AGUIDA MARIA ALVES PEREIRA MORALES

CARACTERIZAÇÃO MOLECULAR DA RESISTÊNCIA À FERRUGEM ASIÁTICA DA SOJA MEDIADA PELO GENE *Rpp4*

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Genética e Melhoramento, para obtenção do título de *Doctor Scientiae*.

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Aprovada em 18 de julho de 2011

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“A fé em Deus nos faz crer no incrível,
ver o invisível e realizar o impossível”

Autor Desconhecido

À minha grande amiga Selma Pereira dos Santos (*in memoriam*),
que sempre será meu exemplo de coragem e determinação.
Sua dedicação pela ciência ficará sempre em minha memória.
Saudades...

Dedico.

Ao meu esposo Alan Alves Pereira.
Obrigada por estar sempre presente na minha vida.
Te amo!

Ofereço.

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Biografia

AGUIDA MARIA ALVES PEREIRA MORALES, filha de Vivaldo Morales e Julia Elisabeth Rodrigues Morales, nasceu em Piraju, São Paulo, em 16 de dezembro de 1981. Possui graduação em Ciências Biológicas pelo Centro Universitário Filadélfia (UniFil), em Londrina, Paraná (conclusão em 2004). Durante a graduação (2002-2004) foi bolsista de Iniciação científica do CNPq no laboratório de Biotecnologia Vegetal da Embrapa-Soja em Londrina, Paraná. No ano de 2005 ingressou no Mestrado em Agronomia com área de concentração em Genética e Melhoramento de Plantas na Universidade Estadual Paulista - Campus Jaboticabal (UNESP/FCAV), em Jaboticabal, São Paulo. Desenvolveu a parte prática da sua dissertação no laboratório de Biotecnologia Vegetal da Embrapa-Soja e concluiu o curso em Fevereiro de 2007. Em Agosto de 2007 ingressou no curso de Doutorado em Genética e Melhoramento da Universidade Federal de Viçosa em Viçosa, Minas Gerais. Teve a oportunidade de desenvolver parte do seu projeto de Tese em parceria com a Embrapa-Soja, Iowa State University (ISU) e Departamento de Agricultura dos Estados Unidos (USDA) em Ames, Iowa, EUA. Defendeu a Tese de doutorado no dia 18 de julho de 2011 na Universidade Federal de Viçosa.

Sumário

Resumo

MORALES, Aguida Maria Alves Pereira, D.Sc., Universidade Federal de Viçosa, Julho de 2011. **Caracterização molecular da resistência à ferrugem asiática da soja mediada pelo gene *Rpp4*.** Orientador: Aluízio Borém de Oliveira. Co-orientadores: Ricardo Vilela Abdelnoor e Ney Sussumu Sakiyama.

Práticas de gerenciamento são essenciais para o controle da ferrugem. O principal método de controle utilizado é a aplicação de fungicida, o qual aumenta substancialmente o custo de produção e são prejudiciais ao ambiente. A prevenção ainda é a melhor maneira de evitar perdas na produção de soja. Alternativas como plantar cultivares resistentes ao fungo também são importantes. O uso de variedades resistentes ou tolerantes é o método mais promissor para o controle da ferrugem asiática, cinco locus dominantes de resistência tem sido descrito na literatura: *Rpp1, Rpp2, Rpp3, Rpp4* e *Rpp5*. Entretanto pouco se sabe sobre a interação molecular desencadeada pela reconhecimento do patógeno pela planta em relação a ferrugem asiática. Entender os mecanismos moleculares envolvidos na resposta de defesa é de primária importância no planejamento de estratégias de controle do estresse e para consequente aumento da adaptação da planta. O *Rpp4* foi mapeado no grupo de ligação G da soja (cromossomo 18) e o sequenciamento desta região no genótipo suscetível Williams82 (Wm82) e resistente (PI459025B) identificou um cluster de genes de resistência CC-NBS-LRR. Meyers et al., (2009) desenvolveu construções para silenciamento induzido por vírus a partir das regiões NBD e LRR dos genes candidatos *Rpp4* no genótipo Wm82 para testar se os genes parálogos são responsáveis pela resistência no genótipo resistente (PI459025B). Neste estudo, o RNA foi extraído de plantas silenciadas *Rpp*4LRR e de plantas controle (Vetor vazio). Análises transcricional de 3 replicatas biológicas foi feita utilizando GeneChip® Soybean Genome Array (Affymetrix®). Um total de 383 genes foram encontrados ser diferencialmente expressos entre plantas *Rpp4* silenciadas e plantas não silenciadas (controle) quando infectadas com *P. pachyrhizi*. Dos 383 genes diferencialmente expressos, 22 foram induzidos e 361 foram reprimidos. Além disso, utilizando a ferramenta Clover (cis-element over representation) e TRANSFAC (transcription factor database) identificamos 33 sitíos para fatores de transcrição presentes nos

promotores dos genes diferencialmente expressos. Finalmente, para elucidar quais os genes são exclusivamentes mediados pela sinalização do *Rpp4*, nós comparamos os resultados de nosso experimento com os resultados de microarranjos oriundos de *Rpp2*, *Rpp3* e *Rpp4* resistente e suscetível. Nós identificamos 101 genes exclusivos. Além disso, com o objetivo de se obter maior informação sobre a função do *Rpp4* nós utilizamos PCR quantitativo em tempo real (RT-qPCR) para analisar a expressão de todos os genes *Rpp4* em diferentes tecidos da planta, diferentes estágios de desenvolvimento e depois da inoculação com *P. pachyrhizi*. Nós desenvolvemos um par de primers no domínio NBD que nós permitiu monitorar a expressão de todos os genes. O sequenciamento direto dos produtos originados no RT-qPCR nos permitiu diferenciar entre os 10 genes. Além disso nós examinamos a ocorrência de splice alternativo do gene *Rpp4* na soja sob efeito da inoculação.

Abstract

MORALES, Aguida Maria Alves Pereira, D.Sc., Universidade Federal de Viçosa, July, 2011. **Molecular characterization of resistance to Asian soybean rust mediated by *Rpp4***. Adviser: Aluízio Borém de Oliveira. Co-advisers: Ricardo Vilela Abdelnoor and Ney Sussumu Sakiyama.

Effective management practices are essential for controlling rust outbreaks. The main control method used is the application of fungicides, which substantially increase the cost of production and are harmful to the environment. Prevention is still the best way to avoid more significant losses in soybean production. Alternatives, such as planting resistant varieties to the fungus, are also important. The use of resistant or tolerant varieties is the most promising method for control of Asian soybean rust. Recently, five single dominant genes to specific soybean rust isolates were described: *Rpp1, Rpp2, Rpp3, Rpp4* and *Rpp5*. However, little is known about the molecular interaction between soybean and soybean rust and on the molecular pathway triggered by pathogen recognition. Understanding the molecular mechanisms involved in defense responses is of primary importance in planning strategies for controlling stress and consequently increasing plant adaptation to limiting conditions. Previously, *Rpp4* was mapped to soybean linkage group G (chromosome 18), and the sequencing of this region in the susceptible genotype Williams 82 (Wm82) and resistant (PI459025B) identified a cluster of CC-NBS-LRR resistance genes. Meyers et al. (2009) developed Virus Induced Gene Silencing constructs from the NBD and LRR regions of the Wm82 *Rpp*4 candidate genes to test whether paralogous genes were responsible for resistance in the resistant parent (PI459025B). In this study, RNA extracted from the same *Rpp4* LRR silenced and empty vector treated plants described by Meyer et al. (2009) was compared. Transcriptome analyses of three independent biological replicates was performed using the GeneChip® Soybean Genome Array (Affymetrix®). A total of 383 genes were found to be significantly differentially expressed between *Rpp4* silenced and no silenced plants infected with *P. pachyrhizi*. Of the 383 differentially expressed genes, 22 were induced, and 361 were suppressed. Using clover (cis-element over representation) software and the TRANSFAC (transcription factor database we identified 33 transcription factor-binding sites

significantly over represented in our differentially expressed genes when compared to all genes in the soybean genome, several with known roles in defense. Finally, to elucidate which genes are exclusively related to *Rpp4*-mediated signaling, we have compared the results of our experiment with microarray results from *Rpp2*, *Rpp3* and *Rpp4* resistant and susceptible reactions. We have identified 101 genes unique to the *Rpp4*-signaling pathway. In addition, in order to obtain more information about *Rpp4* function, we used real time quantitative PCR (RT-qPCR) to analyze the expression of all *Rpp4* genes in different plant tissues, in different stages of development and after inoculation with *P. pachyrhizi*. We have developed a single pair of primers from the NBD domain that allow us to monitor the expression of all ten genes. Direct sequencing of the RT-qPCR product differentiates between the ten genes. In addition we examined the occurrence of alternative splice *Rpp4* gene under inoculation effect.

Chapter I

Review: Advances on molecular studies of the interaction soybean - Asian rust

Revisão: Avanços dos estudos moleculares da interação da soja - ferrugem asiática

Abstract

Effective management practices are essential for controlling rust outbreaks. The main control method used is the application of fungicides, which substantially increase the cost of production and are harmful to the environment. Prevention is still the best way to avoid more significant losses in soybean production. Alternatives, such as planting resistant varieties to the fungus, are also very important. The use of resistant or tolerant varieties is the most promising method for control of Asian soybean rust. Recently, five single dominant genes to specific soybean rust isolates were described; *Rpp1, Rpp2, Rpp3, Rpp4* and *Rpp5*. However, little is known about the molecular interaction between soybean and soybean rust and on the molecular pathway triggered by pathogen recognition. Understanding the molecular mechanisms involved in defense responses is of primary importance in planning strategies for controlling stress and consequently increasing plant adaptation to limiting conditions.

Key words: *Phakopsora pachyrhizi*, resistance, plant-pathogen, molecular biology.

Resumo

Práticas efetivas são necessárias para o controle da ferrugem. O principal método de controle utilizado é a aplicação de fungicidas, o que aumentará substancialmente o custo de produção e são prejudiciais ao meio ambiente. A prevenção ainda é a melhor maneira de evitar mais perdas significativas na produção de soja. Alternativas, como o plantio de variedades resistentes ao fungo, também são importantes. O uso de variedades resistentes ou tolerantes é o método mais promissor para o controle da ferrugem asiática da soja. Recentemente, cinco genes de resistência a ferrugem da soja foram descritos *Rpp1, Rpp2, Rpp3, Rpp4* e *Rpp5*. No entanto, pouco se sabe sobre a interação molecular entre a planta de soja e o fungo da ferrugem asiática e as rotas desencadeadas na planta pelo reconhecimento do patógeno. Compreender os mecanismos moleculares envolvidos nas respostas de defesa é de primordial importância no planejamento de estratégias para controle do estresse e, consequentemente, para aumentar a adaptação das plantas a condições limitantes.

Palavras chave: *Phakopsora pachyrhizi*, resistência, planta-patógeno, biologia molecular.

Asian Soybean Rust

Asian Soybean Rust (ASR) is caused by *Phakopsora pachyrhizi* Syd. & Syd; uredial anamorph; *Malupa sojae* (syn. *Uredo sojae*); Domain Eukaryota; Kingdom Fungi; Phylum Basidiomycota; Order Uredinales; Class Urediniomycetes; Family Phakopsoraceae; Genus *Phakopsora* (Index Fungorum 2010). Rust is considered a polycyclic disease. The fungus is able to complete several generations in a single cycle of the host. Temperatures and humidity that favor the growth and development of soybean plants also favor the development of rust (Zambolim 2006). According to Freire et al. (2008) the South and North American continents were free of *P. pachyrhizi* until 2001. Then *P. pachyrhizi* was first reported in Paraguay (Morel and Yorinori 2002), and became established in Bolivia, Argentina (Rossi 2003) and Brazil (Yorinori et al. 2005) in 2002/2003. In 2004, ASR was reported for the first time in the USA (Schneider et al. 2005). These authors estimated that the disease caused yield losses varying from 10 to 80%.

According to Ono et al. (1992) *P. pachyrhizi* and *Phakopsora meibomiae*, the American rust, have wide host ranges and are able to sporulate on 31 species in 17 genera of leguminous plants. Rust samples taken from wild host plants are able to infect a broad range of plant species in greenhouse environments (Jarvie 2009). Recently, new host species from 25 genera were identified in greenhouse evaluations, including 12 genera that had not been reported previously (Slaminko et al. 2008). The presence of a susceptible host, viable pathogen spores and suitable environmental conditions are prerequisites for the development of a soybean rust epidemic. The optimum temperature for urediniospore germination ranges between 12 and 27°C. Urediniospore germination is greater in darkness and requires a period of leaf moisture. Germination takes about 6 hours in optimum temperature and moisture conditions (Kochman 1977).

First symptoms of ASR could be described as small water soaked lesions that develop into grey, TAN to dark brown, or reddish brown (RB) lesions (uredinia) particularly on abaxial leaf surfaces (Sinclair 1989). The color of the lesion is dependent on lesion age and interaction with the host genotype. RB lesions with little sporulation indicate a resistant reaction, whereas TAN lesions with much sporulation indicate a susceptible reaction. The number of pustules per lesion

increases with lesion age and groups of spores (urediniospores) are expelled from each pustule (uredinia) through a central pore (Sinclair 1989).

The disease destroys leaf tissue resulting in reduced photosynthetic activity, premature defoliation and reduced life cycle. In addition, the premature leaf abscission prevents grain maturation (Sinclair 1989) and rust infection during pod formation or seed fill can cause embryo abortion and pod abscission (Yorinori et al. 2005). The cumulative effect of rust on production translates into lower seed weight and reduces the number of pods and seeds (Sinclair 1989).

P. pachyrhizi forms asexual uredospores on short stalks within a uredium 5-8 days after inoculation on colonized leaves. Uredospores are released from uredia through an ostiole and dispersed by wind. Under appropriate conditions, uredospores germinate a single germ tube and the penetration occurs directly thought epidermis, but can also occur through stomatal openings (Zambolim 2006). Penetration by *P. pachyrhizi* starts with the formation of a funnel-shaped structure, termed the appressorial cone, within the appressorium. This cone is contiguous with the cell wall of the penetration hypha, which is also referred to as the transepidermal vesicle. On penetration, the epidermal cells collapse, become disorganized and show signs of cell death (Panstruga 2003, Mendgen et al. 2006). After penetration, the hypha grows through the epidermal cell and reaches the intercellular space. The primary hypha may branch to form secondary hypha and finally, haustorium mother cells differentiate in close contact with mesophyll cells. The haustorium provides a wide contact surface within the host cell for acquisition of sugars and amino acids through a symport proton gradient (Mendgen et al. 2006).

A recent study depicted a two-year field trial in Brazil where soybean rust was responsible for 37-67% of soybean seed yield losses (Kumudini et al. 2008). This study agreed with yield losses observed in Asia up to 80% (Miles et al. 2003). Effective management practices are essential for controlling rust outbreaks. The main control method used is the application of fungicides, which substantially increase the cost of production and are harmful to the environment (Zambolim 2006). The chemical group most often used for rust control is a mixture of strobilurin and triazole.

Prevention is still the best way to avoid more significant losses in soybean production. One method is to offset the timing of soybean production and pods reach maturity in condition that do not favor *P. pachyrhizi*. In addition, lowering inoculum levels by implementation of a soybean-free period is important. Alternatives such as using resistant varieties to the fungus also are important. However, resistance does not mean that the disease does not occur, but it allows greater stability and efficiency of chemical control (Anuário Brasileiro de Soja 2009). Recently soybean cultivars resistant to the fungus were released in Brazil. These varieties boast characteristics that curb fungal growth and ensure higher production stability, reducing the losses induced by the disease, and the environmental impacts caused by repeated fungicide applications.

Molecular interaction between soybean and ASR

Immunity to *P. pachyrhizi* occurs when no visual lesions are produced by the soybean plant. A resistant response leads to the formation of RB lesions indicating a hypersensitive reaction. A susceptible response occurs when TAN lesions develop indicating fungal growth and development. The genetics of resistance of five single dominant genes to specific soybean rust isolates has been described: *Rpp1, Rpp2, Rpp3, Rpp4* and *Rpp5* (Bromfield and Hartwig 1980, Mclean and Bith 1980, Hartwig and Bromfield 1983, Hartwig 1986, Garcia et al. 2008).

In order to identify new sources of resistance in soybean, Miles et al. (2006) evaluated the entire United States Department of Agriculture (USDA) germplasm collection (16,000 accessions) against a mixture of five *P. pachyrhizi* isolates. After two rounds of evaluation, only 850 accessions were identified with even partial tolerance or resistance reactions to *P. pachyrhizi*, which correlates to less than 5% of USDA germplasm collection.

Resistance alleles, different than those already described in the literature were also identified in several other genotypes (Laperuta et al. 2008, Pierozzi et al. 2008). When the disease was first detected in Brazil, all the described resistance genes were effective against the fungus. However in 2003, a new race of *P.*

pachyrhizi broke the resistance conferred by genes *Rpp1*, and *Rpp3*, while *Rpp2*, *Rpp4* and *Rpp5* remain resistant (Arias et al. 2004).

Although *Rpp2*- and *Rpp4*-mediated resistances have been stable in Brazil (Hartman et al. 2005), single, dominantly inherited *R* gene-mediated resistance against *P. pachyrhizi* has been overcome in nature several times because of the great capacity of the fungus to develop new races. Generally, this scenario of the breakdown of *R* gene-mediated resistance is known as the 'boom and bust' syndrome. In addition to pyramiding known *Rpp* resistance genes into modern cultivars to create a more durable and broad-spectrum disease resistance, the recruitment of novel sources of resistance to *P. pachyrhizi* is desirable (Goellner et al. 2010).

Along with single gene resistance, partial resistance to soybean rust has been described (Hartman et al. 2005). This kind of resistance may be controlled by minor genes and may be expressed as reduced uredinial number and size, a longer latent period, and other components related to fungal reproduction. Recently, the average number of uredinia per lesion and average uredinial diameter were reported to be components of partial resistance in soybean rust and were a reflection of fungal growth in the host tissue (Bonde et al. 2006).

All described *Rpp* genes have been already mapped on soybean chromosomes (Chr), *Rpp1* was mapped on chromosome 18, *Rpp2* on Chr 16, *Rpp3* on Chr 6, *Rpp4* on Chr 18 and *Rpp5* on Chr 3, (Garcia et al. 2008, Hyten et al. 2007, Hyten et al. 2009, Silva et al. 2008b) Additionally, some alleles have been mapped to the same chromosomes, *Rpp1b* was mapped on Chr 18, *Rpp? Hyuuga* on Chr 6 (Chakraborty et al. 2009, Monteros et al. 2007)

Despite the physical location of the *Rpp* genes and the recent release of the soybean genome (Schmutz et al. 2010), none of them have been cloned yet. However, significant progress has been made towards cloning *Rpp4*, which has remained the most stable when challenged against isolates from different parts of the world (Bonde et al. 2006, Yamaoka et al. 2002). Sequencing of the mapped region in the susceptible genotype Williams 82 identified a cluster of three CC-NBS-LRR (coiled-coil, nucleotide-binding site, leucine-rich repeats) resistance genes within a 2cM region on chromosome 18, which shows sequence similarly to the lettuce RGC2 family of resistance genes (Meyer et al. 2009). In addition, VIGS

(Virus-induced gene silencing) demonstrated that silencing of the *Rpp4* candidate genes diminished resistance in PI459025B (that carries *Rpp4* resistance allele), confirming that one of the genes in the cluster is responsible for resistance.

There is clear evidence of the evolutionary forces acting on the *Rpp4* locus. Differences in gene number between Wm82 and PI459025B are likely due to duplication or unequal recombination. In addition, given the similarity of all *Rpp4* candidate genes between genotypes, it is possible that small amino acids differences may play a key role in resistance (Meyer et al. 2009).

Little is known about the molecular interaction between soybean and *P. pachyrhizi* and the defense pathways triggered by pathogen recognition. Understanding the molecular mechanisms involved in defense responses is of primary importance in planning strategies for controlling stress and consequently to increase plant adaptation to limiting conditions. The development of sequencing techniques and gene expression analysis on a large scale, combined with novel bioinformatics tools for data analysis have facilitated the structuring of extremely valuable databases for developing strategies for genetic engineering.

Given the rarity of Asian rust resistance in soybean, few genomic tools are available for examining *P. pachyrhizi* resistance in resistant (*R)* genomes. Therefore, rust resistance research has focused on genotype independent platforms like microarray analyses to identify genes involved in resistance and susceptibility. Van de Mortel et al. (2007) used the soybean affymetrix gene chip to study changes in gene expression in resistant and susceptible genotypes when inoculated with ASR. A biphasic gene response to *P. pachyrhizi* infection was seen in both genotypes. Differences in gene expression between inoculated and mock plants peaked at 12 hours post inoculation (hpi) and returned to almost basal levels by 24 hpi, in both resistant and susceptible genotype. At 72 hpi a second larger wave of defense gene expression could be observed, which was significantly earlier in the resistant than in the susceptible interaction. The early transcriptional response observed in susceptible and resistant plants might represent a general response of soybean to the nonspecific recognition of any pathogen, presumably by interaction with microbe-associated or microbe-induced molecular patterns (MAMPs and MIMPs) (Mackey and Mcfall 2006). By contrast,

the second response likely relates to R-gene detection of *P. pachyrhizi* (Posada-Buitrago and Frederick 2005, Tremblay et al. 2009).

In a similar approach, Panthee et al. (2007) identified genes that might be involved in a defense response against *P. pachyrhizi* by susceptible soybean cv.5601 plants 72h after infection (hai) using microarray. Most of the induced genes had defense and stress related functions such as genes encoding an SA-related protein, heat shock protein (HSP), leaf senescence-associated receptor like kinase, and chalcone synthase. Silva et al. (2008a) identified genes activated during resistant and susceptible interactions with the *P. pachyrhizi* in soybean (PI230970- *Rpp2* resistance). By analysis of cDNA microarrays, they identified 65 transcripts differentially expressed. These genes were involved in the production of reactive oxygen species, phytoalexins and antimicrobial proteins, cell death and senescence, modification, stabilization and protein degradation, control of gene expression and reinforcement of cell wall.

Recently, Pandey et al. (2011) combined the work of Van de Mortel et al. (2007) with VIGS, to screen 140 candidate genes that might play a role in *Rpp2*-mediated resistance toward *P. pachyrhizi*. This study identified 11 genes that compromised *Rpp2*-mediated resistance when silenced, including *GmEDS1*, *GmNPR1*, *GmPAD4*, *GmPAL1*, five predicted transcription factors, an O-methyl transferase, and a cytochrome P450 monooxygenase. Additionally, a large scale transcript profiling approach conducted with soybean plants (accession PI200492) has revealed an up regulation in gene expression for lipoxygenases and peroxidases in an incompatible interaction, suggesting an important function for these genes in *Rpp1*-mediated resistance (Choi et al. 2008).

Using laser capture microdissection, Tremblay et al. (2010) isolated susceptible soybean palisade and mesophyll cells showing signs of infection, extracted the RNA and performed transcriptome profiling. A total of 2,982 genes were differentially expressed, of which 685 were up regulated, and 2,297 were down regulated. Complementary to transcriptional analyses in the host, gene transcript profiling has also been performed with the fungus (Posada-Buitrago and Frederick 2005, Tremblay et al. 2009). A recent study of gene expression within *P. pachyrhizi* germinating spores allowed the identification of 488 unique expressed sequence tags (ESTs). One hundred eighty nine of these ESTs showed significant

similarly (E-value < 10^{-5}) to sequences deposited in the NCBI non-redundant protein database. These genes were assigned putative roles in primary metabolism, gene and protein expression, cell structure and growth, cell division, cell signaling and cell communication (Posada-Buitrago and Frederick 2005). Recently a cDNA library was constructed from uredinia separated from host tissue by laser-captured microdissection (Tremblay et al. 2009). About 80% of identified genes in this study shared no homology to previously described *Phakopsora* genes. This result demonstrates stage-specific gene expression in the development of uredinia.

While the techniques have proven effective at looking at genes downstream of *Rpp* genes, more research is needed to identify potential candidate genes that could be used to engineer sustainable resistance into soybean against *P. pachyrhizi.*

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Chapter II

Combining Transcriptome Analyses and Virus Induced Gene Silencing to Identify Genes in the *Rpp4*-mediated Asian Soybean Rust Resistance Pathway.

Abstract

Five Asian Soybean Rust (ASR) resistance loci have been identified and mapped in soybean genome: *Rpp1*, *Rpp2*, *Rpp3*, *Rpp4* and *Rpp5*. Of particular interest is *Rpp4*, mapped on chromosome 18, which has remained stable and confers resistance against *Phakopsora pachyrhizi* isolates from around the world.

Sequencing of the region harboring Rpp4 in the susceptible Williams 82 (Wm82) and resistant (PI459025B) genotype identified a cluster of CC-NBS-LRR resistance genes. Meyers et al. (2009) developed Virus Induced Gene Silencing (VIGS, Zhang et al. 2009) constructs from the NBD and LRR regions of the Wm82 *Rpp*4 candidate genes to test whether paralogous genes were responsible for resistance in the resistant parent (PI459025B). Twenty-one days after VIGS treatment, plants were inoculated with a spore suspension from *P. pachyrhizi* isolate LA04-1. Fourteen days later, plants were scored for resistance to *P. pachyrhizi.* As expected, the controls (no treatment, mock VIGS inoculation and empty VIGS vector) had no effect on resistance. However, the LRR and NBD constructs silenced *Rpp4*, leading to a susceptible response and confirming that a member of the R-gene cluster was responsible for resistance. In this study, RNA extracted from the same *Rpp4* LRR silenced and empty vector treated plants described by Meyer et al. (2009) was compared. Transcriptome analyses of three independent biological replicates was performed using the GeneChip® Soybean Genome Array (Affymetrix®). Since the plant samples differed only in the silencing of *Rpp4,* comparisons of these samples. Of the 383 differentially expressed genes, 22 were up-regulated genes sharing homology to known genes such as Pectin acetylesterase, Aspartyl protease, GDP mannose pyrophosphorylase, or phosphatidylinositol transfer protein PDR16. Also, of 361 genes were down-regulated genes identified, many with functions related to defense, disease

resistance and metabolism. Statistical analyses of overrepresented biological process and molecular function gene ontology functional categories highlighted the importance of genes involved in lignin biosynthesis, flavonoid biosynthesis, response to oxidative stress and phenylpropanoid biosynthesis for defense. To identify transcription factor active in the *Rpp4* signaling pathway, we used Clover (cis-element over representation) software and the TRANSFAC (transcription factor database) to identify transcription factor binding sites over-represented in the promoters of our differentially expressed genes. This study allow us also the identification of 33 transcription factor-binding sites significantly over represented in our differentially expressed genes when compared to all genes in the soybean genome, several with known roles in defense. Finally, to elucidate which genes are exclusively related to *Rpp4*-mediated signaling, we have compared the results of our experiment with microarray results from *Rpp2*, *Rpp3* and *Rpp4* resistant and susceptible reactions. We have identified 101 genes unique to the *Rpp4*-signaling pathway.

Key words: Asian soybean rust, soybean, expression.

1. Introduction

Asian Soybean rust (ASR) is caused by the obligate fungus *Phakopsora pachyrhizi* Sydow and was first reported in Brazil in 2001 (Yorinori et al., 2005). The disease is considered polycyclic, since the fungus is able to complete several generations in a single life-cycle of the host. Temperatures that favor the growth and development of soybean also favor the development of rust (Zambolin, 2006). The disease destroys leaf tissue, resulting in reduced photosynthetic activity, premature defoliation and reduced life cycle. The cumulative effect of rust on soybean production translates into lower seed weight and reduces the number of pods and seeds (Sinclair, 1989). Currently, *P. pachyrhizi* is one of the most important economical threats for soybean growers in South America. In Brazil, a recent study documented a two-year field trial that showed that rust was responsible for 37-67% of soybean seed yield losses (Kumudini et al., 2008).

Five major sources of *P. pachyrhizi* resistance have been identified in soybean: *Rpp1, Rpp2, Rpp3, Rpp4, Rpp5* (Bromfield and Hartwig, 1980; Mclean and Bith, 1980; Hartwig and Bromfield, 1983; Hartwig 1986; Silva et al., 2008; Garcia et al., 2008). Each of these loci has been genetically mapped on the soybean chromosome (Chakraborty et al., 2009, Garcia et al., 2008, Hyten et al., 2007, Hyten et al., 2009, Monteros et al., 2007, Silva et al., 2008). Futher, comparisons made between molecular markers and the recently released soybean genome (Schmutz et al., 2010) identified clusters of candidate resistance gene (R-gene) homologs. However, identifying the actual resistance genes has remained difficult. The most progress has been made in characterizing the *Rpp4* locus. Thus far, this locus has remained the most stable when challenged against isolates from different parts of the world (Yamaoka et al., 2002; Bonde et al., 2006). Sequencing of the 2 cM region in the susceptible genotype 'Williams82' (Wm82) identified a cluster of three coiled-coil nucleotide-binding site leucine-rich repeats (CC-NBS-LRR) resistance genes with similarity to the lettuce RGC2 family of NBS-LRR resistance genes (Meyer et al., 2009). Expression analyses of these genes in the resistant (PI459025B) and susceptible (Williams 82) parents revealed large differences in gene number and expression. Virus induced gene silencing using constructs developed from the Williams 82 *Rpp4* candidate genes silenced *Rpp4*

mediated resistance in the resistant parent PI459025B, indicating *Rpp4* is a member of the same gene cluster.

Recently transcriptomic techniques have been successful in characterizing soybean pathogen interactions to identify changes in host gene expression following inoculation. In soybean, transcriptomic approaches have identified genes involved in susceptibility and resistance against soybean cyst nematode (*Heterodera glycines), Phytophthora* stem and root rot (*Phytopthora sojae),* soybean mosaic virus (*Pseudomonas syringae)*, soybean aphid (*Aphis glycines)* and Asian soybean rust (*P. pachyrhizi) (*Alkharouf et al., 2006; Ithal et al., 2007; Moy et al., 2004; Zabala et al., 2006; Zou et al., 2005; van de Mortel et al., 2007). Standardized microarray platforms provide inexpensive, genotype independent means to associate gene expression with gene function. Further, microarray analyses accelerate the understanding of host pathogen interactions, because a large fraction of the genome can be analyzed simultaneously and different bioinformatics methods can be used to identify related groups of genes that are activated or repressed in various regulatory pathways (Kato-Maeda et al., 2001).

Transcriptomic approaches have been used by several groups to characterize resistance and susceptibility to *P. pachyrhizi*. Panthee et al. (2007; Panthee et al., 2009) used the Affymetrix® GeneChip® Soybean Genome array to examine *P. pachyrhizi* susceptibility in three stages of development of cultivar 5601T. Tremblay et al. (2010 a,b) used laser capture microdissection to characterize soybean mesophyll and palisade cells from *P. pachyrhizi* infected and uninfected leaves of Williams 82 (susceptible), Microarray analyses of RNA collected from these samples revealed induction of defense-related genes and repression of genes involved in plant metabolism. Transcriptomic approaches have also been used in an attempt to find novel sources of resistance. Soria-Guerra et al. (2010) used the soybean Affymetrix® chip to examine resistant and susceptible *Glycine tomentella* leaves, infected and mock-infected with *P. pachyrhizi*. Transcripts for genes involved in the phenylpropanoid pathway were up-regulated early during rust infection. Similarly, genes coding for proteins related to stress and defense responses such as glutathione-S-transferases, peroxidases, heat shock proteins, and lipoxygenases were consistently up-regulated following infection.

Perhaps the most comprehensive transcriptomic studies thus have been on *Rpp2* mediated defense. Van de Mortel et al. (2007) examined a seven day time course of ASR infection in resistant (mediated by *Rpp2)* and susceptible genotypes. A biphasic response to *P. pachyrhizi* was seen in both genotypes. At 12 hours post inoculation with ASR, both genotypes had induction of basal defense. However, 24 hours after infection, defense gene expression returned to mock-inoculated levels. At 72 hours post infection, a second round of defense gene expression occurred in the resistant genotype, likely due to *Rpp2*-mediated signaling. While this secondary defense response was also detected in the susceptible interaction, it did not occur until 96 hours post infection and never at the same magnitude observed in the resistant parent. Surprisingly, while greater levels of defense-related gene induction were observed in the resistant parent, greater numbers of differentially expressed genes were observed in the susceptible parent.

This work was followed by Pandey et al. (2011) who used virus induced gene silencing to try and disrupt the *Rpp2*-mediated signaling pathway in an *Rpp2* resistant genotype. The authors identified 140 candidate genes that could potentially be involved in *Rpp2-mediated* defense signaling using the work of van de Mortel et al. (2007) and soybean orthologs of known defense signaling genes and transcription factors. Eleven genes were identified in the *Rpp2*-mediated signaling pathway, required for *Rpp2*-mediated resistance. These included four soybean orthologs of known defense genes (*GmEDS1, GmNRP1, GmPAD4* and *GmPal1),* five predicted transcription factors (*GmWRKY36, GmWRKY40, GmWRKY45, GmDBTF* and *GmMYB84*), an O-methyl transferase (*GmO-MT*) and a cytochrome P450 (*GmCYP83E12*). Combining their results with data from other plant-pathogen systems allowed the characterization of *Rpp2* signaling cascade, even though *Rpp2* has yet to be cloned.

In this study we have combined the power of genomic, transcriptomic and virus induced gene silencing approaches to characterize the *Rpp4* signaling cascade. Previously, we silenced *Rpp4* using a candidate gene approach. Now, we are using microarray analyses to compare silenced plants and identify components of the *Rpp4* signaling pathway.

2. Materials and methods

2.1. Silencing of *Rpp*4 via virus induced gene silencing

The VIGS plants used in our analyses are the same plants described by Meyer et al. (2009). In brief, a portion of the LRR domain of the *Rpp4* candidate genes from Williams 82 was cloned into RNA2 of the BPMV VIGS vector (Zhang et al., 2009). Co-bombardment of BPMV RNAs 1 and 2 on Wm82 leaves was used to generate inoculum for further experiments. After three weeks, BMPV infected tissue was collected, lyophilized and shipped to the Foreign Disease-Weed Science Research Unit at Fort Detrick, Maryland. At Fort Detrick, the resistant soybean genotype PI459025B was grown in a growth chamber, and two weeks after germination, plants were rub inoculated with test VIGS constructs. Each construct was tested on six plants. Three weeks later, plants were inoculated with *P. pachyrhizi* isolate LA04-1. Two weeks later plants were evaluated for resistance. Controls included no treatment, mock inoculation and empty BPMV constructs. Three independent replicates of the experiment were performed. After the completion of each replicate experiment, leaves were collected from three LRR-BPMV VIGS plants and three empty-vector BPMV plants, all infected with *P. pachyrhizi*. This provided three biological replicates and three technical replicates to use for microarray analyses. Leaves were flash frozen in liquid nitrogen and stored at -80 C.

2.2. RNA extraction and isolation

The frozen leaf tissue described above was ground with a morter and pestle. RNA was extracted from the *Rpp4* LRR silenced plants and the empty vector BPMV plants using the Qiagen Plant RNeasy kit (Qiagen, no. 74903). RNA samples were subsequently treated with DNase (Ambion, AM1907). RNA concentration and quality were determined using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE) and by RNA Nano LabChip® on a 2100 Bioanalyzer (Agilent Technologies, Inc., Palo Alto, CA).

2.3. Microarray analyses

Labeling, hybridization, and scanning were performed at the Iowa State University GeneChip Facility. Labeled target cRNA was synthesized from 5 µg of total RNA using the GeneChip® One-Cycle Target Labeling and Control Reagents kit (Affymetrix, Santa Clara, CA) according to manufacturer's instructions. Fragment cRNA (10 µg) were hybridized to GeneChip® Soybean Genome Array (Affymetrix®, Santa Clara, CA) according to manufacturer's instructions. cRNA quality was verified on an Agilent 2100 BioAnalyzer equipped with an RNA Nano LabChip®. Microarrays were scanned with a GCS3000 7G scanner (Affymetrix, Santa Clara, CA).

2.4. Statistical analysis and array processing

Raw expression values from the .CEL files generated during array processing were read into R (R development core team 2006). The data was analyzed in the 'affy' background and corrected using the RMA function, normalized using the invariant set and summarized using the median polish command. Only perfect match probes were considered. Expression values were fit to a linear model using the limma package and a contrast matrix comparing treatments was applied. Expression values were corrected for multiple testing by an empirical Bayesian correction (eBayes) and fdr (false discovery rate). Genes differentially expressed between the *Rpp4* silenced plants and the empty vector treated plants were identified by a fold change equal to or greater than 1 or -1 and a P-value equal to or less than 0.05. Since the data is in log2 form, a fold change of 1 equals a 2-fold difference in expression between samples.

2.5. Annotation of differentially expressed probes

The differentially expressed genes were annotated using the SoyBase Affymetrix GeneChip® Soybean Genome Array Annotation page (version2) as described by van de Mortel et al. (2007, http://soybase.org/AffyChip). In short, the target sequences from which each of the ~37,000 probes were designed were

compared to the predicted cDNAs from the soybean whole genome assembly (version 1.0,Schmutz et al., 2010) using BLASTN (Altschul et al., 1997). If matching soybean cDNAs could not be identified or multiple cDNAs from multiple genes could not be distinguished, the Affymetrix consensus sequence was used in place of the soybean cDNA for further analyses. The soybean predicted cDNAs, and when necessary the Affymetrix consensus sequences, were compared to the UniProt protein database (version June 2008, Apweiler et al., 2004) and predicted cDNAs from the *A. thaliana* genome (version 8, The Arabidopsis Information Resource, www.arabidopsis.org) using BLASTX (E<10-6, Altschul et al., 1997). TAIR Gene Ontology (GO) terms (Berardini et al., 2004) were assigned based on the top *A. thaliana* sequence identified. Fisher's exact test (Fisher, 1966) with a Bonferroni correction (Bonferroni, 1935) was used to identify overrepresented Gene ontology categories or transcription factor classes.

2.6. Bioinformatics analysis of cis-elements

Each of the differentially expressed genes identified was assigned to a cDNA from the whole soybean genome assembly (Schmutz et al., 2010) using BLASTN (Altshul et al., 1997, E<10-30, percent identity >95). Using the coordinates of the corresponding soybean cDNA, custom perl scripts were used to extract 1000 bases of promoter sequence from whole soybean genome assembly. Clover (Frith et al., 2004), in combination with the TransFAC transcription factor matrix (Wingender et al., 1996), was used to identify overrepresented transcription factor binding sites in the promoters of the differentially expressed genes. As a background control, the results were compared to 1000 bases of promoter sequence from all predicted soybean genes excluding transposable elements.

In order to find novel transcription factor binding sites in the promoters of the differentially expressed genes, we used MEME (Multiple EM for Motif Elicitation, Bailey and Elkan, 1994). For MEME analysis, motif width was set from 6 to 9 basepairs, distribution of motifs was set for any number of motifs and the maximum number of motifs to report was set to 10. Since the soybean microarray chip only represents 37,000 transcripts, we used MAST (Motif Alignment & Search tool, Bailey and Gribskov, 1998) to search 1000 bases of promoter sequence from all

predicted soybean genes excluding transposable elements. This would identity genes not present on the array that could also be differentially expressed in response to *Rpp4.*

3. Results

3.1. Comparisons of gene expression in PI459025B in *Rpp*4 silenced plants and empty vec tor treated plants, each inoculated with *P. pachyrhizi.*

We previously developed VIGS constructs from the *Rpp4* locus in the susceptible parent, Williams 82 (Meyer et al., 2009). Based on mapping and sequencing data, we hypothesized that *Rpp4* in PI459025B was a member of NBS-LRR cluster characterized in Williams82. To test this hypothesis, we developed VIGS constructs from the LRR domain of the Williams82 R-genes. PI459025B plants, carrying *Rpp4*, were treated with the VIGS constructs and tested for changes in resistance to *P. pachyrhizi.* The LRR VIGS constructs silenced *Rpp4,* leading to susceptibility to *P. pachyrhizi* (Meyer et al., 2009), Mock treated plants and empty VIGS vector treated PI459025B plants maintained resistance to *P. pachyrhizi.* In this study, leaf tissue from these same experiments was collected and frozen to allow future studies of the *Rpp4*-signaling pathway.

3.2. Gene Expression from *Rpp4* silenced plants

RNA from the LRR-VIGS and empty vector VIGS PI459025B plants, both inoculated with *P. pachyrhizi* was hybridized to the Affymetrix soychip and analyzed for differential gene expression. These plants came from the same genotype (PI459025B), inoculated with the same *P. pachyrhizi* isolate, but treated with different VIGS constructs. Before *P. pachyrhizi* inoculation, but after treatment with the VIGS constructs, these plants should differ only in the expression of *Rpp4.* However, once inoculated with *P. pachyrhizi,* LRR-silenced plants cannot mount *Rpp4*-mediated defense. In contrast, the empty vector treated plants still have *Rpp4* expression and can mount a defense to *P. pachyrhizi.* By comparing RNA

from *Rpp4* silenced (LRR VIGS construct) and non-silenced plants (treated with empty vector), we should be able to identify genes downstream of *Rpp4* in the signaling pathway controlling resistance to *P. pachyrhizi.*

A total of 383 genes were significantly differentially expressed (P-value < 0.05) between *Rpp4*-silenced and control silenced plants, each inoculated with *P. pachyrhizi*, being 22 up-regulated, and 361 down regulated. Most of the up-regulated genes show similarity to genes encoding known proteins such as Pectin acetylesterase, Aspartyl protease, GDP mannose pyrophosphorylase, phosphatidylinositol transfer protein PDR16, among others, while several of the down-regulated genes identified share sequence similarity with genes encoding known proteins were related to defense, disease resistance and metabolism (Table 1).

Table 1. List of the most greatly induced and suppressed annotated genes in *Rpp4* silenced plants 14 days after inoculation (dai) by ASR (p-value< 0.05).

Probe set	Gene annotation	Fold change	P-value
Up-Regulated			
Gma.5599.1.A1_at	hypothetical protein	2.00196	7.50E-07
GmaAffx.78729.1.S1_at	SUGAR-1-PHOSPHATE GUANYL TRANSFERASE	2.06924	1.14E-06
GmaAffx.68386.1.S1_at	unknown protein	2.07344	2.50E-07
Gma.14098.1.A1_at	PENTATRICOPEPTIDE REPEAT-CONTAINING PROTEIN	2.13734	5.30E-07
Gma.13925.1.A1_at	ALDOSE-1-EPIMERASE	2.20873	5.35E-06
Gma.5963.1.S1_at	SUGAR TRANSPORTER	2.22862	1.96E-06
GmaAffx.86638.1.S1_at	unknown protein	2.24613	9.20E-07
GmaAffx.85211.1.S1_at	SERINE/THREONINE-PROTEIN KINASE WNK (WITH NO LYSINE)-RELATED	2.24767	1.08E-05
GmaAffx.88028.1.A1_at	unknown protein	2.28299	4.20E-07
Gma.18082.1.S1_at	hypothetical protein	2.30806	1.10E-07
GmaAffx.79275.1.S1_s_at	CYTOCHROME P450	2.30924	5.87E-06
GmaAffx.35140.1.S1_at	Phosphatidylinositol transfer protein PDR16 and related proteins	2.31658	8.89E-06
GmaAffx.61395.1.A1_at	unknown protein	2.35599	1.69E-05
Gma.2961.1.S1_at	GLUCOSYL/GLUCURONOSYL TRANSFERASES	2.46152	3.20E-06

Gma.6498.1.A1_at	Aspartyl protease	2.54087	2.15E-05
GmaAffx.53274.1.S1_at	unknown protein	2.57811	6.10E-07
Gma.7454.1.S1_a_at	hypothetical protein	2.65087	2.27E-06
Gma.1007.2.S1_at	unknown protein	2.78284	3.00E-07
GmaAffx.4935.2.S1_at	unknown protein	2.78974	1.20E-07
GmaAffx.48606.1.S1_at	unknown protein	3.24535	7.10E-07
GmaAffx.4935.1.S1_at	Pectin acetylesterase and similar proteins	4.09302	3.85E-06
Gma.4755.1.S1_at	unknown protein	4.38654	6.95E-05

Down-regulated

GmaAffx.93635.1.S1_s_at	Cystein-rich secretory protein (CRISP/SCP/TPX1)-related	-11.26476	3.71E-05
GmaAffx.77637.1.S1_at	chalcone and stilbene synthases	-9.83177	7.64E-05
Gma.10150.1.A1_at	Iron/ascorbate family oxidoreductases	-8.99014	7.17E-05
GmaAffx.92564.1.S1_at	hypothetical protein	-8.59062	6.15E-05
GmaAffx.92558.1.S1_s_at	Iron/ascorbate family oxidoreductases	-7.50755	3.90E-06
Gma.14338.1.A1_at	hypothetical protein	-7.28335	0.00013166
Gma.3604.4.S1_s_at	caffeoyl-CoA_O-methyltransferase	-6.74917	6.30E-07
GmaAffx.57966.1.S1_at	PAR1 protein	-6.54803	3.68E-06
GmaAffx.18868.1.S1_s_at	NADH:flavin oxidoreductase/12-oxophytodienoate reductase	-6.41068	1.27E-06
Gma.2586.1.S1_at	unkown protein	-6.20006	3.46E-05
Gma.17873.1.S1_s_at	hypothetical protein	-5.91209	0.0002849
GmaAffx.92410.1.S1_s_at	Flavonol reductase/cinnamoyl-CoA reductase	-5.56145	7.20E-07
Gma.15958.1.S1_at	hypothetical protein	-5.43274	6.70E-05
Gma.79.4.S1_s_at	hypothetical protein	-5.40399	5.79E-05
Gma.9072.1.S1_at	chalcone and stilbene synthases	-5.21972	2.69E-06
Gma.1269.1.S1_at	alcohol dehydrogenase	-5.10924	5.40E-07
Gma.10820.1.S1_at	Hydroxyindole-O-methyltransferase and related SAM-dependent methyltransferases	-5.09748	5.14E-06
Gma.3988.1.S1_at	Glyoxalase	-5.07278	5.26E-05
GmaAffx.92479.1.S1_s_at	alcohol dehydrogenase	-5.05661	5.42E-06
Gma.16913.1.S1_s_at	hypothetical protein	-5.01768	8.10E-05
GmaAffx.74923.1.S1_at	Iron/ascorbate family oxidoreductases	-4.90309	4.60E-07
GmaAffx.42893.1.A1_at	Reductases with broad range of substrate specificities	-4.89001	3.90E-07
GmaAffx.23591.1.S1_at	unkown protein	-4.87330	6.58E-06

GmaAffx.91071.1.S1_at	Reductases with broad range of substrate specificities	-4.86401	1.20E-07
GmaAffx.90009.1.S1_s_at	hypothetical protein	-4.82904	1.41E-05
GmaAffx.82647.1.S1_at	Peroxidase/oxygenase	-4.78257	6.57E-05
GmaAffx.7258.1.S1_s_at	unkown protein	-4.72723	1.47E-05
GmaAffx.88105.1.S1_at	hypothetical protein	-4.68377	5.92E-06
GmaAffx.83910.1.S1_at	NADH:flavin oxidoreductase/12-oxophytodienoate reductase	-4.57422	1.07E-05
GmaAffx.92070.1.S1_at	caffeoyl-CoA_O-methyltransferase	-4.49304	1.20E-07
GmaAffx.50446.1.S1_at	unkown protein	-4.45622	0.00023923
GmaAffx.50670.1.A1_at	Kynurenine aminotransferase, glutamine transaminase K	-4.44107	4.35E-06
GmaAffx.84342.1.S1_x_at	unkown protein	-4.41364	0.00021469
GmaAffx.21548.1.S1_at	UDP-glucuronosyl and UDP-glucosyl transferase	-4.36898	3.44E-06
GmaAffx.4716.1.S1_at	Flavonol reductase/cinnamoyl-CoA reductase	-4.33782	3.10E-07
Gma.6211.1.S1_at	unkown protein	-4.33512	2.65E-06
Gma.4716.2.S1_at	unkown protein	-4.31655	9.07E-05
Gma.16709.2.S1_s_at	Cytochrome P450 CYP2 subfamily	-4.24676	1.21E-06
GmaAffx.92894.1.S1_s_at	Peroxidase/oxygenase	-4.20954	1.45E-05
Gma.169.1.S1_at	hypothetical protein	-4.20737	2.77E-05
Gma.3473.1.S1_at	Molecular chaperone (small heat-shock protein Hsp26/Hsp42)	-4.15882	8.75E-05
Gma.17851.1.S1_at	unkown protein	-4.14922	5.21E-05
GmaAffx.86629.1.S1_at	Glycosyl hydrolases	-4.14421	1.50E-07
Gma.6549.1.S1_at	Defense-related protein containing SCP domain	-4.13849	8.02E-06
GmaAffx.83919.1.S1_at	Hydroxyindole-O-methyltransferase and related SAM-dependent methyltransferases	-4.06296	6.27E-05
Gma.144.1.S1_at	unkown protein	-4.06067	1.41E-06
Gma.169.1.S1_x_at	Glycosyl hydrolases	-4.04885	1.01E-05
GmaAffx.215.1.S1_at	ATPase	-4.03547	3.75E-06
GmaAffx.18940.1.S1_at	carbonate dehydratase	-4.02311	3.36E-06

In order to understand the pathways affected by *Rpp4* silencing and to place the differentially expressed genes in a biological context, we examined the biological process and molecular function gene ontology (GO) terms (Ashburner et al., 2000) assigned to each differentially expressed gene. We then used Fisher's exact test with a Bonferroni correction to identify GO functional classes that were

significantly overrepresented in our differentially expressed gene list when compared to all genes represented on the soybean array. We identified sixteen GO biological process categories significantly overrepresented in our data set (Table 2). Of these, three were associated with defense or stress responses. Similarly, we identified 13 overrepresented molecular function GO categories including methyl transferases, peroxidases, and enzymes involved in redox reactions, lignin sythesis and flavonoid biosynthesis.

Table 2. Significantly (P<.05) overrepresented Gene Ontology (GO) biological process and molecular function terms found in *P. pachyrhizi* regulated probe sets as determined by Fisher's exact test and Bonferroni correction.

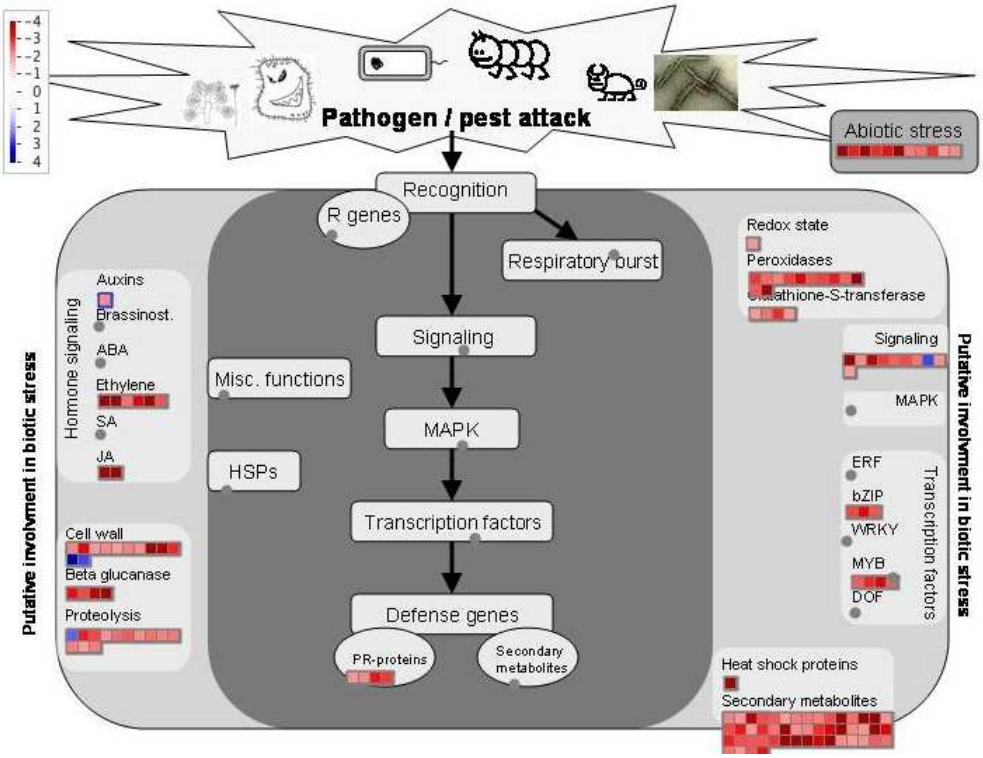
GO Term	GO Description	Corresponding probe sets present on array	Number of *P. pachyrhizi*-responsive probe sets	P-value following Bonferroni correction
GO Biological Process				
GO:0009809	Lignin biosynthetic process	109	17	0.00E+00
GO:0009813	Flavonoid biosynthetic process	102	19	0.00E+00
GO:0006979	Response to oxidative stress	402	29	1.40E-07
GO:0010422	Regulation of brassinosteroid biosynthetic process	8	6	1.87E-07
GO:0001561	Fatty acid alpha-oxidation	6	5	2.15E-06
GO:0016131	Brassinosteroid metabolic process	11	6	2.90E-06
GO:0009807	Lignan biosynthetic process	23	7	2.40E-05
GO:0009699	Phenylpropanoid biosynthetic process	49	9	4.62E-05
GO:0010224	Response to UV-B	86	11	1.03E-04
GO:0009411	Response to UV	62	9	3.67E-04
GO:0051347	Positive regulation of transferase activity	4	3	4.10E-03
GO:0051555	Flavonol biosynthetic process	50	7	6.30E-03
GO:0009435	NAD biosynthetic process	6	3	1.99E-02

GO:0009827	Plant-type cell wall modification	15	4	2.26E-02
GO:0006334	Nucleosome assembly	89	8	4.39E-02
GO:0010260	Organ senescence	18	4	4.84E-02
GO Molecular Function				
GO:0016614	Oxidoreductase activity, acting on CH-OH group of donors	8	6	3.69E-08
GO:0042409	Caffeoyl-CoA O-methyltransferase activity	18	6	2.24E-05
GO:0004601	Peroxidase activity	118	12	3.00E-05
GO:0005199	Structural constituent of cell wall	61	9	4.01E-05
GO:0047763	Caffeate O-methyltransferase activity	34	7	7.95E-05
GO:0045430	Chalcone isomerase activity	14	5	1.69E-04
GO:0045548	Phenylalanine ammonia-lyase activity	10	4	1.23E-03
GO:0008987	Quinolinate synthetase A activity	4	3	1.67E-03
GO:0010283	Pinoresinol reductase activity	4	3	1.67E-03
GO:0008171	O-methyltransferase activity	60	7	4.07E-03
GO:0004553	Hydrolase activity, hydrolyzing O-glycosyl compounds	228	13	5.95E-03
GO:0016682	Oxidoreductase activity	19	4	2.04E-02
GO:0004338	Glucan 1,3-beta-glucosidase activity	2	2	2.81E-02

We also placed our differentially expressed genes in a biological context using the MapMan (Thimm et al., 2004) visualization tool to examine different aspects of plant biology (Figure 1). Of the 383 differentially expressed genes identified in our experiment, 137 were related to biotic stress including genes involved in hormone signaling, cell wall structure, pathogenesis-related proteins, stress-related genes, secondary metabolites, redox and transcription. Redox related genes included a thioredoxin, twelve peroxidases and four glutathione-s-

transferases. Among the transcription factors, we found a heat shock protein, four protein with Myb domain and three bZip transcription factors (Figure 1 A). Analyzing the secondary metabolism overview we identified 71 genes related to phenylpropanoid, lignin, lignan, and flavonoid pathways (Figure 1B). Twenty-genes were related to the phenylpropanoid synthesis and included genes such as phenylalanine ammonia lyase (PAL-1), phenylalanine ammonia lyase (PAL-2), 4-coumarate: CoA ligase-3 (4Cl3), caffeoyl-CoA 3-O-methyltransferase, ferulate-5-hydroxylase, and cinnamyl-alcohol dehydrogenase. Twenty genes were identified belonging to the ligin and lignan pathways and include the O-methyltransferase family 1, O-methyltransferase family 2 protein, phenylalanine ammonia lyase and ferulate-5-hydroxylase. Analysis of the flavonoid pathway identified eight genes including chalcone flavonone isomerase, chalcone synthase 7 and chalcone synthase 4.

A)



B)

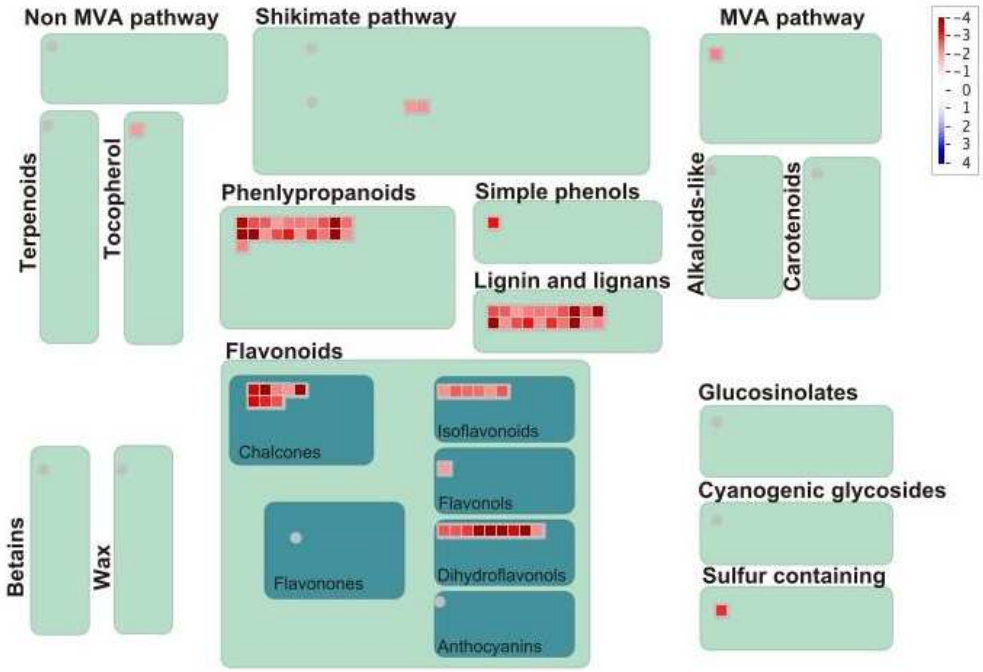


Figure 1. MapMan visualization of *the Rpp*4 regulated probes identified in *the Rpp*4 VIGS microarray experiment. A) Biotic stress overview, 137 genes identified, **B)** Secondary metabolism overview, 71 genes identified. The expression level of each probe is associated with a specific color, red- down regulated genes, blue- up regulated genes.

Comparison of the overrepresented gene ontology terms to the results of Mapman revealed the two approaches were complimentary. The gene ontology approach was much more stringent and identified individual pathways important in resistance. The Mapman approach tied these pathways together to form a general picture of defense.

3.3. Bioinformatics analysis of cis-elements

In order to understand how differentially expressed genes in the *Rpp4*-signaling pathway were regulated, we wanted to analyze their promoters for known transcription factor binding sites. Of the 383 differentially expressed genes identified by microarray, we were able to identify 1000 bases of promoter sequence for 255 genes. We used the program Clover (cis-element over representation, Frith et al., 2004) in conjunction with the TRANSFAC transcription factor database (Wingender et al., 1996) to identify known transcription factor

binding sites over represented (P<0.05) in the promoters of differentially expressed genes when compared to promoters of all genes (minus transposable elements) in the soybean genome. From this analysis, we identified 33 transcription factor binding sites (TFBD) significantly over represented in the differentially expressed. Many of the transcription factors binding sites were related to defense including MYB80, MYBBAS1, MYB.PH3, and CRF-2 (Table 3).

Table 3. List of Transcription Factor Binding Sites identified in promoters of *Rpp4*- regulated genes.

Nomenclature	Raw Score	p-value	Reference	Function
CPRF-3	47.2	0	Weisshaar et al., 1991	Involved in light-induced gene expression
TGA1b	34.6	0	Niggewe et al., 2000	auxin, salicylic acid, light (disease resistance)
LIM1	212	0	Wang et al., 2009	involved in lignin biosynthesis
OCSBF-1	7.18	0	Singh et al., 1990	expression during plant development
HBP-1b	38	0	Tabata et al., 1991	phosphorylation is required for DNA-binding (histone genes)
ROM	74.8	0		unknown
HBP-1a	53.9	0	Tabata et al., 1991	involved in the cell cycle-dependent expression of Wheat core histone genes
TAF-1	65.9	0	Oeda et al., 1991	transcriptional activator
CPRF-3	45.3	0	Weisshaar et al., 1991	involved in light-induced gene expression
TGA1b	38.8	0	Niggewe et al., 2000	auxin, salicylic acid, light (disease resistance)
AtMYB-84	22.8	0	Martin and Paz-Ares, 1997	Protein REGULATOR OF AXILLARY MERISTEMS 3 (disease response -PAL)
P	35.1	0.001		unknown
EmBP-1b	22.4	0.001	Carlini et al., 1999	may be involved in mediating ABA-response
C1	76.9	0.002	Piazza et al., 2001	anthocyanin biosynthesis
RITA-1	14.4	0.002	Izawa et al., 1994	seed development
PCF2	57.9	0.003	Kosugi and Ohashi, 1997	bind to site in the promoter proliferating cell nuclear antigen (PCNA) gene.
CG1	72.5	0.004	Staiger et al., 1990	light-inducible expression (chalcone Synthase promoter)
CPRF-2	37.6	0.006	Kircher et al.,	may be involved in the activation of

			1999	phenylpropanoid biosynthetic gene and in early plant defense response
TGA1a	21.5	0.006	Niggewe et al., 2000	auxin, salicylic acid, light (disease resistance)
CPRF-1	46.9	0.007	Weisshaar et al., 1991	involved in light-induced gene expression
CPRF-2	32.8	0.007	Kircher et al., 1999	CPRF-2 is transported from the cytosol into the nucleus upon irradiation due to action of hytochrome photoreceptors PhyA and PhyB
Alfin1	218	0.008	Bastola et al., 1998	may play a role in the regulated expression of PRP2 in alfafa roots and contribute to salt tolerance in these plants
TAF-1	40	0.009	Oeda et al., 1991	transcriptional activator
OSBZ8	35.9	0.01	Mukherjee et al., 2006	induced by Abscisic acid, increase after dehydration
HBP-1a	35.9	0.015	Tabata et al., 1991	Involved in the cell cycle-dependent expression of wheat core histone genes
Opaque-2	19.2	0.02	Schmidt et al., 1990	involved in the regulation of seed storage protein synthesis
MYBAS1	30.6	0.025	Yang et al., 2001	induced by water deficit stress
GBP	39.8	0.026		unknown
TRAB1	34.3	0.03	Hobo et al., 1999	involved in ABA-regulated transcription
TGA1a	9.38	0.032	Niggewe et al., 2000	auxin, salicylic acid, light (disease resistance)
ATHB-9	-3.29	0.033	Prigge et al., 2005	Probable transcription factor involved in the determination of adaxial-abaxial polarity in ovule primordium (UniProt)
MYB80	-3.45	0.046	Li et al., 1999	disease response
ZAP1	-0.691	0.047	Pater et al., 1996	transcriptional activator

Not surprisingly, not all transcription factors binding sites were equally represented in the promoters of the differentially expressed genes (Figure 2). For example, LIM1 and ALFIN1 were identified 204 and 191 times respectively. In contrast, AtMYB84 was found 53 times. Each of these transcription factors is involved in stress and defense responses.

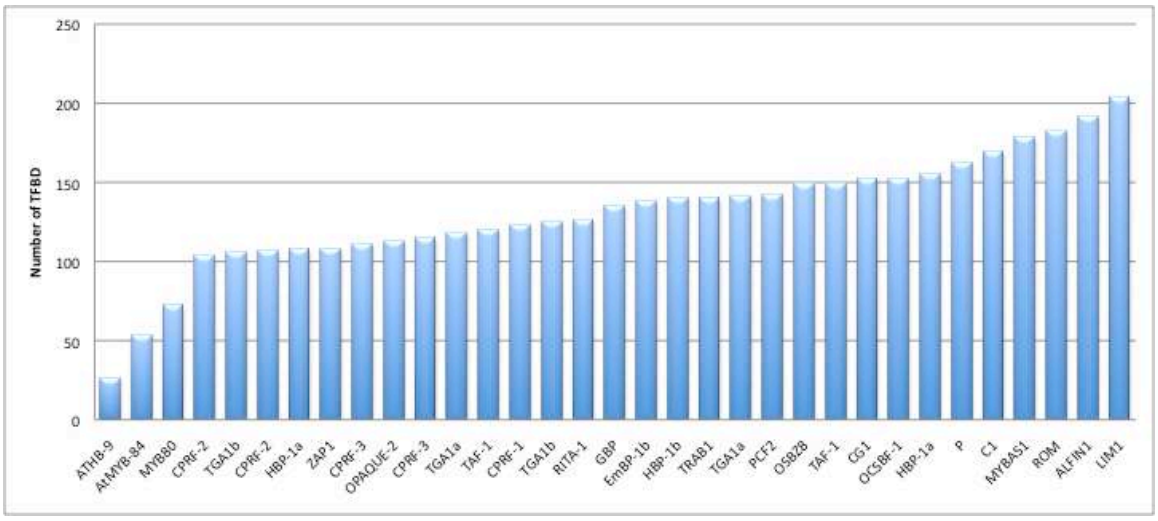


Figure 2. Number of transcripiton factor binding sites found in the promoters of *Rpp4*-regulated genes. Each of the differentially expressed genes identified was assigned to a cDNA from the whole soybean genome assembly using BLASTN. Custom Perl scripts were used to extract 1000 bases of promoter sequence for all differentially expressed genes. Clover and Transfac were used to identify overrepresented transcription factor binding sites (TFBD) in the promoters. Thirty-three TFBD were identified, with varied frequency.

3.4. MEME and MAST analysis

While Clover can be used to identify previously characterized transcription factor binding sites, it cannot identify novel sites. Therefore, we used MEME (Multiple EM for Motif Elicitation, Bailey et al., 2009) to identify novel transcription factor binding sites from the 255 promoters of our differentially expressed genes. In brief, MEME searches for conserved sequence elements shared across multiple promoter sequences. By modifying different parameters within MEME, we could alter the length and number of motifs identified. Using this approach, we identified five motifs with a P-value more significant that P<0.0001 (Figure 3).

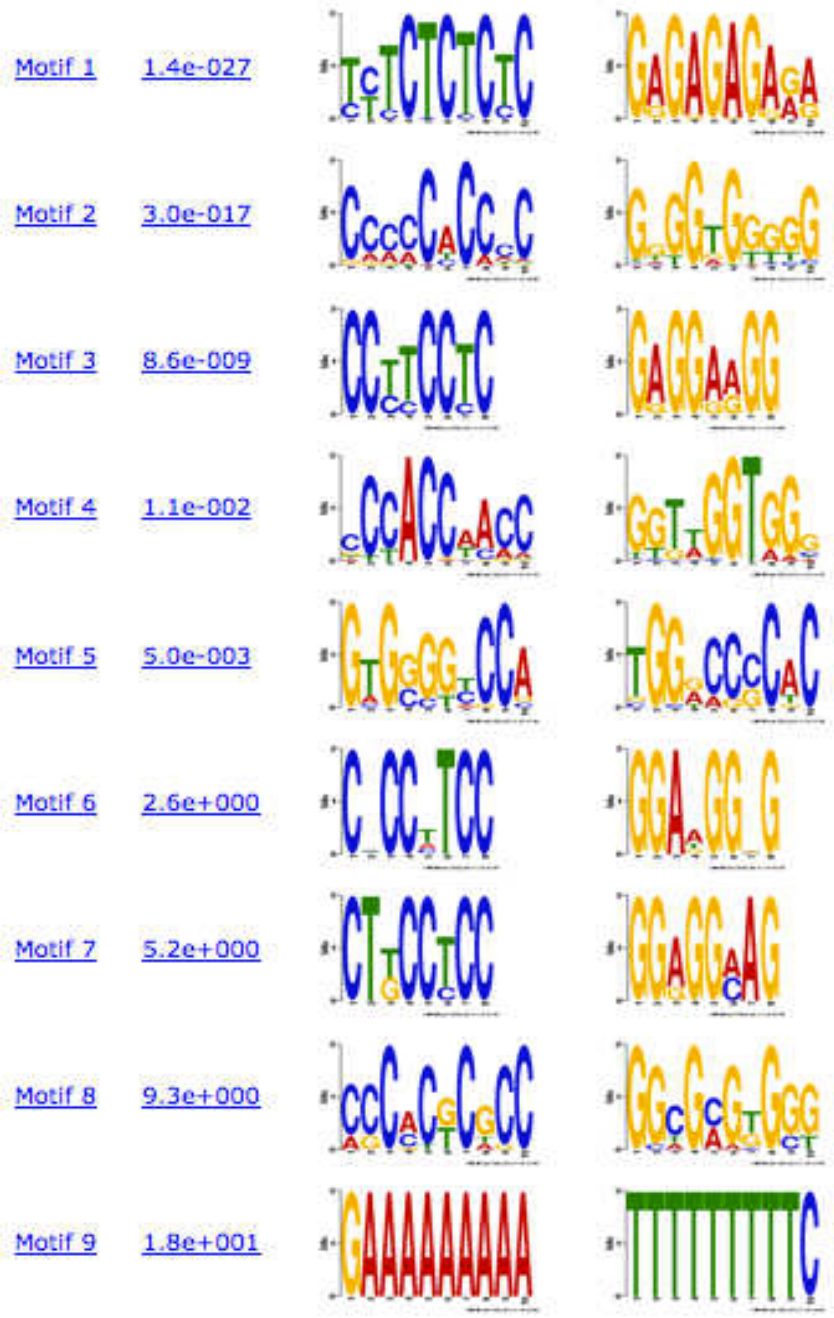


Figure 3. Motif Overview. We used MEME (Bailey and Elkan, 1994) to find probable transcription factor binding sites in soybean genes. The Figure shows 9 motifs identified with the respective motif number, p-value and logo of protein motif. Logos are use to visualize conserved nucleotides in the motif. Only motifs with P-values greater than 0.05 were used for further analysis.

Next, we used the five significant putative transcription factor binding sites identified by MEME to analyze all promoter sequences in the soybean genome using MAST (Motif Alignment & Search Tool, Bailey and Gribskov, 1998). This

would identify additional genes not present on the soybean genome array that could be downstream of *Rpp4*. Using MAST to analyze our five significant motifs, we identified 667 promoters in the soybean genome containing one or more motifs (Table 4).

Table 4. Identification of the frequency of motif by MAST.

Motif ID	Number of times a motif was found with a single promoter	Total number of time a motif was found
Motif 1	551	1280
Motif 2	489	775
Motif 3	290	409
Motif 4	275	349
Motif 5	361	185

The frequency of the motifs varied greatly, for example, Motif 1 was found in 551 different promoters while Motif 5 was found only in 167 promoters. The number of times a motif was found with a single promoter also varied, Motif 1 was found anywhere from 0 to 18 times while Motif 5 was found from 0 to 2 times. The next step in the analysis will be to annotate the gene corresponding to the identified promoters to see if they have roles in defense.

3.5. Unique genes identified in microarray *Rpp4* silenced plants.

Recently, several labs have published work on *P. pachyrhizi* resistance and susceptibility in soybean. However, little is known on the overlap of molecular responses to *P. pachyrhizi* conditioned by different resistance genes. van de Mortel et al. (2007) measured gene expression over a seven day time-course in mock- inoculated and infected leaves of a soybean accession (PI230970) carrying the *Rpp2* resistance gene and a susceptible genotype (Embrapa 48) (Table V). Schneider et al. (unpublished) measured gene expression across twelve days in a single soybean genotype (PI46312) carrying the *Rpp3* resistance gene, using a compatible and incompatible *P. pachyrhizi* isolate, while Freeman et al.

(unpublished) measure gene express across twelve days using two soybean genotypes (PI450925B (*Rpp4)* and Williams 82) and a single *P. pachyrhizi* isolate. These experiments identified 894, 8447, and 5,806 differentially expressed genes associated with the resistance response governed by *Rpp2*R*, Rpp3*R and *Rpp4*R, respectively. Similarly, 1516, 1827 and 5,365 differentially expressed genes were associated with the susceptible response (*Rpp2*S, *Rpp3*S and *Rpp4*S). To try to elucidate which genes are exclusively related to *Rpp4* VIGS experiment, we have overlapped the differentially expressed genes identified by microarray *Rpp4* silenced plants with other microarray data from different genotypes carrying genes for resistance as *Rpp2*, *Rpp3* and *Rpp4* and susceptible genotypes reactions (Table 5).

Table 5. List of genotypes and timepoints used to overlap the differentially expressed genes identified by microarray *Rpp4* silenced plants.

R- gene	Resistant interaction	Susceptible interaction	Timepoints (hours after inoculation)
*Rpp*2	PI230970/Brazil *P. pachyrhizi* isolate	Embrapa48/Brazil *P. pachyrhizi* isolate	6, 12, 18, 24, 36, 48, 72, 96, 120, 168
*Rpp*3	PI462312, *P. pachyrhizi* HW94-1	PI462312, *P. pachyrhizi* TW80-2	12, 24, 72, 144, 216, 288
*Rpp*4	PI459025B, *P. pachyrhizi* HW94-1	Williams, *P. pachyrhizi* isolate HW94-1	12, 24, 72, 144, 216, 288
*Rpp*4 VIGS	PI459025B, empty vector silenced, LA04-1	PI459025B, *Rpp*4 silencing vector silenced, LA04-1	336

Comparing all the microarray data on one by one basis, we identified 302 (*Rpp2*R), 284 (*Rpp2*S), 162 (*Rpp3*R), 254 (*Rpp3*S), 148 (*Rpp4*R) and 137 (*Rpp4* S) differentially expressed genes unique to the *Rpp4* VIGS experiment. When all the microarray experiments were combined (*Rpp2*R*, Rpp2*S, *Rpp3*R, *Rpp3*S *Rpp4*R, and *Rpp4*S) and compared to the *Rpp4* VIGS microarray data, 101 unique differentially *Rpp4* VIGS genes were identified. Using the MapMan tool, we could see that 24 of these genes were related to biotic stress including thioredoxins, peroxidases, protein kinases and transcription factors. In addition, seven genes were identified in secondary metabolism being two with phenylpropanoid, one with lignin/lignan and four with flavonoid pathways, respectively.

4. Discussion

In this study, we combine the power of transcriptomics and virus induced gene silencing to characterize genes involved in the *Rpp4*-mediated Asian Soybean Rust resistance pathway. Our experiment uses a single soybean genotype (PI459025B) with two different VIGS vectors previously described by Meyer et al. (2009). The first vector is a BPMV silencing vector lacking a silencing target. This construct does not alter the expression of *Rpp4*, so PI459025B remains resistant to *P. pachyrhizi.* The second construct, developed from the LRR of the *Rpp4* candidate genes in Williams 82, is able to silence the expression of *Rpp4* in PI459025B, leading to susceptibility to *P. pachyrhizi.* Following silencing and *P. pachyrhizi* inoculation, these plants differ only in the expression of *Rpp4* and genes downstream of *Rpp4* in the resistance pathway. By isolating and comparing RNA from both plants, we have identified 383 genes downstream of *Rpp4* important in *Rpp4*-mediated defense. Of these, 101 were unique and had not been identified in previous microarray experiments (van de Mortel et al. 2007, Schneider et al. (unpublished), Freeman et al. (unpublished). These genes corresponded to several different biological pathways including transcription factors related to biotic stress (AtbZIP9 and bZIP61), genes involved in cell wall structure (UDP-D- galactose 3 epimerase) and secondary metabolites (phenylpropanoids (OMT1), flavonoids (chalcone synthase) and dihydroflavonoids (cinnamyl-alcohol- dehydrogenase).

In general, bZIP proteins bind DNA as dimmers mediated by the leucine zipper domain (ZIP), a heptad repeat of leucine or other hydrophobic amino acids creating an amphipathic helix. BZIP transcription factors regulate diverse biological processes including pathogen defense, light and stress signaling, seed maturation and flower development. In the model plant *Arabidopsis thaliana*, 75 bZIP proteins have been identified and classified into 10 groups (Jakoby et al., 2002). A homolog of ATbZIP 9 from *Arabidopsis thaliana* was identified to be unique to *Rpp4* VIGS mediated response in this experiment. ATbZIP9 belongs to group C (Jakoby et al., 2002) which includes the maize BZIP transcription factor Opaque2 and parsley CPFR2. The information available on Opaque2 and closely related monocot genes

indicates that they regulate seed storage protein production by interacting with the PBF protein (Vicente-Carbajosa et al., 1997). Few publications have focused on the biological function of group C bZIPs. ATbZIP 10 also belongs to group C and was shown to be involved in oxidadtive stress response, particularly during defense against the biotrophic pathogen *Hyaloperonospora parasitica* (Kamida et al., 2006). Recently a microarray analysis revealed 231 genes differentially expressed between two genotypes (WT and an ATbZIP-9 mutant) leading to some possible connections between ATbZIP9 and energy metabolism, abiotic stresses, jasmonic acid, ethylene and salicylic acid signaling (Vilela et al., 2009). A promoter fusion with GUS revealed that ATbZIP9 expression is restricted to the phloem of all organs analyzed. ATbZIP9 mRNA accumulation was also shown to be repressed by glucose and induced by abscissic acid and cytokinin (Silveira et al., 2007). In addition, in vitro phosphorylation experiments show that ATbZIP9 is phosphorylated, suggesting a signaling role in the cell (Kircheler et al., 2010).

Analysis of the secondary metabolism overview of MapMan identified seven genes unique to the *Rpp4 VIGS* experiment. These genes were related to phenylpropanoid, flavonoid and lignin/lignan pathways. Flavonoids are involved in plant defense response through production of various phytoalexins and cell wall reinforcing metabolities. Flavonoids are synthesized in plants via the flavonoid branch of the phenylpropanoid and acetate-malonate metabolic pathways (Buer et al., 2010). Chalcone synthases (CHS) are key enzymes in the flavonoid biosynthesis pathway. Probes related to CHS were identified in *Rpp4* VIGS microarray and also by Van de Mortel et al. (2007). Van de Mortel et al. identified 10 probes related to chalcone biosynthetic process in both resistant and susceptible genotypes. In general, expression of these genes increased significantly during early infection in both soybean genotypes. However, during later infection, gene expression diverged, but with distinct kinetics in the two interaction types. Increased expression of these genes occurred at least 1 day earlier in resistant plants compared with the susceptible plants. A cDNA clone encoding CHS was isolated from *Populus trichocarpa* by reverse transcription-polymerase chain reaction (RT-PCR) and semi-quantitative RT-PCR. Analysis revealed that this gene was abundantly expressed in the leaves and stems, while its expression was drastically reduced in the roots. In addition, transcript

abundance of the gene was stimulated by 2.5-fold within 24 h of wounding treatment. Promoter analysis confirmed that the gene promoter was capable of directing expression of the GUS reporter in both wounded and unwounded leaves of transgenic plants, indicating that the gene promoter is systemically responsive to wounding stimuli (Sun et al., 2011).

In plants, the phenylpropanoid pathway has a role in defense (Subramanian et al., 2005). In this category we identified genes such as cinnamyl-alcohol dehydrogenase (CAD). CAD catalyses the conversion of the cinnamyl aldehydes to cynnamyl alcohols, this is the last step in the synthesis of monolignols before their polimerization in cell walls (Ma, 2010). High levels of 1bCAD1 mRNA were found in the roots of sweet potato. The 1bCAD1 gene transcripts were highly induced by cold, wounding and reactive oxygen species (Kim et al., 2010). Interestingly, analyses of transcriptional regulation of the 1bCAD1 promoter-GUS revealed that 1bCAD1 promoter expression was strong in the roots, but barely detectable in the cotyledons. The identification of CAD in our microarray analysis implies that successful defense against *P. pachyrhizi* involves modification and fortification of cell walls.

Many of the probes we identified were related to proline-rich extensins. Extensins (HRGPs) play an essential role in biotic and abiotic stress responses due to their abilities to cross-link and strengthen the cell wall. The plant cell wall has been established as one of the most important structures of plants as it harbors many vital functions for the plant. Besides providing stability to the plant and counterbalancing internal turgor pressure, it offers protection from injury and pathogen attack. In addition, cell wall-mediated resistance in plants forms the first line of defense against pathogens (Deepak et al., 2010).

Cell wall-associated defenses include effective transport and secretion of defense compounds in response to elicitor perception. Oxidative phenolics formed from amino acids residues within extensins and are known to be one of the most important factors which contribute to the strengthening of cell wall. Pathogen-derived elicitor treatment induced cross-linking of HRGPs in bean and soybean resulting in a rapid insolubilization of pre-existing Hyp-rich structural proteins in the cell walls (Bradley et al., 1992). In several plants, HRGPs accumulated to a higher extent in resistant cultivars than in susceptible ones. Further, these proteins

accumulated substantially in intracellular spaces and in papillae which are known to be physical barriers formed in response to pathogen infection (Basavaraju et al., 2009). The expression of HRGP genes have been studied in several species including soybean (*PRP3)* and members of the HGRP family from *Brassica napus* (Datta and Marcus 1990, Evans et al., 1990). Through our analysis we conclude that the *Rpp4* signal pathway mediates the expression of proline-rich extensin genes to promote cross-linking leading to strengthening of the cell wall.

Another probe identified in this experiment is homologous to NDR1 (Non race-specific disease resistance 1). NDR1 was first identified in a genetic screen aimed at identifying genetic loci required for disease resistance in Arabidopsis in response to infection by *P. syringae* (Century et al., 1995, 1997). NDR1 is a plasma membrane, glycophosphatidyl-inositol (GPI)-achored protein required for activation of disease resistance signaling mediated by members of the largest class of disease resistance proteins in Arabidopsis (Coppinger et al., 2004). The mechanism of NDR1 function in disease resistance signaling remains unknown, however, previous work has addressed the genetic requirement for NDR1 in the activation of resistance signaling mediated by the coiled coil (CC) NB-LRR class of resistance proteins.

Two-component systems (TCS) contain two multi domain proteins including a histidine kinase and a response regulator (RRs). Together, they control many biological processes including cell division, cell growth and proliferation and responses to environmental stimuli. RRs act as phosphorylation-actived switches that catalyze the transfer of the phosphoryl group to a conserved Asp in its own regulatory domain. In our experiment, we identified a homolog of ATARR9, recently reported to be involved in stress signaling. The recent completion of the soybean genome sequence allowed Mochida et al., (2010) to identify a total of 49 RRs in soybean, including both the authentic and the pseudo-RRs. In addition, they classify the soybean RRs into type-A GmRR, type-B GmRR, Type-C GmRR and pseudo-GmRR categories. ATARR9 belong into type-A class. The phylogenetic tree developed from the RRs collected from Arabidopsis, rice and soybean indicates closed relationship among the type-A RRs of the three species, which might suggest similar functions for the soybean type-A GmRR (Mochida et al., 2010).

5. Conclusion

This study revealed parts of the metabolic pathways potentially activated by the *Rpp4* locus. These findings provide new insights into the complex changes in plant gene expression that occur globally in response to *P. pachyrhizi*. Some of these genes can serve as potential targets for genetic improvement of soybean plants for enhanced rust resistance.

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Chapter III

Expression Analyses of Candidate Resistance Genes in the *Rpp4* Asian Soybean Rust Resistance Locus

Abstract

Five ASR resistance genes have been identified in soybean: *Rpp1*, *Rpp2*, *Rpp3*, *Rpp4* and *Rpp5*. Of particular interest is *Rpp4*, which has remained stable and confers resistance against *P. pachyrhizi* isolates from around the world. *Rpp4* was mapped to soybean linkage group G (chromosome 18), 1.9cM from simple sequence repeat (SSR) marker Satt288 (Silva et al., 2008). Sequencing of this region in the susceptible genotype Williams 82 (Wm82) identified a cluster of three coiled-coil nucleotide bind site leucine rich repeat (CC-NBS-LRR) resistance genes (Meyer et al., 2009). We sequenced a >607 kb region of the *Rpp4* locus in the resistant mapping parent PI459025B. Ten CC-NBS-LRR resistance genes have been identified in this region. In order to obtain more information about *Rpp4* function, we used real time quantitative PCR (RT-qPCR) to analyze the expression of all ten genes in different plant tissues, in different stages of development and after inoculation with *P. pachyrhizi*. We have developed a single pair of primers from the NBD domain that allow us to monitor the expression of all ten genes. Direct sequencing of the RT-qPCR product differentiates between the ten genes. In addition to examining gene expression directly, we are also interested in determining if alternative splicing of intragenic duplications can create additional sequence diversity at the RNA level.

1. Introduction

Soybean [*Glycine max* (L.) Merrill] is one of the most important crops in Brazil. Last season total production was over to 72 million tons, an all-time record (Conab, 2011). A number of biotic and abiotic factors have affected soybean production. Asian soybean rust (ASR) is a soybean disease caused by *Phakopsora pachyrhizi* Sydow, a fungal pathogen that has an unusually broad host range, infecting over 95 plant species from more than 42 genera (Bromfield 1984, Ono et al., 1992, Slaminko et al., 2008). Susceptible soybean plants infected with virulent isolates of *P. pachyrhizi* are characterized by tan-colored lesions and sporulating uredinia, predominantly on the abaxial leaf surfaces (tan phenotype). Plants producing dark reddish-brown (RB) lesions at the site of infection in response to avirulent isolates of the pathogen are resistant (Bromfield 1984, Bonde et al., 2006, Garcia et al., 2008).

Scientists have screened over 16,000 soybean accessions from U.S. germplasm for resistance or tolerance to soybean rust (ASR) (Miles et al., 2006). Five ASR resistance genes have been identified in soybean: *Rpp1*, *Rpp2*, *Rpp3*, *Rpp4* and *Rpp5* (Bromfield and Hartwig, 1980; Mclean and Bith, 1980; Hartwig and Bromfield, 1983; Hartwig 1986; Silva et al., 2008; Garcia et al., 2008). In addition, two more loci, *Rpp1b* (Ray et al., 2009) and *Rpp?* (Hyuuga) (Monteros et al., 2007), have been identified. It is unclear if these are tightly linked genes or novel alleles of *Rpp1 and Rpp3*. Each of these genes has been genetically mapped (Chakraborty et al., 2009, Garcia et al., 2008, Hyten et al., 2007, Hyten, et al., 2009, Monteros, et al., 2007, Silva, et al., 2008) and comparisons to the soybean genome (Schmutz et al., 2010) identified clusters of candidate resistance gene (R-gene) homologs. Of the known resistance sources, *Rpp4* has remained the most stable when challenged against isolates from different parts of the world (Yamaoka et al., 2002; Bonde et al., 2006). *Rpp4* was mapped to soybean (chromosome 18) linkage group G, 1.9cM from simple sequence repeat (SSR) marker Satt288 (Silva et al., 2008). Sequencing of this region in the susceptible genotype Williams 82 (Wm82) identified a cluster of three coiled coil nucleotide binding site leucine rich repeat (CC-NBS-LRR) resistance genes (Meyer et al., 2009). Simple satellite repeat markers (SSRs) developed from the Wm82 *Rpp4* locus and polymorphic between

Wms82 and PI459025B, were screened against the same segregating population used to originally map *Rpp4* (Silva et al., 2008). These markers placed *Rpp4* in the middle of the R-gene cluster (Meyers et al., 2009) in PI459025B.

To test whether an orthologous gene in PI459025B was responsible for resistance, Meyer et al. (2009) took advantage of a relatively new tool for soybean genomics: virus-induced gene silencing (VIGS) (Zhang et al., 2009). Bean Pod Mottle Virus (BPMV)-based vectors have been used successfully for sequence-specific gene silencing in soybean (Zhang et al., 2010). Using a portion of the NBD or LRR domains from the Wm82 *Rpp4* candidate genes, Meyer et al. (2009) were able to silence resistance demonstrating that orthologs genes were responsible for resistance. A >607 kb region of the *Rpp4* locus in the resistant mapping parent PI459025B has now been sequenced. Revealing ten CC-NBS-LRR resistance genes in this region. In order to identify the gene or genes corresponding to *Rpp4,* we have used real time quantitative PCR (RT-qPCR) to analyze the expression of all ten genes in different plant tissues, in different stages of development and after inoculation with *P. pachyrhizi*. The intragenic duplications suggested alternative splicing might play a role in creating additional sequence diversity. To test whether alternative splicing occurs, primers were designed.

2. Materials and methods

2.1. Pathogen isolation and plant inoculation

P. pachyrhizi was obtained from urediniospores harvested in the greenhouse collected from BRSMS-Bacuri (susceptible) leaves at Embrapa-Soja, in Londrina, Paraná, Brazil in September 2009. Spore suspensions were made using sterile distilled water containing 0.01% Tween-20 (vol/vol). Urediniospores were quantified and diluted to a final concentration of 1.1 x 104 spores/mL. Three plants from the resistant genotype (PI459025B) per pot with three replicates (pots) were inoculated. Sterile distilled water containing 0.01% Tween-20 was used for mock inoculation on three pots of plants to monitor the infection. After inoculation, plants were kept in a greenhouse where temperatures were maintained at 25°C

during the day and 20°C during the night under a 12h photoperiod. Tissues from leaves (stage V3), flowers (stage R2), and roots (stage V3) were harvest 12 hours after inoculation and seeds (stage R7) were harvested at the end of the experiment. All samples were immediately frozen in liquid nitrogen and stored at -80°C.

2.2. RNA extraction, isolation and DNase-treatment.

Prior to RNA extraction, all samples were ground in liquid nitrogen, and RNA was extracted using 1 mL of Trizol reagent (Invitrogen, Carlsbad, CA, U.S.A) at Embrapa Soja, Londrina, PR- Brazil. RNA samples were stored as pellets in 1mL of ethanol 75% at -80°C and shipped to Iowa State University, where the RNA samples were centrifuged and resuspended in 50μL of Nuclease-free water (Applied Biosystems). In order to remove contaminating DNA, RNA samples were treated with Turbo DNA-free (Ambion, Cat#AM1907) according to the manufacture's directions and the quality analysis and quantification were performed by agarose-gel analysis and a NanoDrop ND-1000 spetrophotometer (Therm Scientific) respectively.

2.3. Efficiency curve

Efficiency curves of three soybean genes, b-actin (Gmβ-actin, Genbank accession no. GMU6050), Glyceraldehyde 3-phosphate dehydrogenase (GmGADPH, Genbank accession no. DQ224371.1) and a ribosomal gene (GmRNAr18S, Genbank accession no. X02623.1) were run on all RNA samples to determine the best reference gene for quantitative PCR (RT-qPCR) (Table 1). The use of these primers for real-time quantitative PCR has been previously documented (Stolf-Moreira et al. 2011).

Table 1: Primer sequences of the normalizer used in the reactions of RT-qPCR and expected size of amplicons generated

Primer ID	Primer sequences	Expected size of fragments (bp)
Gm b-actin Fw	5'GAGCTATGAATTGCCTCTGG3'	118
Gm b-actin Rw	5'CGTTTCATGAATTCCAGTAGC3'	
Gm GAPDH Fw	5'GTGGAGACCCATTGGAGGAA3'	118
Gm GAPDH Rw	5' TGGTTTGCTGCTGGTAATGGTA3'	
Gm RNAr18S Fw	5'AAACGGCTACCACATCCAAG3	155
GM RNAR18S RW	5'CCTTCAATGGATCCATCGTTA3'	

Real time PCR analysis were performed in a Stratagene Mx3000P and the SuperScriptTM III Platinum® SYBR® Green One-Step RT-qPCR kit (Invitrogen, Carlsbad, CA, U.S.A) was used according to the manufacturers' protocols with 600ng, 300ng, 150ng, 50ng, 10ng of total RNA, 300nM final concentration of primers in the following RT-qPCR program: 5min at 60°C, 10 min at 95°C, PCR cycling at 95°C for 15 s, 62°C for 30 s, and data collection for 15s at the extension temperature of 72°C for 40 cycles. The calculation of primer amplification efficiency and cycle threshold (Ct) determinations were achieved using the miner algorithm (Zhao and Fernald, 2005), corresponding to the formula E=[10-1/slope], used to calculate the reaction efficiency. The calibration curve was established graphically by plotting Ct values as a function of log dilutions of cDNA. The GeNorm (Pattyn et al., 2003) was used for the analysis of gene expression stability and rank. The best reference gene for soybean was selected by GeNorm application (Vandesompele et al., 2002). This application calculates a gene stability value (M) and a normalization factor (NF) based on the geometric mean of the expression values of the set of control genes tested.

2.4. Relative quantification of candidate R-genes in the *Rpp4* locus

ClustalW (Thompson et al., 1994) was use to align the predicted transcripts of the eight full-length *Rpp4* candidate genes from PI459025B (Figure 1). Given the high nucleotide identity shared between genes, we were unable to design gene-specific primers for RT-qPCR that would allow direct comparison of gene

expressions levels. Therefore, primers were developed from a conserved region in nucleotide binding domain (NBD). The primers sequences were absolutely conserved (100% nucleotide identity) in all eight genes and resulted in an amplification product of 304 bases in length. By cloning and sequencing the amplification product, we could identify single nucleotide polymorphisms that would distinguish each of the genes and would allow determination of primer efficiency for each gene. Genomic DNA of PI459025B (50ng) was used to test primer efficiency for each gene during PCR, since all eight genes would be represented an equal number of times. PCR, cloning and sequencing were performed using the following reagents: Hi-Fi platinum Taq DNA polymerase (Invitrogen, no. 10342-053), reactions were run using the following cycling parameters: 94°C for 1 min, 30 cycles at 94 °C for 15s, 60°C for 30s, 68°C for 1min and a final incubation at 72°C for 2 min. PCR products were cloned according to the manufacturer's recommendations. Three hundred random clones were chosen at random for full sequencing. Plasmid DNA was isolated using the miniprep solution (Qiagen, P1[no. 19051], P2[no. 19052], and P3[no. 19053]), 96-well unifilters and uniplates (Whatman, nos. 7770-0062 and 7701-1750), and ABI Big Dye version 3.1 chemistry protocol and Hi-Di formamide (Applied Biosystems, nos. 431320 and 4337457). Plasmid DNA was sequenced using an Applied Biosystems 3730 DNA Analyzer with a 96-capillary array. To determine efficiency for a given gene, we divided the number of clones assigned to a particular gene by the total number of clones sequenced.

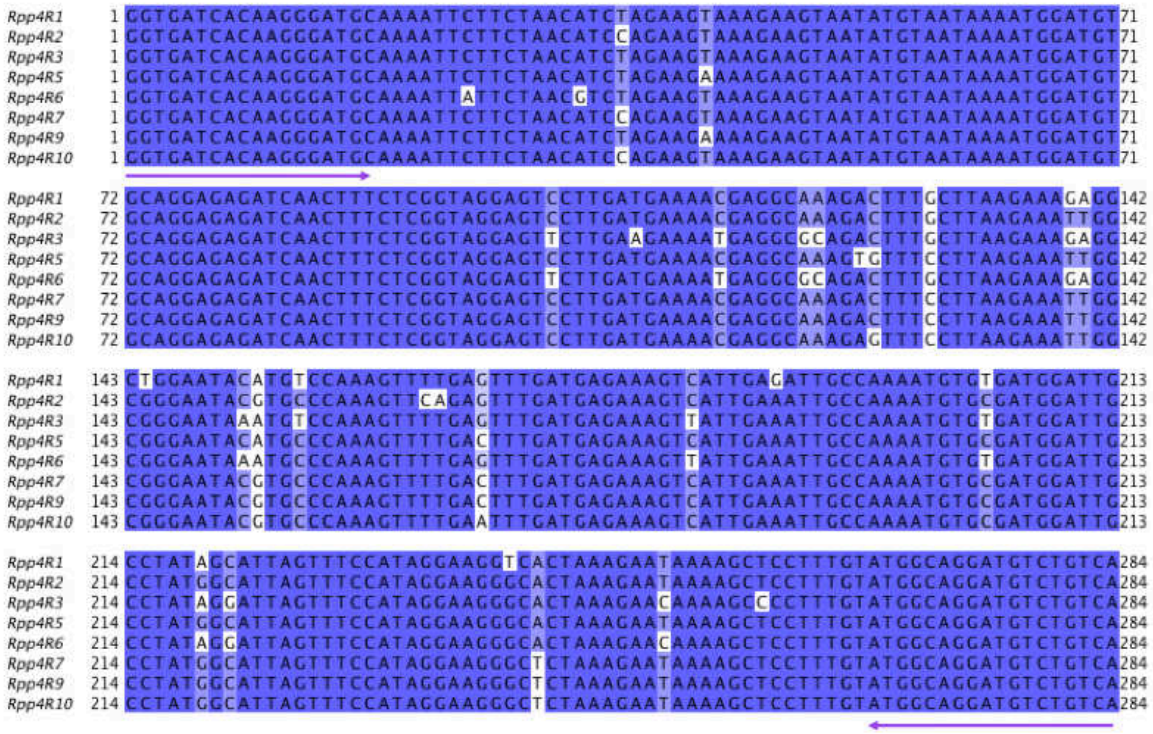

Figure 1. Primer development for *Rpp4* expression analyses. A portion of the NBD domain of the *Rpp4* candidate genes was used to develop primers o monitoring *Rpp4* gene expression. We have developed a single pair of primers (purple arrows) at of all eight full-length genes. Direct sequencing of the RT-qPCR product differentiates between the eight genes in a given tissue.

The same primers were used for RT-qPCR, while we could not use traditional methods to distinguish between genes during RT-qPCR, the sequencing method above was used to distinguish genes in the RT-qPCR product. For each tissue, we used three biological replicates containing pooled RNA from three plants. The water-inoculated samples were used as calibrators for each tissue. The SuperScript™ III Platinum® SYBR® Green One-Step RT-qPCR kit (Invitrogen, Carlsbad, CA, U.S.A) was used according to the manufacturers' protocols with 100ng of total RNA, 300nM final concentration of primers in the following RT-qPCR program: 5min at 60°C, 10 min at 95°C, PCR cycling at 95°C for 15 s, 62°C for 30s, and data collection for 15s at the extension temperature of 72°C for 40 cycles. The PCR was run in a Stratagene Mx3000P followed by a dissociation curve, taking a fluorescent measurement at every degree between 55°C and 95°C. The expression of all eight *Rpp4* genes together was normalized to the Gmβ-actin

(Genbank accession no. GMU60500), which was not differentially expressed in response to *P. pachyrhizi*.

The fold change was calculated from the differences in threshold cycle (Ct) using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). For each tissue, over 120 clones were sequenced and assigned to one of the eight *Rpp4* candidate genes. Since the RT enzyme lacks proofreading activity, RT sequences were considered a match at 99% identity, provided that the base change did not match the genomic sequence of any of the *Rpp4* candidate genes.

2.5. Analysis of Alternative Splicing

BLASTN (Altshul et al., 1997) was used to identify internal repeats within the *Rpp4* candidate genes. Intragenic duplications were visualized using WebACT (Abbott et al., 2006). To test whether alternative splicing of the intragenic duplications occurs, primers were designed from the 5' end of the repeat unit (F-5'ATTCCATCTIATGTACTTCCTT 3') and the final exon of the predicted gene (R-5'GCTTGAATTGATGCAAATCAGG 3'), outside of the repeat unit. The same RNA samples described in real time analyses were used for this analysis. The LongAmp® *Taq* DNA Polymerase (BioLabs, Cat# M0323S) was used according to the manufacturers' protocols with 1μl of cDNA and 300nM final concentration of primers in the following PCR program: 3 min at 94°C, 40 cycles for PCR cycling at 94°C for 15s, 52,5°C for 30s, 65°C for 3 min and final extension at 65°C for 10min.

3. Results

We previously sequenced a >607 kb region of the *Rpp4* locus in the resistant mapping parent PI459025B (Figure 2). FGENESH (www.softberry.com), BLASTX (Altschul et al. 1997) against the Uniprot protein database (Apweiler et al. 2004), and BLASTN (Altshul et al. 1997) against the soybean transposable element database (SoyTEdb, Du et al. 2010) were used to identify protein coding genes. Ten CC-NBS-LRR resistance genes were identified in this region. Eight full-length (18-40 kb) CC-NBS-LRR resistance genes were identified with two

additional R-gene fragments (*Rpp4R4F* and *Rpp4R8F*). *Rpp4R1, Rpp4R2* and *Rpp4R6* contain frameshift mutations leading to truncated and likely nonfunctional proteins. Exon boundaries and intron splice sites were predicted using the NetPlantGene Server (Hebsgaard et al., 1996) and BLASTX comparisons with Uniprot protein database (Apweiler et al. 2004).

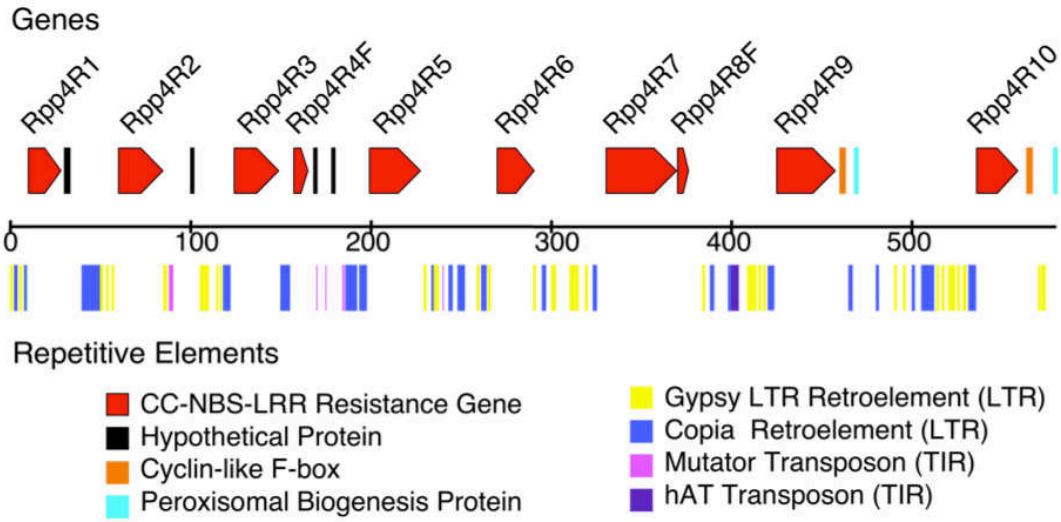

Figure 2. The *Rpp4* locus in PI459025B. FGENESH (www.softberry.com) and BLASTX (Altschul et al.,1997) against the Uniprot protein database (Apweiler et al., 2004) were used to annotate genes (above the ruler) in the *Rpp4* locus. Repetitive elements (below the ruler) were annotated using SoyTEdb (Du et al., 2010).

In order to obtain more information about *Rpp4* function, we wanted to use real time quantitative PCR (RT-qPCR) to analyze the expression of the eight full-length genes from PI459025B in different plant tissues, in different stages of development and after inoculation with *P. pachyrhizi*. Prior to measuring the expression of the *Rpp4* genes of interest, we needed to identify a reference gene with a constitutive expression pattern that did not change in different tissues or treatments. Gmβ-actin, GmGAPDH and GmRNAr18S were all tested for their potential use as reference genes as suggested by Stolf-Moreira et al. (2011). Based on our analyses (Figure 3), Gmβ-actin, GmGAPDH and GmRNAr18S showed efficiencies of 98, 103, 89% respectively. *Gmβ-actin* was chosen as the reference gene in relative quantification analysis because this gene had the lowest M value in GeNorm analysis and therefore is more stable than other genes.

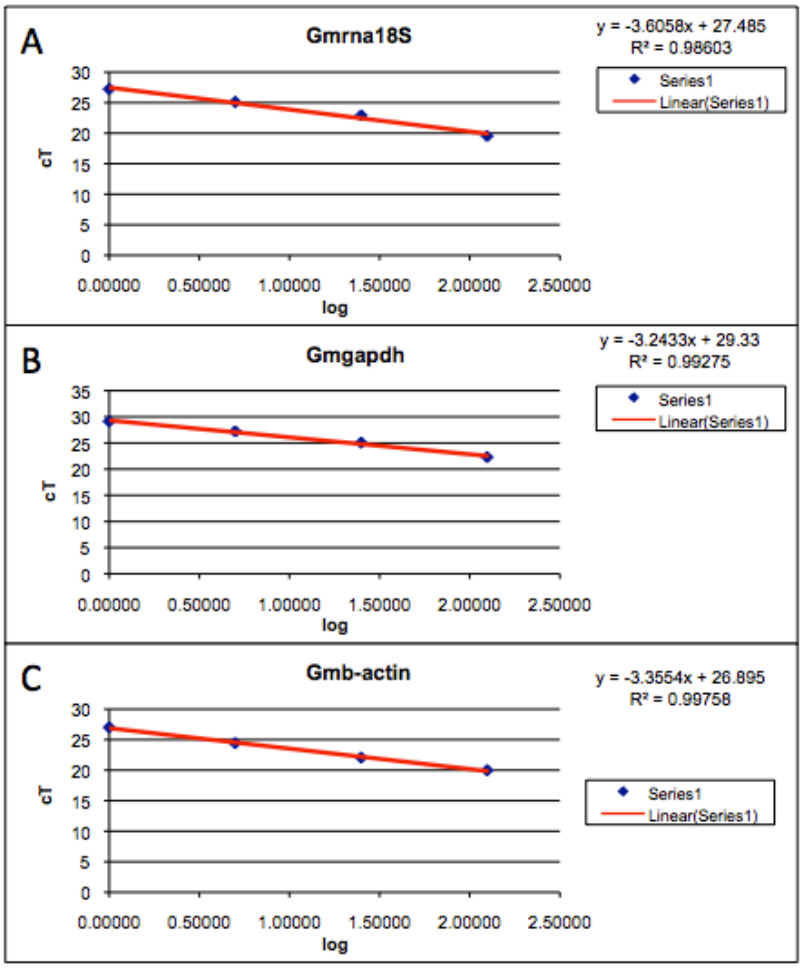

Figure 3. Efficiency curve from reference genes. The calibration curve was established graphically by plotting Ct values as a function of log dilutions of cDNA. **A)** Gmrna18S-E=89%, **B)** Gmgapdh-E=103%, **C)** Gmβ-actin-E=98%

In order to accurately quantify gene expression gene-specific primers are ideal. When comparing across multiple related genes, designing gene-specific primers becomes more difficult. In this case all ten *Rpp4* candidate genes, shared between 90 and 99% nucleotide identify. Designing ten pairs of gene-specific primers from the same location in each gene was not possible. If primers were designed from different locations within the genes or with different amplicon sizes, differences in amplification efficiency would affect the results of gene expression analyses. Therefore, a single primer pair was designed from a conserved region of the NBD. Primer sequences matched the eight full-length genes with 100% nucleotide identity. In addition, amplicon sizes were the same for all eight genes.

Use of these primers in RT-qPCR would monitor the expression of all eight full-length genes at once. Direct sequencing of RT-qPCR products would identify individual SNPs or SNP combinations specific to each gene and allowed us to determine which genes were expressed in a given tissue. Prior to RT-qPCR, the primers were tested on genomic DNA to determine the amplification efficiency of the primers for each gene.

Previously, Meyer et al. (2009) reported that *Rpp4* homologs were induced in leaves of the resistant parent PI459025B relative to the susceptible parent Williams82. However no significant differences in gene expression were observed between *P. pachyrhizi* inoculated tissue and mock inoculated tissue within a soybean genotype. No additional plant tissues were analyzed. Therefore, we chose to monitor the expression of the *Rpp4* homologs in several plant tissues including *P. pachyrhizi* inoculated and mock inoculated leaves, roots and flowers and seeds (Figure 4). We observed differential expression of *Rpp4* candidate genes in all tissues relative to mock-treated samples except root tissue. In flowers and seeds, *Rpp4* candidate gene expression was reduced by *P. pachyrhizi* inoculation. A 1.9 fold reduction was observed in flowers and an even greater reduction, 8.4 fold, was observed in seeds, both relative to mock plants. In leaves, expression was increased 1.35 fold by *P. pachyrhizi* inoculation. Since R genes are expressed at low levels we couldn't be able to analyze it in root tissue.

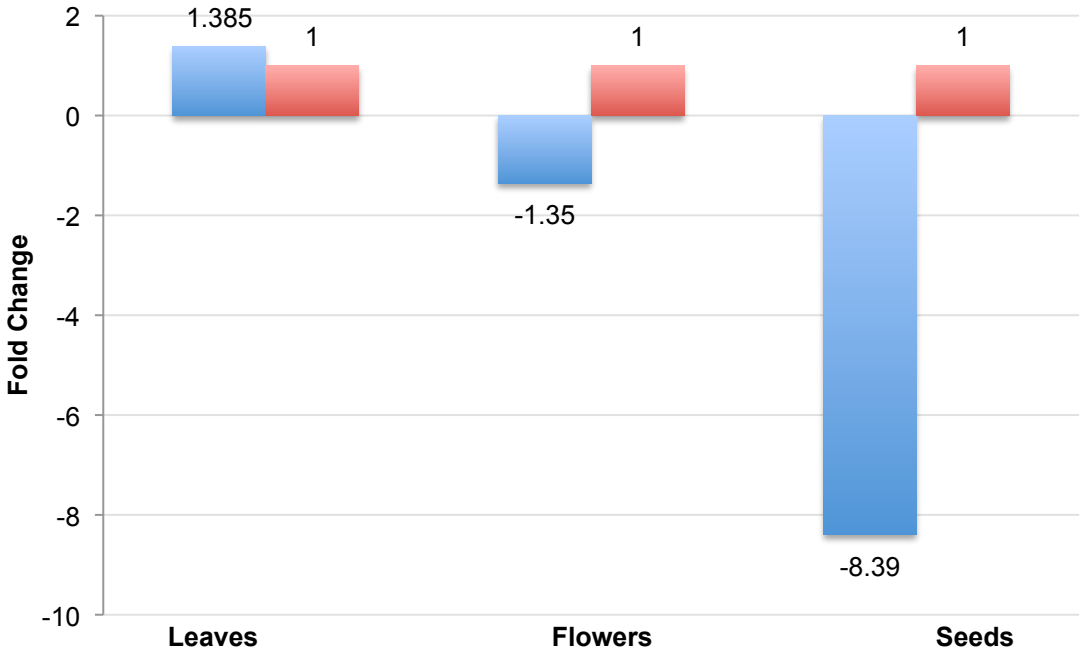

Figure 4. Relative quantification of *Rpp4* candidate gene expression. RT-qPCR was used to obtain more information about *Rpp4* expression in different tissues and 12 hours after inoculation with either *P. pachyrhizi* or water (mock). We observed differential expression of *Rpp4* candidate genes in all tissues. In Blue inoculated samples and in red mock samples.

To determine which *Rpp4* candidates were contributing to the expression detected by RT-qPCR, we purified and cloned all the real time PCR products, to monitor the expression of individual genes. Over 120 clones were sequenced from each sample and clones were assigned to one of the eight *Rpp4* candidate genes based on identifying SNPs or SNP combinations. Once clones were assigned to individual genes, gene counts were adjusted for different gene amplification efficiencies (Table 2).

The genes *Rpp4R1, Rpp4R2* and *Rpp4R6* contain a frameshift mutation leading to truncated and likely non-functional protein. However, the genes *Rpp4R2* were expressed only in root while *Rpp4R6* were expressed in flower, root and seed. Based on these results, we believe that *Rpp4R3*, *Rpp4R7* and *Rpp4R9,* which are heavily expressed in leaves, are candidates for *Rpp4.*

Table 2. Cloning of RT-qPCR products from *Rpp4* candidate genes. For each tissue, over 120 clones were sequenced and assigned to one of the eight *Rpp4* candidate genes. Following gene assignment values were adjusted to reflect different amplification efficiencies. We present the expression of eight individual *Rpp4* candidate genes as a percentage of all expressed genes within a given sample.

***Rpp4* gene**	*P. pachyrhizi* **Leaves**	*P. pachyrhizi* **Flowers**	*P. pachyrhizi* **Roots**	*P. pachyrhizi* **Seeds**
R1	0	0	0	0
R2	0	0	80	0
R3	36	12	0	0
R4	-	-	-	-
R5	-	60	0	74
R6	0	5	16	21
R7	45	9	2	2
R8	-	-	-	-
R9	19	14	2	3
R10	0	0	0	0

While expression of R-genes in the appropriate tissue is required for defense, little is known about the impact of alternative splicing on R-gene function. Analyses of R-genes in the *Rpp4* locus revealed extreme variation in the number of exons and the predicted protein coding sequence. While *Rpp4R1* contained 10 exons was 18,295 bp in length, *Rpp4R9* contained 14 exons and was 30,370 bp in length and *Rpp4R7* contained 18 exons and was 39,146 bp in length. To understand these vast differences, BLASTN (Altshul et al. 1997) and WebACT (Abbott et al., 2006) were used to visualize intragenic duplications within *Rpp4* candidate genes (Figure 6A, only *Rpp4R9* is shown). All of the candidate genes except fragments *Rpp4R4F* and *Rpp4R8F* contained a variable number of internal duplications of exons 5 and 6. Close examination of the candidate gene sequences demonstrates the extent of the duplications (Figure 6B) (only *Rpp4R9* is shown). Within the LRR domain, the end of intron 4 through the start of intron 6 has been duplicated head to tail four times. While the duplication affected the same two exons, duplication size varied from 1027 bases to 7754 bases, depending on intron size. In each case, the duplication preserved exon splice sites, suggesting

alternative splicing of these genes was possible. We used CLUSTALW (Thompson et al., 2994) to align the repeat units and the terminal exon of the LRR.

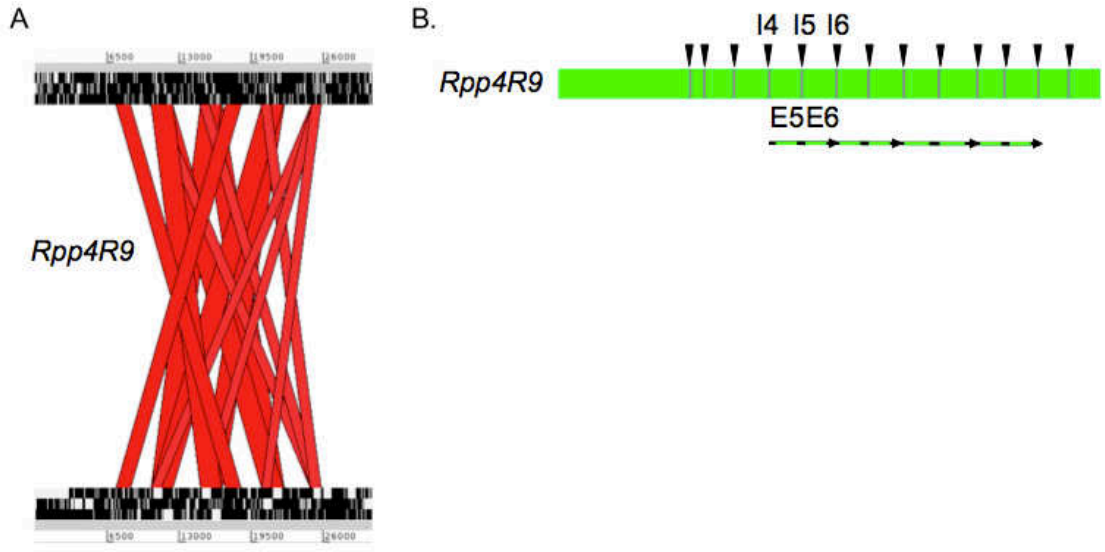

Figure 5. The *Rpp4* candidate genes contain large duplications. A) WebACT was used to visualize intragenic duplications within *Rpp4* candidate genes (only *Rpp4R9* is shown). All of the candidate genes except *Rpp4R1* and fragments *Rpp4R4F* and *Rpp4R8F* contain these duplications. **B)** Close examination of the candidate gene sequences demonstrates the extent of the duplications (only *Rpp4R9* is shown).

We designed primers from the 5' end of the repeat unit and the final exon of the predicted gene. Based on our analyses, we predicted genes 3,7,9, would have amplified with amplification product sizes ranging from 1027 to 3000bp. Our results, shown in Figure 6, show two interesting phenomena. First, we saw different alternative splice products in different tissues. Second, the splicing pattern changes in response to *P. pachyrhizi* inoculation. These differences are most apparent in the leaf samples.

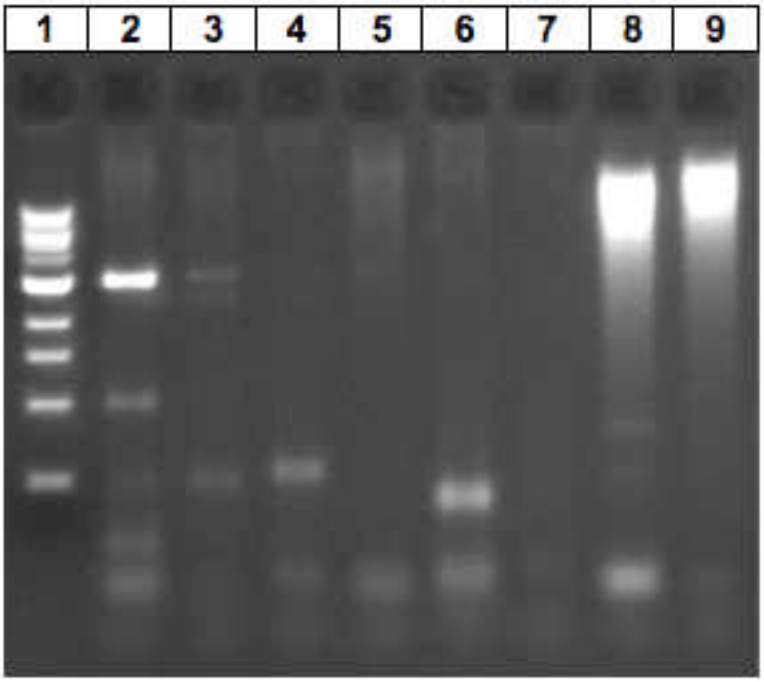

Figure 6. Alternative splicing of *Rpp4* candidate genes in different plant tissues. We developed primers that span the duplicated regions to determine if alternative splicing does occur. Our results suggest that differential splicing occurs in different tissues and in response to *P. pachyrhizi* inoculation. 1) 1Kb ladder, 2) leaves (*P. pachyrhizi* inoculated), 3) leaves (water inoculation), 4) flower (*P. pachyrhizi* inoculated), 5) flower (water inoculated), 6) seed (*P. pachyrhizi* inoculated), 7) seed (water inoculated), 8) root (*P. pachyrhizi* inoculated), 9) root (water inoculated).

4. Discussion

Many studies on defense and stress mechanisms in plants have been based on gene expression. Transcriptome studies have helped to provide a better understanding of plant stress response. We have been identified ten CC-NBS-LRR resistance genes in the *Rpp4* locus in the resistant mapping parent PI459025B. The sequencing of ten candidate genes was based in Meyer et al., 2009. We used real time PCR to analyze the expression of all ten genes in different plant tissues, in different stages of development and after inoculation with *P. pachyrhizi*. Real time PCR is becoming an important technology for studying host-pathogen interactions. However, proper and highly reliable reference genes are needed for normalization of data. These reference genes, referred to as “housekeeping” genes, are required for cell survival and were assumed to undergo little or no

variation in expression. The most common housekeeping genes are actin, glyceraldehyde-3-phosphate dehydrogenase, ribosomal genes (Stolf-Moreira et al., 2011).

As shown in Figure 3, Gmβ-actin had 98% of efficiency and it can be used to normalize gene expression in soybean plants. The actin gene was often used to normalize the quantification of expression (Bezier et al., 2002; Langer et al., 2002; Thomas et al., 2003, Stolf-Moreira et al., 2011). However, the 18S ribosomal subunit is another example of a commonly used internal control. There are several arguments against the use of rRNA as the internal control. Ribosomal subunit transcription is affected by biological factors and drugs (Vandesompele et al., 2002). Recently, some papers had published using real time PCR to confirm several differentially expressed genes in leaves of *Glycine tomentella* in the presence of the fungal pathogen *P. pachyrhizi* by real time PCR (SORIA-GUERRA et al., 2010 a, b). In addition, classical and real-time fluorescent PCR assays were developed to identify and differentiate between *P. pachyrhizi* and *P. meibomiae* (Frederick et al., 2002).

Gene expression after leaf rust infection was compared in near-isogenic wheat lines differing in the Lr10 leaf rust resistance gene. RNA from susceptible and resistant plants was used for cDNA library construction. In total, 55008 ESTs were sequenced from the two libraries. Several genes from the resistant sample and the susceptible samples collected at different time points after leaf rust infection was confirmed by RT-qPCR analysis (MANICKAVELU et al., 2010 or 20011).

Using real time PCR analyses, we observed differential expression of *Rpp4* candidate genes in all tissue. In flowers and seeds, *Rpp4* candidate gene expression was reduced by *P. pachyrhizi* inoculation. In leaves, expression was slightly increased *P. pachyrhizi* inoculation. This expression could be explained because of fitness costs associated with maintaining R protein-mediated resistance, expression level of R genes have to be tightly regulated. Our results were similar to those found by Meyer et al., 2009 where they used RT-qPCR to determine relative expression levels of *Rpp4C1* to *Rpp4C3* (Wm82) and *Rpp4C1* to *Rpp4C5* (PI459025B) in the susceptible line (Wm82) and the resistant line (PI459025B) following infection with *P. pachyrhizi* and mock inoculation.

Resistance genes candidates (RGC) were reported to be expressed constitutively at very low level before pathogen challenge (Aswati & Thomas, 2007). In cotton, the expression levels of 6 RGC were undetectable before inoculation, whereas all the 6 RGC were induced after *Verticillium dahliae* inoculation (Gao et al., 2006).

Recently, several approach have been done to analysis the gene expression on soybean, like microarray or RNAseq. However, these approaches are often ineffective in characterizing R-gene expression because genes are expressed at such low levels. Many microarray are based on ESTs and since the R-genes are expressed at low levels there are no corresponding ESTs. A perfect example is the *Rpp4* candidate genes, they are not represented on the microarray chip and analysis of available RNAseq data demonstrate they are expressed at low levels.

Alternative Splicing (AS) is a post-transcriptional process that regulates gene expression through increasing protein complexity and modulating mRNA transcript levels (SIMPSON et al., 2010). Different types of AS event include alternative 5' and 3' splice site selection, intron retention, exon skipping and mutually exclusive exon splicing, resulting in the inclusion or exclusion of intronic or exonic sequences (BLACK, 2003; STAMM et al., 2005). Dynamic changes in AS patterns reflect changes in abundance, composition and activity of splicing factors in different cell types and in response to cellular or environmental cues. Research indicates that SA (?) treatment can induce disease-resistant response to bacterial and fungal diseases in some plants (Niu et al., 2007).

Recently, Meyer et al., 2009 saw clear evidence of evolutionary forces acting on the *Rpp4* locus. Differences in gene number between Wm82 and PI459025B are likely due to duplication or unequal recombination. Using WebACT (Abbott et al., 2006) we visualized intragenic duplications within *Rpp4* candidate genes. All candidate genes except *Rpp4R1* and fragments *Rpp4R4F* and *Rpp4R8F* contain these duplications. Given the similarity of all *Rpp4* candidate genes, it is possible that small amino acid differences may play a key role in resistance.

5. Conclusions

This study revealed ten CC-NBS-LRR resistance genes in the *Rpp4* locus. The *Rpp4* genes showed differential expression in all of the tissues studied and responded to ASR infection. Based on high expression levels in leaf tissue we believe that *Rpp4R3*, *Rpp4R7* and *Rpp4R9* are candidates for *Rpp4.* Preliminary evidence suggests tissue-specific alternative splicing may play a role in generating diversity. The only way to characterizing *Rpp4* genes expression and alternative splice is describe in this paper, since the *Rpp4* are expressed at low level, they are not present in Microarray or RNAseq.

These studies will allow a better understanding of the molecular mechanisms involved in the interaction between soybean and *P. pachyrhizi*, and allow the development of technologies that could lead to new alternatives for disease control.

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