other F_2 individuals displayed variable phenotypes, each F_2 individual for this analysis was virulence tested with the same culture used to obtain RNA-Seq data (protocol in Section 2.8) so that an accurate phenotype could be assigned in the DESeq analysis. Four isolates each were placed in

Rps2 -avirulent (F_2-3-11, F_2-5-13, F_2-6-4, F_2-6-17) and -virulent (F_2-3-2, F_2-3-3, F_2-5-1, F_2-6-16) groups and base-mean transcript expression levels compared between the two groups. A large number of genes (96) were identified as differentially expressed between avirulent and virulent groups. All 96 genes were queried for *Avh* gene matches but none were found. One of the genes (ID: 286523) coded for an unknown protein with a potential RXLR motif but no signal peptide. A second gene (ID: 436425) encoded a Crinkler-like protein in *P. infestans* which is second class of secreted effector proteins known alter host responses and promote infection in *Phytophthora* species (Lamour & Kamoun, 2009). Several secreted apoplastic effectors that execute pathogenic functions outside the host cell were also identified with higher expression levels in the virulent group compared to the avirulent group. Three transcripts matched *P. sojae* elicitins (ID: 554314, 355701, 565633), and two transcripts best resembled elicitins in other *Phytophthora* species (ID: 356816, 517422). Elicitins, a type of pathogen-associated molecular pattern (PAMP), can trigger cell by eliciting HR in the host cell (Qutob et. al, 2003; Yu, 1995). This type of plant-pathogen interaction is non-race specific and involves extracellular recognition, as opposed to specifically targeted RXLR effectors which translocate inside the host cell to manipulate host defense responses (cytoplasmic) (Chen et al., 2014). Another apoplastic effector that triggers plant cell death is the necrosis-inducing Nep1-like protein in *P. sojae* which was also identified as a highly expressed gene in the virulent group compared to the avirulent group (ID: 509399). These proteins constitute a large gene family in plant pathogenic oomycetes and are thought to act as positive virulence factors (Gijzen & Nürnberger, 2006; Oome et al., 2014). Many protein-matches from other large gene families implicated in pathogenesis in *P. sojae* and other oomcyetes (Chen et al., 2014) were also largely expressed in the virulent groups: glycoside hydrolases, ATP- binding cassette transporters, proteases and kinases. One gene of particular interest (ID: 472220), henceforth called *candidate 2* (*Cand2*), encoded for a small secreted protein that appears to belong a large gene family in *P. sojae* as it matches to many similar genes in *P. sojae* and other *Phytophthora* species. It was also expressed solely in the avirulent group. Since some of the hits in other species include predicted RXLR proteins, *Cand2* was also considered a candidate *Avr2* gene.

One gene (ID: 338243), overlapped with all three DESeq analyses (*P. sojae* races, parental strains and F_2 isolates). This gene is predicted to encode hydromethylbilane synthase (also called porphobilinogen deaminase) which is involved in tetrapyrrole and heme biosynthesis.

Additionally, six genes were found to overlap within the parental strains and races; no genes in the F_2 group were found to overlap with the parental strains or races (Table 17; Figure 13). In the six overlapping genes, one (ID: 318438) matched to a *P. infestans* Crinkler protein like a similar gene in the F_2 group. Two other genes (ID: 531145, 500696) were found to have conserved glycosidase and serine protease family domains respectively in other *Phytophthora* species. The catalytic and hydrolytic activities of these enzymes are known to play an important role in disease outcome in a variety of plant pathogens (Bellincampi et al., 2004; Figueiredo et al., 2014).

Table 16: RNA-sequencing of avirulent and virulent *P. sojae* strains towards *Rps.*

Groups[1]	Phenotype[2] *Rps2*	Phenotype[2] *rps*	P-value[3]	Total # of reads (million)[4]	Mapped Reads (%)[5]
Parental Strains					
Race 2 (1)	0/30 A	30/30 V	8.5E-18	47	59
Race 2 (2)	0/30 A	30/30 V	8.5E-18	53	61
Race 20 (1)	0/30 A	30/30 V	8.5E-18	46	62
Race 20 (2)	0/30 A	30/30 V	8.5E-18	46	29
Race 17 (1)	30/30 V	30/30 V	n.s	55	60
Race 17 (2)	30/30 V	30/30 V	n.s	47	53
Race 7 (1)	30/30 V	30/30 V	n.s	54	61
Race 7 (2)	30/30 V	30/30 V	n.s	44	59
F_2 progeny (race 2 x race 7)					
F2-3-11	7/30 A	30/30 V	8.7E-11	46	61
F2-5-13	9/30 I	30/30 V	1.8E-09	44	61
F2-6-4	3/30 A	30/30 V	4.6E-14	48	38
F2-6-17	5/30 A	30/30 V	2.7E-12	47	28
F2-3-2	30/30 V	30/30 V	n.s	46	66
F2-3-3	30/30 V	30/30 V	n.s	48	64
F2-5-1	24/30 V	30/30 V	n.s	46	62
F2-6-16	26/30 V	30/30 V	n.s	49	29
Races (catalog)					
Race 1	0/30 A	30/30 V	8.5E-18	47	67
Race 2	0/30 A	30/30 V	8.5E-18	49	68
Race 10	0/30 A	30/30 V	8.5E-18	55	67
Race 12	0/30 A	30/30 V	8.5E-18	56	67
Race 19	0/30 A	30/30 V	8.5E-18	32	67
Race 20	0/30 A	30/30 V	8.5E-18	52	67
Race 25	2/30 A	30/30 V	4.2E-15	41	67
Race 7	30/30 V	30/30 V	n.s	57	67
Race 8	30/30 V	30/30 V	n.s	30	67
Race 9	29/30 V	30/30 V	n.s	51	68
Race 17	30/30 V	30/30 V	n.s	40	68
Race 21	29/30 V	30/30 V	n.s	63	69

[1] Isolates undergone transcriptional profiling via next generation sequencing (RNA-Seq). Parentals have 2 sets of transcript data representing (1) and (2) independent biological replicates. F_2 progeny were selected from confident phenotype scores from 3 different populations (F_2-3, F_2-3, and F_2-6). Races previously deep-sequenced by Mark Gijzen (Agriculture and Agri-Food Canada) and transcript data obtained from private gene catalog.

[2] A = avirulent; I = intermediate; V = virulent. The virulence of each strain was tested by inoculating seedlings containing resistance gene *Rps2* (L76-1988 and L70-6494) and no known resistance gene *rps* (Williams and Haro(1-7)). The number of dead seedlings/total inoculated seedlings is shown, summed from 2 biological replicates. Phenotypes for F_2s and parental strains were directly sampled from the same culture used to extract and sequence RNA.

[3] Determined by Fisher Exact Test (one-tailed) used to compare the frequency of seedling survival between *Rps2* and *rps* (control) cultivars. Significant P- value (< 0.01) indicates isolate is avirulent towards *Rps2* cultivars and virulent towards control *rps*; n.s. = not significant.

[4] Raw reads obtained from Illumina HiSeq 2000 and rounded to the nearest million, after trimming and discarding reads < 20 nt. Read lengths =101 bp.

[5] Percentage of sequence reads mapping to the reference transcriptome, *P. sojae* assembly v3.0 (JGI) with quality Q-score > 30.

Table 17: Significantly differentially expressed transcripts in *Rps2*-avirulent and virulent *P. sojae* isolates (groups: F = F_2s; R= races; P = parental strains).

Gene ID[1]	Group[2]	Transcript Abundance[3]		P_{adj}[4]	E-value	Best protein hit[5]
		Avirulent	Virulent			
318438	R, P	0	702	4.0E-31	0.0	Crinkler family [P. infestans]
305556	R, P	2	162	4.0E-18	2.8E-05	Dentin sialophosphoprotein [Rattus norvegicus]
489962	R, P	184	0	9.0E-18	1.2E-33	CHP [P. infestans]
326636	R	208	4325	7.0E-17	3.4E-20	Zinc- Iron Permease Family [P. infestans]
356110	**R**	**3308**	**86**	**5.0E-14**	**0.0**	**Avirulence homolog (Avh) 172 [P. sojae]**
259475	R, P	1.9	113	2.0E-13	3.1E-14	Conserved hypothetical protein [P. infestans]
316879	R	3	2412	4.0E-08	0.0	Transmembrane protein, putative [P. infestans]
338270	R	75	636	2.0E-07	0.0	CHP [P. infestans]
531145	R, P	0	83.9	3.0E-07	0.0	Unk. [P. ramorum]: Glycosidase family
470032	R	0	27	4.0E-06	1.1E-03	CHP [P. infestans T30-4]
338243	R, P, F	52	549	8.0E-06	3.5E-06	porphobilinogen deaminase [P. infestans]
369654	R	22.1	186	5.0E-05	0.0	Transmembrane protein, putative [P. infestans]
500969	R, P	105	0	5.0E-05	3.5E-17	Serine protease family, putative [P. infestans]
288739	R	473	90	1.0E-04	0.0	Unk./ actin cross-linking domain [P. ramorum]
316879	P	2	1735	4.6E-20	0.0	Transmembrane protein, putative [P. infestans]
357683	P	406	0	6.0E-19	-	No hits
356543	P	176	0	2.5E-14	0.0	Avirulence homolog Avh6/ Avr 1b-1 [P. sojae]
343915	P	205	1	9.9E-14	6.0E-04	Unk. [P. ramorum]
336187	P	1	294	2.1E-13	0.0	Unk. [P. ramorum]
489962	P, R	108	0	9.1E-11	1.2E-33	CHP [P. infestans]
318438	P, R	3	292	1.1E-10	0.0	Crinkler family protein [P. infestans]
305556	P, R	0	100	2.1E-10	2.8E-05	Dentin sialophosphoprotein [R. norvegicus]

Table 17 (continued): Significantly differentially expressed transcripts in *Rps2*-avirulent and virulent *P. sojae* isolates (groups: F = F_2s; R= races; P = parental strains).

Gene ID[1]	Group[2]	Transcript Abundance[3]		P_{adj4}	E-value	Best protein hit[5]
		Avirulent	Virulent			
500969	P, R	88	0	2.1E-10	3.5E-17	Serine protease family, putative [P. infestans]
288739	P	248	1	1.3E-09	0.0	Unkown [P. ramorum]
338243	P, R, F	0	584	2.4E-09	3.5E-06	Porphobilinogen deaminase [P. infestans]
259475	P, R	0	99	8.5E-09	3.1E-14	CHP [P. infestans]
531145	P, R	0	69	2.3E-08	0.0	Unk. [P. ramorum]: Glycosidase family
320898	P	403	1	2.7E-08	0.0	Unk. [P. ramorum]
308341	P	1	109	7.1E-08	9.6E-07	Uncharacterized gene [Herpesvirus saimiri]
405374	P	619	1	2.1E-07	0.0	Unk. [P. ramorum]
298172	P	1	90	6.0E-07	-	No hits
516970	P	49	0	2.3E-06	1.4E-45	hypothetical protein [P. infestans]
308340	P	50	780	1.6E-05	3.0E-06	Vacuolar protein [Cryptococcus neoformans]
382816	P	0	272	2.6E-05	3.4E-26	Unk. [P. ramorum]
556164	P	9	133	9.7E-05	0.0	MtN3-like protein [P. infestans]
475957	P	43	535	1.4E-04	0.0	Unk. [P. ramorum]- centromere family
386565	P	16	258	1.7E-04	0.0	Unk. [P. ramorum]- zinc finger domain
412880	P	1	55	2.6E-04	5.6E-08	CHP [P. infestans]
355212	P	88	803	3.2E-04	4.0E-06	Pyruvate phosphate dikinase [P. cinnamomi]
485436	P	45	381	7.9E-04	0.0	CHP [P. infestans]
523105	F	4	119	1.4E-15	0.0	Unk. [P. ramorum]
556794	F	698	2393	1.5E-13	0.0	CHP [P. infestans]
376660	F	0	84	1.1E-12	8.4E-41	Unk. [P. ramorum]
307534	F	0	139	1.1E-12	0.0	**Unk. [P. ramorum] / Peptidase domain**
509399	F	1488	5433	2.2E-10	0.0	Necrosis-inducing-like protein [P. sojae]
318922	F	0	114	2.7E-10	0.0	Unk. [P. ramorum]

Table 17 (continued): Significantly differentially expressed transcripts in *Rps2*-avirulent and virulent *P. sojae* isolates (groups: F = F_2s; R= races; P = parental strains).

Gene ID[1]	Group[2]	Transcript Abundance[3]		P_{adj}[4]	E-value	Best protein hit[5]
		Avirulent	Virulent			
561454	F	801	2811	2.7E-10	0.0	Glycoside hydrolase, putative [P. infestans]
313290	F	761	3598	1.3E-09	0.0	Unk. [P. ramorum]/ phosphatase domain
560301	F	17183	60514	6.3E-09	0.0	Unk. protein [Phytophthora capsici]
308710	F	60	557	1.2E-08	0.0	Carbohydrate esterase, putative [P. infestans]
356816	F	25196	77300	1.9E-08	0.0	Beta-elicitin cryptogein [P. cryptogea]
555072	F	237	1061	7.5E-08	0.0	Glycoside hydrolase [P. infestans]
254010	F	2266	7745	1.6E-07	0.0	glucosylceramidase, putative [P. infestans]
264618	F	2535	7422	1.7E-07	0.0	Fatty acid desaturase, putative [P. infestans]
517422	F	1412	5062	4.3E-07	0.0	Elicitin protein [P. parasitica]
248487	F	15	130	5.0E-07	0.0	Transmembrane protein, putative [P. infestans]
288545	F	27709	75277	8.7E-07	0.0	Elicitin protein [*Phytophthora sojae*]
308093	F	1375	11501	8.7E-07	0.0	CHP [P. infestans]/ phosphatase domain
502698	F	211	634	2.3E-06	0.0	Unk. [P. ramorum]/ zinc finger domain
506688	F	36	152	4.8E-06	0.0	Unk. [P. ramorum]/ zinc finger domain
491552	F	15	99	6.1E-06	0.0	Unk. [P. capsici]
355971	F	3347	9411	9.3E-06	0.0	Long chain fatty acids, putative [P. infestans]
361323	F	19727	52705	9.3E-06	0.0	Annexin Family [P. infestans]
557185	F	2351	5466	2.3E-05	0.0	Serine protease S33, putative [P. infestans]
286523	F	1392	570	2.4E-05	0.0	Unk. [P. capsici] RXLR motif; no signal peptide
557232	F	621	1876	2.4E-05	0.0	Serine protease S10, putative [P. infestans]
344205	F	16	84	2.6E-05	0.0	Protein kinase, putative [P. infestans]
249854	F	206	509	2.6E-05	0.0	CHP [P. infestans]
563836	F	146	486	2.7E-05	0.0	Unk. [P. ramorum]/ glycoside hydrolase
527314	F	87	261	2.7E-05	0.0	Unk. [P. ramorum]/ zinc finger domain

Table 17 (continued): Significantly differentially expressed transcripts in *Rps2*-avirulent and virulent *P. sojae* isolates (groups: F = F_2s; R= races; P = parental strains).

Gene ID[1]	Group[2]	Transcript Abundance[3]		P_{adj}[4]	E-value	Best protein hit[5]
		Avirulent	Virulent			
546913	F	1559	4826	3.2E-05	0.0	Unk. [P. ramorum]/ hydrolase domain
550978	F	13929	42012	3.5E-05	0.0	Cation Symporter [P. infestans]
554314	F	320	1176	3.5E-05	0.0	Elicitin-like protein SOL11B [P. sojae]
315514	F	457	1230	3.7E-05	0.0	Galactosidase, putative [P. infestans]
357010	F	5157	14461	4.0E-05	0.0	Unk. [P. capsici]
338243	F, P, R	70	395	4.2E-05	3.5E-06	Porphobilinogen deaminase [P. infestans T30-4
551718	F	294	1511	4.6E-05	0.0	Glycoside hydrolase, putative [P. infestans]
357793	F	1780	3985	4.6E-05	0.0	Delta-6 desaturase [P. citrophthora]
314512	F	52	8	5.3E-05	0.0	Tenascin-like protein [P. infestans]
320049	F	0	22	5.8E-05	0.0	Unk. [P. ramorum]
246230	F	624	1456	6.0E-05	0.0	Beta-glucan associated, putative [P. infestans]
544332	F	252	840	6.0E-05	0.0	Galactosidase [P. infestans]
493321	F	1413	3896	6.7E-05	0.0	Sulfate Permease Family [P. infestans]
383265	F	517	1264	6.8E-05	3.5E-17	CHP [P. infestans]
481896	F	3	35	7.0E-05	6.0E-23	Hypothetical protein [P. infestans]
557086	F	2794	7030	7.0E-05	0.0	CHP [P. infestans]
532808	F	16	89	7.6E-05	0.0	Glycoside hydrolase, putative [P. infestans]
499969	F	13	73	8.4E-05	0.0	Folate-Biopterin Transporter [P. infestans]
526644	F	61	413	8.8E-05	0.0	Major Facilitator Superfamily [P. infestans]
357523	F	37	0	8.8E-05	0.0	Hypothetical protein [P. infestans]
551511	F	396	988	8.8E-05	0.0	Dicarboxylate symporter [P. infestans]
492030	F	85	263	9.9E-05	0.00	Unk. [P. ramorum]
567778	F	4398	9734	1.0E-04	0.0	ATP-binding Cassette protein [P. infestans]
320287	F	321	1659	1.2E-04	0.0	Acyl-CoA desaturase [P. infestans]
509924	F	23	127	1.2E-04	0.0	Mitochondrial precursor [P. infestans]

Table 17 (continued): Significantly differentially expressed transcripts in *Rps2*-avirulent and virulent *P. sojae* isolates (groups: F = F_2s; R= races; P = parental strains).

Gene ID[1]	Group[2]	Transcript Abundance[3]		P_{adj}[4]	E-value	Best protein hit[5]
		Avirulent	Virulent			
472220	**F**	**379**	**0**	**1.2E-04**	**0.0**	**CHP / large gene family [P. sojae]**
499018	F	470	1894	1.2E-04	0.0	Beta glucosidase, putative [P. infestans]
494282	F	343	772	1.4E-04	0.0	Unk. [P. ramorum]
561468	F	2033	4847	1.5E-04	0.0	Unk. [P. ramorum]
547012	F	964	2049	1.5E-04	0.0	Unk. [P. ramorum]
526251	F	717	1489	1.5E-04	2.3E-32	ATP-binding Cassette [P. infestans]
481377	F	263	1889	1.9E-04	0.0	CHP [P. infestans]/ SET domain
492989	F	16	67	2.0E-04	0.0	Hypothetical protein [Botryotinia fuckeliana]
317035	F	1052	2494	2.1E-04	0.0	Unk. [P. ramorum]
255079	F	267	105	2.1E-04	0.0	CHP [P. infestans]
535439	F	302	800	2.2E-04	0.0	Cation-transporting ATPase, putative [P. infestans]
549007	F	7747	16253	2.3E-04	0.0	Acyl-CoA desaturase [P. infestans]
337191	F	1124	513	2.3E-04	7.7E-25	Unk. [P. capsici]
350946	F	128	324	2.4E-04	0.0	Metal Ion Transporter Family [P. infestans]
557223	F	73	241	2.5E-04	0.0	Serine protease family S10, putative [P. infestans]
512862	F	81	1	2.8E-04	0.0	Thrombospondin type 1 [P. cinnamomi]
336396	F	133	0	2.8E-04	0.0	Short chain dehydrogenase, putative [P. infestans]
490600	F	279	677	2.9E-04	0.0	Unk. [P. ramorum]/ zinc finger domain
473436	F	36	103	3.1E-04	0.0	Unk. [P. ramorum]
505731	F	107	349	3.2E-04	0.0	Unk. [P. ramorum]
506548	F	53	223	3.3E-04	0.0	Acetyl-coenzyme A synthetase [P. infestans]
372537	F	69	249	3.3E-04	0.0	Protein kinase [P. infestans]
566561	F	249	969	3.4E-04	0.0	Nucleoside Transporter [P. infestans]

Table 17 (continued): Significantly differentially expressed transcripts in *Rps2*-avirulent and virulent *P. sojae* isolates (groups: F = F_2s; R= races; P = parental strains).

Gene ID[1]	Group[2]	Transcript Abundance[3]		P_{adj}[4]	E-value	Best protein hit[5]
		Avirulent	Virulent			
513216	F	2214	891	3.6E-04	0.0	Mitochondrial protease, putative [P. infestans]
524255	F	375	839	4.1E-04	0.0	Unk. [P. ramorum]
304558	F	267	644	4.2E-04	7.5E-09	Hypothetical protein [Volvox carteri f. nagariensis]
556173	F	11	115	4.3E-04	0.0	Nmra-like family protein, putative [P. infestans]
550689	F	3795	7972	5.7E-04	0.0	ATP-citrate synthase [P. infestans]
287801	F	322	770	6.2E-04	0.0	Glycoside hydrolase, putative [P. infestans]
517114	F	26	111	6.3E-04	0.0	ATP-binding Cassette [P. infestans]
352771	F	1005	1986	7.4E-04	0.0	Lysophospholipid acyltransferase [P. infestans]
512002	F	416	853	8.2E-04	0.0	Urease, putative [P. infestans]
355701	F	1060	2587	8.8E-04	2.2E-32	Elicitin-like protein SOL1A [P. sojae]
507075	F	69	259	8.9E-04	0.0	Unk. [P. ramorum]
306182	F	34	121	8.9E-04	7.0E-10	Protein kinase, putative [P. infestans]
565633	F	1594	3761	9.2E-04	0.0	Transglutaminase elicitor precursor [P. sojae]
555070	F	1313	2814	9.2E-04	0.0	Polysaccharide lyase, putative [P. infestans]
436425	F	33	3	9.2E-04	1.3E-14	Crinkler-like CRN11 protein [P. infestans]
485174	F	53	174	9.3E-04	0.0	Mtn3-like protein [P. infestans]
553764	F	4295	9683	9.8E-04	0.0	Synthase, putative [P. infestans]
486067	F	880	1854	9.9E-04	0.0	Unk. [P. ramorum]

[1] Transcript identification number (ID) obtained from *Phytophthora* genome assembly v3.0 (JGI).

[2] The three *P. sojae* groups are as follows: R = races (avirulent: races 1, 2, 10, 12, 19, 20 and 25; virulent: races 7, 8, 9, 17, 21), P = parental strains (avirulent: race 2 and race 20; virulent: race 17 and race 7) and F = F_2 isolates (avirulent: F_2-3-11, F_2-5-13, F_2-6-4, F_2-6-17; virulent: F_2-3-2, F_2-3-3, F_2-5-1, F_2-6-16).

[3] Normalized base mean counts of reads (mapping quality >20) that were mapped to one unique location for avirulent and virulent *P. sojae* isolates, calculated from RNA-Seq data using BWA v0.6.2- r126.

[4] Adjusted P-value corrected for false discovery rates.

[5] Transcript ID queried for protein matches and/or the presence of conserved family domains using Blast Tools (NCBI/JGI). Unk = Unknown (protein); CHP = Conserved Hypothetical Protein.

(Bold) Candidate genes selected for co-segregation tests with *Avr2* expression.

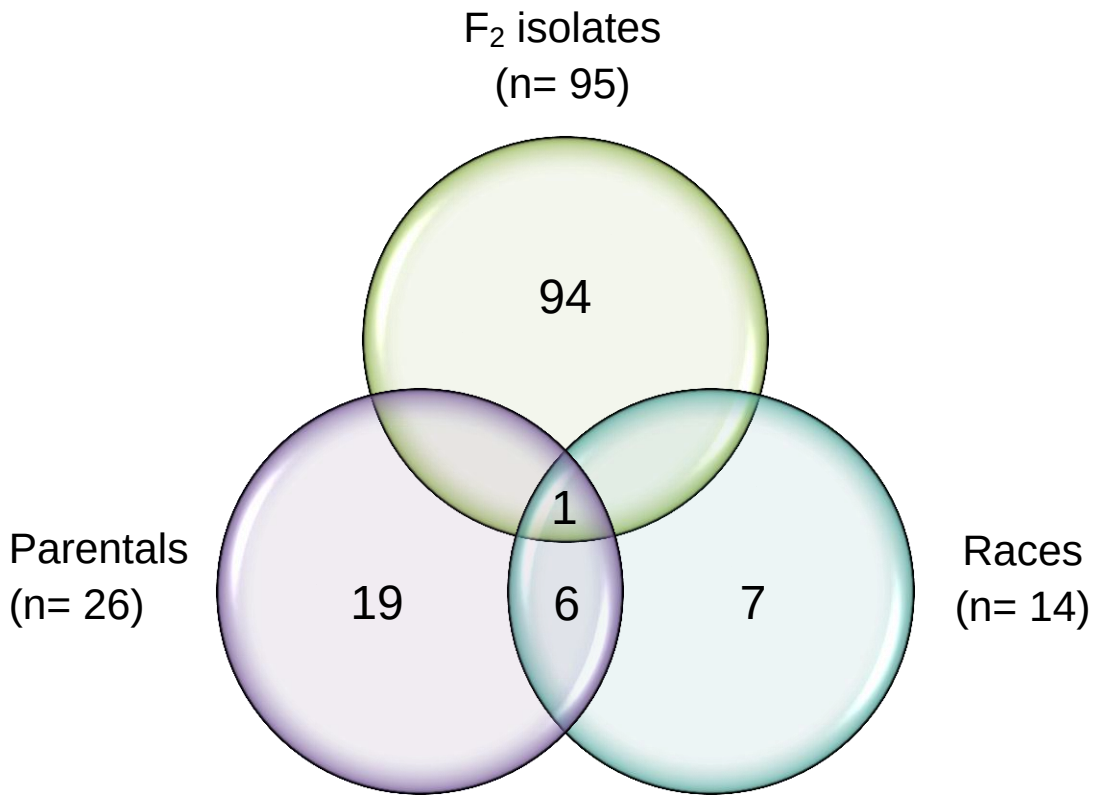

Figure 13: Venn diagram of differential transcript expression data from *P. sojae* races, F_2 isolates and parental strains.

The number of significantly differentially expressed transcripts (n) is shown within the colored sets along with overlapping transcripts in the subsets. All differential expression numbers were generated by Bioconductor package using RNA-Seq data. Races = (races 1, 2, 7, 8, 9, 10, 12, 17, 19, 20, 21, 25), F_2 isolates = (F_2-3-11, F_2-5-13, F_2-6-4, F_2-6-17, F_2-3-2, F_2-3-3, F_2-5-1, F_2-6-16) and parental strains = (race 2, 20, 17,7) Gene information can be found in Table 16.

3.6 Candidate genes do not co-segregate with *Rps2* virulence phenotypes

Avirulence homolog *Avh172* (ID: 356110), found to be differentially expressed in *P. sojae* strains avirulent towards *Rps2*, was tested for co-segregation with *Avr2* in race 2 x race 7 F_1 progeny (F_1-1, 3, 5 ,6 ,7, 8), in eight selected F_2 progeny with reliable avirulent (F_2- 3-11, 5-13, 6-4, 6-17) and virulent (F_2- 3-2, 3-3, 5-1, 6-16) phenotypes, and in 12 *P. sojae* races (avirulent: race 1, 2, 10, 12, 16, 19, 20, 25; virulent: race 7, 8, 9, 17, 21). Purified gDNA was amplified using primers designed around the *Avh172* CAPs marker and digested using *BaeG*I restriction enzyme. Results show that the *Avh172* DNA marker does not co-segregate with the *Rps2* virulence phenotype in the F_1 or the F_2 cultures. In addition, restriction patterns of all F_2 individuals correspond to that of their F_1 parentages (Figure 14).

In contrast to F_1 and F_2 progeny, *P. sojae* races exhibited consistent virulence phenotypes towards *Rps2* throughout this study. Amongst the races, the *Avh172* CAPs marker co-segregated with the *Rps2*-virulence phenotype. However, RT-PCR analysis for *Avh172* expression shows that this gene is expressed in most *P. sojae races*; including *Rps2*-virulent ones (Figure 14B). The F_1 and F_2 cultures tested for the *Avh172* CAPs marker were also analyzed for *Avh172* expression by RT-PCR. Results show that the *Avh172* transcript level was variable among the progeny but not associated with *Rps2* virulence phenotype.

Expression of the candidate gene, *Cand2* (ID: 472220), was tested for co-segregation with virulence towards *Rps2* by RT-PCR (shown in Figure 15). Results show that transcript expression for the *Cand2* gene did not co-segregate with virulence phenotype in F_1 and F_2 progeny. Interestingly however, *Cand2* transcripts were detectable in all the virulent *P. sojae* but not in the avirulent race types, with one exception (race 1). In addition, two bands were amplified in each RT-PCR reaction, indicating that this gene may be duplicated in the genome.

Thus, the two candidate genes *Avh172* and *Cand2* did not appear to co-segregate with the *Rps2* virulence phenotype in the F_1 and F_2 cultures, but each appears in associated with the trait in the *P. sojae* races.

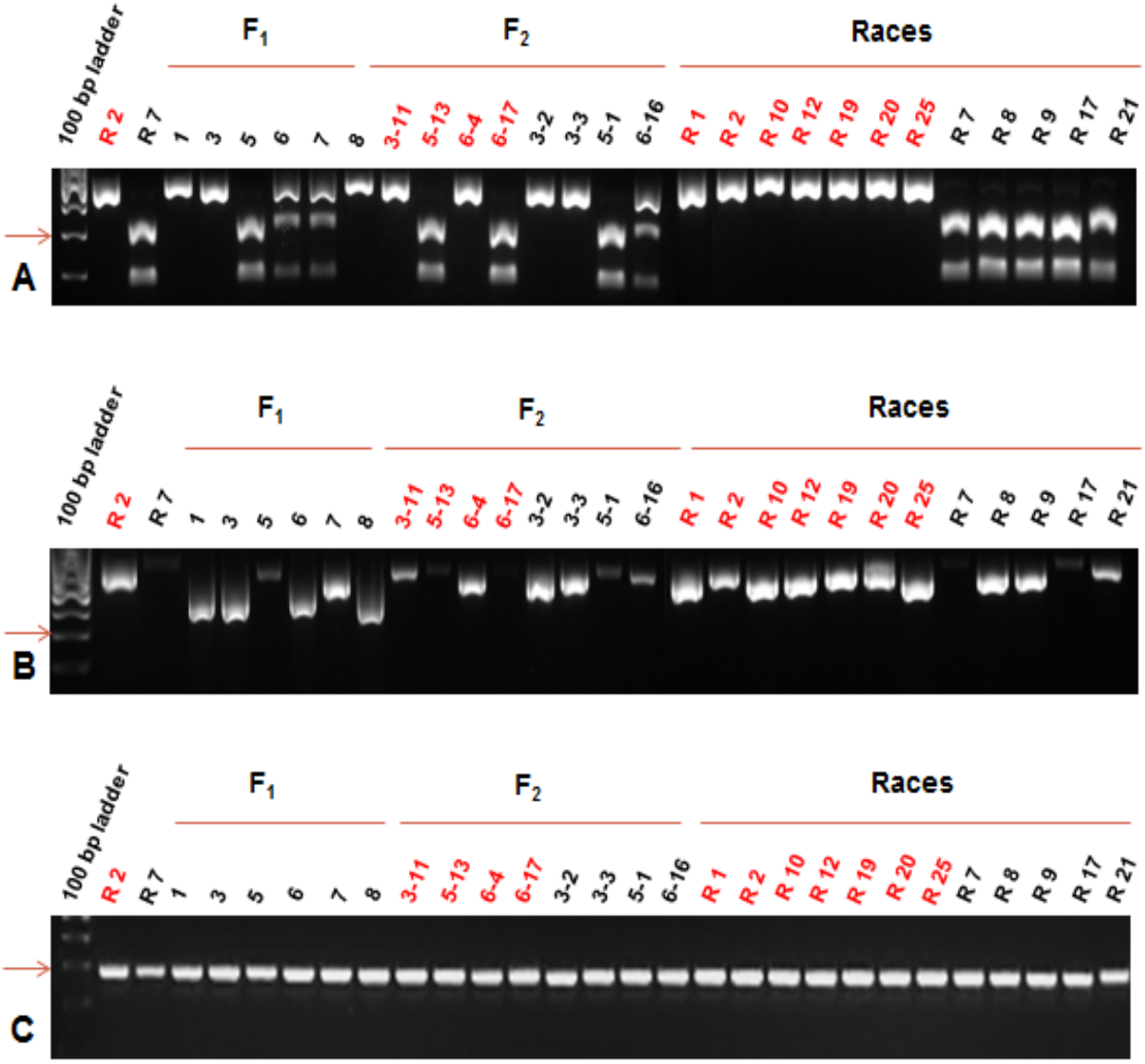

Figure 14: Candidate gene (*Avh172)* screening in *P. sojae* races and F_1/F_2 populations (race 2 x race 7).

(A) CAPs digestion of gDNA using *Avh172* marker (B) RT-PCR of cDNA using *Avh172* primers. (C) RT-PCR of cDNA using *P. sojae Actin* primers as a control for transcript expression. R= race. F_1 and F_2 progeny derived from *P. sojae* race 2 x race 7 cross. Avirulent isolates are labelled red, virulent isolates are labelled black. The F_1 cultures F_1-3, F_1-5, and F_1-6 were originally scored as avirulent to *Rps2* (see Table 7) but phenotypes had switched to virulent by the time this experiment was conducted. Arrow represents 300 bp on ladder. Marker size details in Table 3.

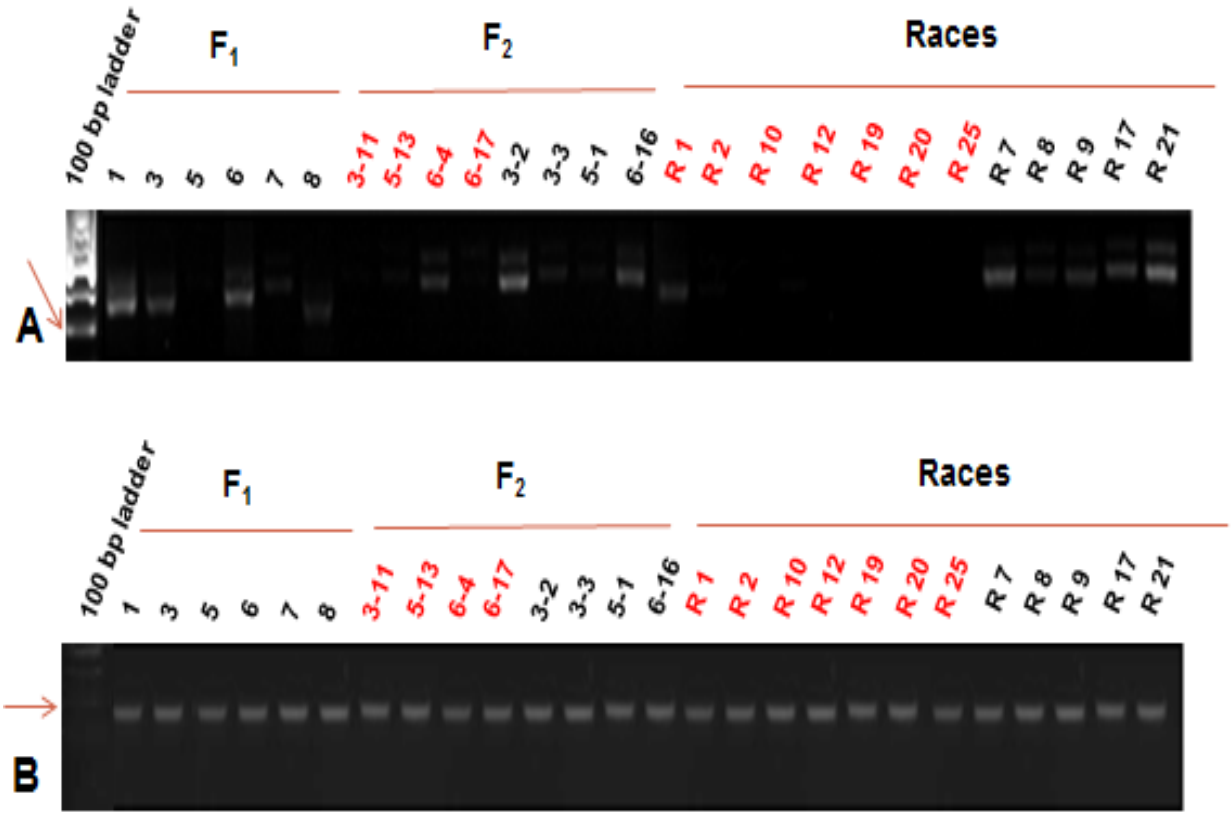

Figure 15: Candidate gene (*Cand2)* transcript screening in *P. sojae* races and F_1/F_2 populations (race 2 x race 7).

(A) RT-PCR of cDNA using *Cand2* primers. (B) RT-PCR of cDNA using *P. sojae Actin* primers as a control for transcript expression. R= race. F_1 and F_2 progeny derived from *P. sojae* race 2 x race 7 cross. Avirulent isolates are labelled red, virulent isolates are labelled black. The F_1 cultures F_1-3, F_1-5, and F_1-6 were originally scored as avirulent to *Rps2* (seeTable 6) but phenotypes had switched to virulent by the time this experiment was conducted. Arrow represents 300 bp on ladder. Marker size details in Table 3

4 Discussion

4.1 Outcrossing limitations

Identification and characterization of *P. sojae Avr* genes is a vital step to determine the still largely unknown mechanisms in involved in host-resistance, virulence targets, and pathogen diagnostics. This knowledge is important for crop protection. The primary objective of this study was to identify the predicted avirulence gene that is responsible for triggering resistance in soybean *Rps2* cultivars, thereby named the *Avr2* gene. *P. sojae*, a homothallic oomycete, offers an advantage over other heterothallic plant pathogens by enabling the use of F_2 progeny for genetic mapping of avirulence traits. All known *P. sojae Avr* genes have been identified based on mapping approaches that traced the segregation of the avirulence gene in F_2 progeny using phenotyping and genetic markers (Na et al., 2013). Other advantages of homothallic species in genetic analyses are the rarity of recessive lethal mutations and the fact that the majority of strains retain pure-breeding states and are mostly homozygous due to repeated self-fertilization (Tyler et al., 1995). A major hindrance encountered in this study was the generation of F_1 populations from outcrossing *P. sojae* strains. Low numbers of hybrids were obtained because most oospores resulted from self-fertilization events. Low rates of oospore germination were also observed. This is not unusual as many studies involving oospore manipulations have been limited by low frequency germination (Hord & Ristaino, 1991). In addition, germination is usually asynchronous in laboratory settings, most likely due to the lack of signals by plant and other environmental factors that induce sporulation in nature. Aside from these conditions, slight changes to optimal nutrition, light, humidity and incubation time can drastically affect the success of meiosis events and sporulation in the lab which varies among *Phytophthora* species (Judelson & Blanco, 2005). Many sporulation and germination trials using optimized conditions were done on both genetic crosses used in this study to acquire F_1 progeny. However, very few F_1 progeny were recovered. Previous mapping approaches have utilized race 2 and race 7 as parents in genetic crosses (Qutob et al., 2009; Tyler et al., 1995) and successfully demonstrated their sexual compatibility through the creation of F_1 and F_2 progeny. During this study I noticed that race 7 has a weak mycelial growth rate compared to race 2 which could explain why many isolated oospores resulted from race 2 self-fertilizations (Figure 5). Parents of the second cross, race 10 and race 17, have never successfully crossed, to my knowledge. Germination frequency

for this cross was much higher than the previous but produced no F_1 hybrids. The vast majority of oospores were race 10 self-fertilized progeny. Like the previous cross, this indicates that race 10 likely outcompetes race 17 in growth and sporulation during the co-cultivation conditions used to generate oospores.

4.2 Non-Mendelian inheritance of *Avr2*

Based on the gene-for-gene interaction widely used in plant pathology, *Avr2* is predicted to exist in *P. sojae* strains that are avirulent towards *Rps2* cultivars. This gene was expected to segregate as a single dominant allele following Mendel's Law, as observed for many other *P. sojae Avr* genes. Virulence assays of *P. sojae* strains show clear, qualitative variation and support the existence of an *Avr2* effector eliciting hypersensitive defense. However, the results from the cross *P. sojae* race 2 x race 7 indicate that this trait is not inherited in a Mendelian fashion. The six F_1 progeny that were isolated from race 2 x race 7 cross segregated for virulence towards *Rps2*. This finding is unusual because *Avr* genes are normally dominant, and heterozygous F_1 progeny are expected to uniformly avirulent. Segregation in the F_2 progeny was also unusual and did not match any kind of Mendelian model. Putting aside the phenotypic switching of the progeny, which is discussed later, F_2 populations derived from *Rps2*-virulent F_1s were true breeding and uniformly virulent, whereas F_2 populations derived from *Rps2*-avirulent F_1s segregated for the trait. Although unusual, these results are similar to a recent study on the inheritance of the expression and virulence determined by the *Avr*3a gene (Shrestha, 2014). Earlier studies have also observed unexpected inheritance patterns for other *Avr* genes in *P. sojae* (Tyler et al., 1995; Whisson et al., 1994)

The cause of the unusual inheritance and segregation of *Rps2*-virulence in the *P. sojae* cross race 2 x race 7 is not known. Though the majority of *P. sojae* strains are predicted to be homozygous (Tyler et al., 1995), it is possible that one or both parents of the cross are heterozygous for loci that control this trait. Gene conversion occurring in hybrid progeny is another possible explanation that could account for the unusual segregation results. However, the phenotype switching of the progeny suggest that epigenetic factors may control expression of the predicted *Avr2* gene and cause the unusual segregation patterns and phenotypic instability. Epigenetic control of *Avr* gene expression has been proposed as a mechanism that enables plant pathogens

to adapt to changes in host immunity (Dong et al., 2011; May et al., 2002; Na et al., 2014; Qutob et al., 2009; Qutob et al., 2013). Therefore, there are exceptions to the "single dominant" allele model for *Avr* genes behaviour in *P. sojae*.

4.3 Candidate *Avh* gene selection using sequence polymorphic data

The *P. sojae* genome encodes a large number of *Avh* genes. This superfamily of rapidly evolving RXLR effector proteins is a distinguishing feature of *Phytophthora* species (Jiang et al., 2008). Sequence polymorphisms in *Avh* genes are prevalent and, in many cases, have been shown to underlie changes in virulence. In fact, other *P. sojae Avr* genes have been identified based on sequence variants in pre-selected candidate genes (Dong et al., 2009; Dong et al., 2011; Na et al., 2013). The 29 *Avh* genes selected as *Avr2* candidates for this study were identified from existing comparative sequence and expression data of *Avh* genes in specific *P. sojae* strains. Five *Avh* genes (*Avh437, Avh264, Avh172, Avh163, Avh29*) in race 17 and race 7 contain high levels of sequence polymorphisms in comparison to the reference strain race 2. These genes were of special interest because they displayed amino acid polymorphisms which can affect recognition by plant *R* genes by altering the protein surface on a conserved backbone or through other overall differences in protein structure (Dodds et al., 2006). The *Avh172* gene emerged as a prime candidate for *Avr2* because it was also identified from the RNA-Seq analysis of *Rps2*-virulent versus avirulent strains. However, none of the candidate *Avh* genes co-segregated with the *Rps2*-virulence trait in the progeny from the race2 x race 7 cross. This result is disappointing but it is clear that the candidate gene approach is not a fail-safe method for identifying new *Avr* genes. Gene, transcript, and protein predictions based on sequence data are helpful but nonetheless are incomplete and hypothetical. It is also possible that the *Avr2* gene does not encode an RXLR effector protein and thus would not be on a list of *Avh* gene candidates. Although all known *Avr* genes from *Phytophthora* species encode RXLR effectors, the *ATR5* avirulence gene from the oomycete *Hyaloperonospora arabidopsidis* encodes an effector protein that lacks this motif (Bailey et al., 2011). The uncertainties of choosing genes *a priori* can be avoided by *de novo* mapping approaches, which are a more robust and unbiased method of *Avr* gene identification. In addition, deep sequencing and bioinformatics technologies offer new a useful methods to assist *Avr* gene identification, as illustrated here and in earlier studies (Na et

al., 2014). Four candidate genes markers (*Avh8, Avh270, Avh124, and Avh232*) were randomly selected and tested for co-segregation with the *Rps2*-virulence trait in F_2 individuals. These F_2 isolates were selected based on the consistent phenotype scores despite the high degree phenotypic variations observed in other progeny. As marker co-segregation analyses rely on reliable phenotypic data, these negative co-segregation data could indicate that the candidate does not correspond to *Avr2* or that phenotype scores were incorrect.

4.4 Possible epigenetic control of *Avr2* gene expression

Epigenetic inheritance refers to heritable alterations in gene expression that do not occur as a result of DNA sequence mutations. Factors such as DNA methylation, modifications to DNA-binding proteins or non-coding DNA or RNA splicing factors can cause stochastic variation in the expression of genes, even in clonally reproducing populations. Epigenetic variation can contribute to phenotypic diversity and be subject to the forces of natural selection (Baquero, 2013). Epigenetic mechanisms are reversible and typically offer more rapid rates of change than DNA mutations (Kasuga & Gijzen, 2013; Soyer et al., 2014). For instance, epigenetic mutations (cytosine methylation) in the model plant *Arabidopsis thaliana* occur nearly 1000 times faster than those derived from single-base-pair DNA substitutions and are determined to underlie phenotypic variation (Becker et al., 2011). Some epigenetic modifications have the potential to become permanent genetic mutations if they are evolutionarily advantageous to the organism; however, the mechanisms involved in the conversion are not well understood (Gijzen et al., 2014). Epigenetic modifiers can alter chromatin and DNA structure and impact the flow of genetic information (Kasuga & Gijzen, 2013). In plant pathogenic organisms, epigenetic control of effector gene expression has recently drawn interest. For example, chromatin de-condensation by removal of histone H3 lysine 9 methylation is reported to be a transcriptional determinant of effector gene expression during primary infection in fungi (Soyer et al., 2014). Epigenetic systems in *P. sojae* are proposed to play a major role in gain of virulence changes observed for *Avr* genes encoding effector proteins (Dean et al., 2014; Gijzen et al., 2014; Hofer, 2013; Na et al., 2014). *Avr* genes are often positioned in repetitive, transposon-rich regions which increase the likelihood of gene duplication, recombination and mutations that lead to amino acid variations in effector proteins. Moreover, the close association between *Avr* genes and

transposons can potentially facilitate the transfer of epigenetic switches used to regulate transcription. Epigenetic regulators, such as histone methyltransferases, are also known to be embedded in repetitive, transposon-rich segments and to be highly polymorphic (Gijzen et al., 2014; Vleeshouwers & Oliver, 2014).

The instability of the *Rps2*-virulence trait in the F_1 and F_2 populations developed from crossing race 2 x race 7 could be explained by stochastic epigenetic changes in transcription of *Avr2*. Shan et al. (2004) suggest that the loss of transcription of *Avr* genes may be common in *Phytophthora* species, and serve as a means to defeat *R*-gene mediated resistance. Moreover, epigenetic changes in *Avr2* transcript and expression could be the cause of changing virulence phenotypes (avirulence to virulence, and vice-versa) reported in *P. sojae* and *P. infestans* (Rutherford et al., 1985; Samen et al., 2003). Stably inherited gene silencing, which can account for the consistently virulent F_2 populations derived from virulent F_1s (F_1-1, F_1-7 and F_1-8), has also been reported for *P. sojae Avr* genes (Jiang et al., 2006; Na et al., 2014; Qutob et al., 2013). Thus, although *Avr* gene silencing is a fascinating epigenetic phenomenon, it could be responsible for the unusual results reported here and make the mapping of *Avr2* extremely difficult. Exploring new crosses using different parental strains may offer a way forward for future work on *Avr2*.

Conclusions

In summary, the *P. sojae Avr2* gene could not be identified by the methods used in this study. Genetic crosses were performed on strains that differ in virulence towards *Rps2* soybean plants in order to determine the inheritance of *Avr2*, which was expected to segregate as a single, dominant allele. In one attempted cross (race 10 x race 17), no hybrid oospores were obtained. A total of six F_1hybrids were recovered from a second cross (race 2 x race 7) and used to create F_2 populations. Results show that virulence towards *Rps2* is inherited in an unusual manner that does not follow typical Mendelian patterns. The instability of the virulence trait in the F_1 and F_2 progeny suggest that stochastic epigenetic changes may control the expression of *Avr2*.

Candidate *Avh* genes potentially encoding *Avr2* were selected based on polymorphism frequencies (SNPs and amino acid mutations) that differ between virulent strains (race 7 and race 17) and avirulent strain (race 19) in comparison to the *P. sojae* reference strain race 2 (also avirulent). Of the 29 candidates selected, *Avh*172 was especially promising because of polymorphic pattern that it displayed and its differential expression in *Rps2*-virulent and –avirulent *P. sojae* strains. However, the *Avh172* gene did not co-segregate with the virulence trait in the F_1 and F_2 progeny. Expression of the *Avh172* transcript was also tested, but again, no association with virulence to *Rps2* was discovered. A second candidate gene, *Cand2*, was identified from the RNA-Seq analysis and chosen for further study because it coded for a small secreted protein with a potential link to the RXLR effector family. However, expression of *Cand2* in the F_1 and F_2 progeny did not correlate with *Rps2* virulence phenotypes. Although these methods of pre-selecting candidate genes have been used with some success in the past to identify *Avr* genes they can be flawed due to the reliance on hypothetical and incomplete data sets. In addition, the unusual inheritance pattern of the virulence trait made it difficult to track *Avr2* by conventional genetic mapping techniques. Therefore, attempting new crosses using different *P. sojae* parental strains could be helpful in future quests for *Avr2*.

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