DISCOVERY AND DEPLOYMENT OF SOYBEAN RUST (*PHAKOPSORA PACHYRHIZI*) RESISTANCE (*RPP*) GENES

by

ZACHARY RAYMOND KING

(Under the Direction of Henry Roger Boerma)

ABSTRACT

Soybean rust (SBR), caused by the fungal pathogen *Phakopsora pachyrhizi* is an economic threat to soybean production, especially in the Americas. Host plant resistance is important for SBR management.

PI 567068A possesses a resistance gene on chromosome 18 near the *Rpp6* locus of PI 567102B, determined by bulked segregant analysis (BSA). Linkage mapping placed *Rpp6* between 5,916,005 to 5,961,229 bp and the resistance gene from PI 567068A between 5,961,229 to 6,122,387 bp (Wm82.a1). PI 567068A reacted differentially to two international *P. pachyrhizi* isolates when compared with PI 567102B. Both PI 567068A and PI 567102B carry the same haplotype designated as *Rpp6/Rpp[PI567068A]*. The result indicated that PI 567068A possesses a novel allele that is either at the *Rpp6* locus or at a new locus.

Four near-isogenic lines (NILs) of G00-3213 were developed by backcrossing rust resistance genes *Rpp1*, *Rpp2*, *Rpp3*, or *Rpp4* into an elite, maturity group VII soybean line, G00-3213 and were designated as G00-3213*Rpp1*, G00-3213*Rpp2*, G00-3213*Rpp3*, and G00-3213*Rpp4*. Each NIL exhibited resistance to the GA12 *P. pachyrhizi* bulk isolate equivalent to that of the original resistant sources, and similar agronomic qualities to G00-3213.

Marker-assisted selection has become a valuable tool to select value-added quantitative trait loci. A reliable, inexpensive, high-throughput DNA extraction protocol for soybean seed and leaf samples that does not generate hazardous waste was developed,

allowing for the leverage of SNP genotyping platforms, including the SimpleProbe Assay and KASPar v4.0 SNP Genotyping System. This methodology allows one to run up to 150 SNP markers from a single seedchip or leaf with a 95% success rate.

Germplasms with putatively novel *Rpp* genes, PI 594796, PI 433972, and PI 605823, were identified. Using BSA with SoySNP50K Infinium Chips, the putative resistance locus from PI 605823 was located on Chr 19. PI 476905A has the *Rpp6/Rpp[PI567068A]* haplotype, yet has a unique *P. pachyrhizi* isolate pattern. The resistance of PI 605791A was mapped to the *Rpp4* locus, and its haplotype matches the novel PI 423972 (*Rpp4[PI423972]*) haplotype associated with the resistance allele described here. PI 605791A has a different isolate reaction pattern compared to both PI 459025B (*Rpp4*) and PI 423972.

INDEX WORDS: DNA isolation, Comparative genomics, *Glycine max*, Haplotype analysis, Linkage mapping, *Phakopsora pachyrhizi* resistance gene (*Rpp*), Seed chipping, Single nucleotide polymorphism (SNP), Soybean rust (SBR), Super bulked segregant analysis, Quantitative trait locus (QTL) mapping

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ZACHARY RAYMOND KING

BS with Honors, SUNY College of Environmental Science and Forestry, 2008

MS, University of Georgia, 2011

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ZACHARY RAYMOND KING

Major Professor:	H. Roger Boerma
Committee:	Zenglu Li
	David Walker
	James Buck
	Scott Jackson

Electronic Version Approved:

Suzanne Barbour
Dean of the Graduate School
The University of Georgia
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DEDICATION

I dedicate this work in my Father's memory, William Dean King. You gave me my first microscope and inspired me to love plants, animals, and above all else, the natural world. Thank you for always believing in me and encouraging me to do my best.

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CHAPTER 1

LITERATURE REVIEW

Introduction to soybean rust (SBR) caused by the fungus *Phakopsora pachyrhizi*

Soybean rust (SBR) is caused by the obligate fungal pathogens *Phakopsora pachyrhizi* (Sydow and Sydow) (Sydow and Sydow 1914), and *P. meibomiae* (Arthur) (Arthur, 1917), which are capable of infecting plants predominantly in the subfamily Papilionoideae, including soybean (*Glycine max* L. Merrill) (Ono et al., 1992). These basidiomycete fungi persist predominantly in the anamorph stage, and also produce teliospores, with morphological characteristics that allow them to be differentiated from one another (Ono et al., 1992; Saksirirat and Hoppe, 1991). Advances in molecular analyses such as polymerase chain reaction (PCR) and analysis of the internal transcribed spacer sequence or real time PCR can rapidly and reliably identify and discern whether *P. pachyrhizi* or *P. meibomiae* is present on a diseased host (Frederick et al., 2002).

P. meibomiae was first observed in the Caribbean Islands, and Central and South America, and is less virulent on soybean than *P. pachyrhizi*, which was first discovered in Japan in 1902 (Bonde et al., 2006; Bromfield, 1984; Bromfield and Hartwig, 1980). For the purpose of this review, *P. pachyrhizi*, the more aggressive and economically important species responsible for the disease SBR will be the focus. In May 1994, SBR was found on the Hawaiian Islands Oahu, Kauai, and Hawaii (Killgore, 1994). Shortly after, SBR was found in Kenya and Uganda (1996), and likely spread south via windblown urediniospores to Zimbabwe in 1998 (Kawuki et al., 2003; Levy, 2005). In the 2001-2002 season, *P. pachyrhizi* was confirmed in Paraguay and

rapidly expanded to Brazil (Yorinori et al., 2005). By November of 2004, SBR was found near Baton Rouge, LA and in five other locations in central and south Louisiana shortly thereafter (Schneider et al., 2005). Urediniospores were likely brought into the continental USA by Hurricane Ivan (Isard et al., 2005).

In the USA, more than 72 isolates from 12 states have been collected from infected kudzu and soybean plants (Twizeyimana and Harman, 2012). Using a set of differential soybean lines (seven distinct resistant lines and one susceptible genotype), three distinct *P. pachyrhizi* pathotypes are now recognized, as well as six different aggressiveness categories, as determined by the number of uredinia present per square centimeter of the host's leaf tissue (Twizeyimana and Harman, 2012).

Symptoms and signs of soybean rust and environmental effects on infection

SBR first appears as small TAN or reddish-brown (RB) lesions on the abaxial portion of leaves located near the bottom portion of the canopy on soybean (Fig. 1.1). It has been observed that when temperatures reach ≥37°C, the production of urediniospores is inhibited, which may be a limiting factor for the spread of SBR to the northern regions of the USA each year from the southern regions of the USA (Bonde et al., 2012).

On susceptible kudzu accessions, the disease caused by *P. pachyrhizi* is more prevalent in partially shaded areas (Dias 2008; Dias et al., 2011; Sconyers et al., 2006). Increased solar radiation in controlled laboratory settings is negatively correlated with *P. pachyrhizi* urediniospore survival (r = -0.83), and SBR disease severity was greater in the lower canopy of all field plots (Young et al., 2012). Koch and Hoppe (1987) have documented that the germ tubes of urediniospores exhibit negative phototropism, which is induced by light in the blue

spectrum, especially at 441 nm. Additionally, it was found that as ultraviolet light intensity increased, germ-tube length declined, causing a 40% reduction in growth due to either delayed germination or suppression of growth. The germination of urediniospores is also strongly negatively correlated (r = -0.91) with increasing solar irradiance (MJ m^{-2}). This prediction is now integrated into the USDA's SBR aerobiological model (http://sbrusa.net, verified 13 May, 2012) (Isard et al., 2006). These data are supported by Buck et al. (2010), who demonstrated that when *P. pachyrhizi* urediniospores were placed in direct, midmorning sunlight for 2.5 hours, no germination or germ tube elongation were recorded when compared to control samples placed in the dark. When urediniospores samples were subjected to 24 hours of cool, white fluorescent light (600 µmol $s^{-1}m^{-2}$), an 11.4% reduction in germination, and 9.3% reduction in germ tube length were observed as compared to the control. The differences in observations may be due to one or several unknown factors including, but not limited to, the intensity of light, light spectra, and temperature differences. In this study, experiments that exposed urediniospores to sunlight ranged up to 11°C higher than fluorescent light experiments in some instances, therefore the increased temperature may have also played a role in the reduction of urediniospore germination (Buck et al., 2010).

Dias (2008) studied the disease occurrence in soybean plants exposed to different daylight conditions in Florida as well as the relationship between cloudiness and disease level in both Brazil and South Africa. Decreased sunlight intensity and duration were generally significantly correlated with both higher disease occurrence and severity in the field (Dias, 2008; Dias et al., 2011). The data of Dias et al. (2011) strongly suggest that increased disease presence and severity in shady areas are not correlated with higher germination of urediniospores or

increased post-infection processes. More likely, it is believed that sunlight intensity and duration plays a key role in host-pathogen interaction processes, yet the mechanism remains unknown.

Yield losses due to SBR

The potential for severe yield and economic losses due to the rapid spread of *P. pachyrhizi* makes SBR a highly destructive foliar disease of soybean (Livingston et al., 2004; Sinclair, 1989; Sinclair and Hartman, 1999). Yield losses due to SBR have been extensive, and are due in part to premature defoliation, fewer filled pods, and reduced seed weight (Bromfield, 1984; Melching et al., 1989; Ogle et al., 1979). When research plots have been severely infected with *P. pachyrhizi,* the oil content of soybean has been significantly reduced compared to soybean plots protected with mancozeb fungicide (Ogle et al., 1979). The disease can develop quickly when conditions are favorable, such as frequent rain, long periods of dew, and temperatures ranging from 15°C to 29°C (Melching et al., 1989). Infection can significantly reduce both yield and seed quality. Ogle et al. (1979) observed 60 to 70% yield losses in Queensland in unprotected plots due to reduced pods per plant, fewer filled pods per plant, and a higher frequency of seed abortions.

From 2002 to 2003, SBR related yield losses in Brazil exceeded 60%, which collectively in Mato Grosso, southern Goias, and Bahia accounted for a reduction of 3.4 million tons of soybean (~$759 million US) (Yorinori et al., 2005). In 2003, SBR infected 14.8 million ha of soybean fields in Brazil causing approximately 2 billion dollars (U.S.) in losses from control methods and yield decline (Yorinori et al., 2005). When effects of SBR infection on soybean yield were assessed over 2 years (2005 and 2006) at Londrina, Brazil, mean yield reductions were 67 and 37% when infection occurred at the R2 and R5 stage of development (Fehr et al.,

1971), respectively, compared to the fungicide treated control (Kumudini et al., 2008). Greater yield reductions caused by infection during the R2 developmental stage is attributed in part by a higher reduction in photosynthetic leaf area, which was evident from the defoliated controls in the 2006-2007 growing season. Soybean maturity therefore is a major factor in SBR infection and subsequent disease development. In 2005 to 2006 in the southeastern USA (Alabama, Florida, Georgia, North Carolina, South Carolina, and Virginia) SBR was detected primarily at the R4 stage (2 cm pods) (Fehr et al., 1971) or later, accounting for 97 and 88% of all cases, respectively (Christiano and Scherm 2007; Sconyers et al., 2006).

However, U.S. SBR related yield losses have been moderate, which is in part due to the use of sentinel plots that were once present in over 30 of the continental states and serve to monitor for rust using early maturing soybean cultivars or other susceptible legumes (e.g., pigeon pea (*Cajantis cajan* (L.) Huth)) (Roberts, 2006). The period from detection in a sentinel plot to the spread of the fungus to any neighboring field was at least a month in 2005 and 2006, thereby allowing ample time for soybean growers to protect their fields with fungicides (Christiano and Scherm, 2007). For these reasons the sentinel plot program has been overwhelmingly successful for the timely application of protective fungicides, saving soybean growers an estimated $209-299 million dollars per year since 2005 (Hershman et al., 2011).

Soybean rust has not come close to causing the yield losses that were predicted to have occurred in the USA due to disease outbreaks. This is in part because the USA took proactive measures to create tools to monitor the spread of SBR each year as it emerges from the southern regions of the USA where it overwinters. The USDA Integrated Pest Management Pest Information Platform for Extension and Education (IPM-PIPE) provides tools such as the monitoring of strategically placed sentinel plots to monitor the disease

(http://sbr.ipmpipe.org/cgi-bin/sbr/public.cgi) (Isard et al., 2006; Sikora et al., 2014). When the collective yield losses of soybean due to SBR were approximated for Georgia, South Carolina, Alabama, Louisiana, North Carolina, Arkansas, Oklahoma, and Texas collectively from 2005 to 2007 the disease is estimated to have incurred 53.65 million metric tons of yield losses (Wrather and Koenning, 2009).

Over wintering of *Phakopsora pachyrhizi* on hosts including kudzu (*Pueraria* spp.)

It is known that *P. pachyrhizi* can infect over 150 plant species from 53 genera. These species are either native to the USA or have been introduced; although not all infected hosts were observed producing uredinia capable of sporulation (Hartman et al., 2011; Lynch et al., 2006a, 2006b; Rytter et al., 1984; Slaminko et al., 2008a, 2008b). To date, all hosts identified belong to the subfamily Papilionoideae within the family Fabaceae, where *Glycine* species are classified (Hartman et al., 2005; Ono et al., 1992; Rytter et al., 1984).

P. pachyrhizi is an obligate biotroph, and therefore requires a living host to overwinter in areas where the temperature remains above 4°C; although urediniospores can survive short freeze and thaw cycles (Bromfield, 1984; Jurick et al., 2008). In the USA the potential for overwintering is possible due to large invasive stands of kudzu (*Pueraria* spp.), introduced to the USA in 1876 (Blaustein, 2001). Kudzu is a perennial, leguminous species with trifolioliate leaves and a woody stem and sprawling growth habit that forms dense patches. In 1997, 121 years after its introduction to the USA, the USDA declared kudzu a “noxious weed” (Blaustein, 2001).

Differences in susceptibility and resistance of kudzu host plants collected and challenged with *P. pachyrhizi* isolates Alabama 04-1, Brazil 01-1, and Louisiana 04-1 revealed genetic

differences among kudzu patches across the southeastern USA (Bonde et al., 2009). This is supported by several kudzu plant introductions over time and gene flow amongst different kudzu biotypes (Bonde et al., 2009; Jordan et al., 2010; Sun et al., 2005). Bonde et al. (2009) observed genetic diversity between different kudzu accessions in their lesion reaction type to *P. pachyrhizi* isolates; some kudzu accessions showed resistance (RB lesions) or immunity (IM) and other accessions were susceptible (TAN lesions) to a set of eight distinct and diverse isolates.

Kudzu has been estimated to cover approximately 2.8 million hectares of the USA, with the dominant species identified as *Pueraria montana* (Lour.) Merr. variety *lobata* (Willd.) (Forseth and Innis, 2004). In parts of Florida, kudzu provides a year-round host for *P. pachyrhizi*, and kudzu is the only known species in the USA that provides over-wintering; although Florida Beggarweed (*Desmodium tortuosum*) may also provide refuge (Jurick et al., 2008; Sconyers et al., 2006). In below freezing (0°C) temperatures, urediniospores within the leaf litter of defoliated kudzu hosts retained the ability to germinate; however, no detectable infectively of the inoculum was observed. (Jurick et al., 2008) It is believed that *P. pachyrhizi* is overwintering each year on patches of kudzu, which act as primary inoculum to facilitate yearly epidemics of SBR that move north from southern Florida (Jurick et al., 2008).

Genetic mapping of resistance to *Phakopsora pachyrhizi*

Genetic resistance of soybean cultivars to *P. pachyrhizi* is the preferred means of disease control, and in theory is the most sustainable solution to controlling SBR both economically and environmentally, primarily due to the reduction of fungicide applications to infected soybean fields (Hartman et al., 2005; Lemos et al., 2011). At least seven dominant and two recessive alleles at six loci that condition resistance to a limited set of rust isolates are recognized by the

Soybean Genetics Committee: *Rpp1*, *Rpp1-b*, *Rpp2*, *rpp2*, *Rpp3*, *Rpp4*, *Rpp5*, *rpp5*, and *Rpp6* (Table 1.1; Fig. 1.1; Fig. 1.2) (Bromfield et al., 1980; Bromfield and Hartwig, 1980; Chakraborty et al., 2009; Garcia et al., 2008; Hartwig, 1986; Hartwig and Bromfield, 1983; Li et al., 2012; McLean and Byth, 1980).

Soybean lines with *Rpp1* (PI 200492) can react with an immune response (no lesions) to certain *P. pachyrhizi* populations or isolates (Hartwig and Bromfield, 1983; Walker et al., 2014a; 2014b). When infected with *P. pachyrhizi,* specifically three of the bulk isolates collected in Georgia (GA07, GA08, and ,GA12) lines with *Rpp2*, *Rpp3*, *Rpp4*, and *Rpp6* can exhibit variable dark, reddish-brown resistance lesions on their leaves (Fig. 1.1 & 1.2; Table 1.1) that have few or in some cases no uredinia (Hartwig, 1986; Hartwig and Bromfield, 1983; Walker et al., 2014a; 2014b). The susceptible phenotype caused by *P. pachyrhizi* infection is characterized by TAN lesions with high levels of sporulation (Fig. 1.1) (Bromfield and Hartwig, 1980; Miles et al., 2006).

Rpp1 was fine-mapped to linkage group (LG)-G (chromosome (Chr) 18) between simple sequence repeat (SSR) markers BARC_Sct_187 and BARC_Sat_064, an interval of less than 1 cM (Hyten et al., 2007) equivalent to approximately 156-kb. Fine mapping *Rpp1* has allowed molecular markers associated with the *Rpp1* locus to be employed by breeders to more effectively incorporate this gene into U.S. cultivars (Hyten et al., 2007). More recently, the fine-mapped location of *Rpp1*, along with the release of genotype data for approximately 50K SNPs across the 20 chromosomes of soybean (SoySNP 50K; Song et al., 2013), has identified a unique haplotype for the *Rpp1* locus (Harris et al., 2015). Chakraborty et al. (2009) mapped and subsequently confirmed the presence of a different resistance allele at the *Rpp1* locus, which has been designated as *Rpp1-b*, contributed by PI 594538A. *Rpp1-b* confers resistance characterized

by RB lesions to the Zimbabwe isolate (ZM01-1), but may have limited use in the USA, as the source of *Rpp1-b* develops TAN lesions when challenged with the Georgia 2007 bulk *P. pachyrhizi* isolate (GA07) (Chakraborty et al., 2009; Walker et al., 2014b). Differential isolate phenotyping differentiated the sources of *Rpp1* (PI 200492) from *Rpp1-b*, as the source of *Rpp1* is susceptible to the Zimbabwe 2001 *P. pachyrhizi* isolate (ZM01-1) and developed TAN lesions. Furthermore, Ray et al. (2009) has described two additional sources of *Rpp* genes at the *Rpp1* locus: PI 587886 and PI 587880A. Based on the evaluation of PI 587886, PI 587880A, PI 594538A (*Rpp1-b*), and PI 200492 (*Rpp1*) inoculated with eight international *P. pachyrhizi* isolates, the resistance genes in PI 587886 and PI 587880A are likely the same allele, or potentially allelic variants with PI 594538A (*Rpp1*-b) as determined by their reaction responses that were nearly identical (Ray et al., 2009). The only notable exception was when challenged with the Australian *P. pachyrhizi* isolate AU79-1, some plants of PI 587886 and PI 587880A showed both an immune response and RB lesions, whereas PI 594538A responded with RB lesions (Ray et al., 2009). The eight isolates tested by Ray et al. (2009) clearly distinguished the resistance of PI 200492 (*Rpp1*) from that of PI 587886, PI 587880A, and PI 594538A (*Rpp1-b*), highlighting the usefulness of isolate panels. Further evaluations, such as allelism tests, fine mapping, or gene cloning, could determine if the locus identified in PI 587886 and PI 587880A contains a unique allele of or the *Rpp1-b* allele.

Garcia et al. (2008) mapped the resistance locus of PI 230970 (*Rpp2*) to LG-J (Chr 16). The *Rpp2* locus has since been fine-mapped by Yu et al.,(2014) to a 188.1-kb interval on the 'Williams 82' reference genome (Glyma.Wm82.a2). Garcia et al. (2008) reported an additional source of resistance at the *Rpp2* locus contributed by PI 224270. This recessive *Rpp* allele has been designated as *rpp2[?](PI224270)*. An allelic test on the F_2 generation from a PI 224270 x

PI 230970 cross demonstrated that no segregation occurred, indicating the two alleles *Rpp2* and *rpp2[?](PI224270)* are probably allelic (Garcia et al., 2008). PI 224270 and PI 230970 both showed similar levels of resistance when challenged with the bulk Georgia isolates GA08, where both had the RB/I type of resistance with no sporulation. When challenged with GA12, both PI 224270 and PI 230970 developed RB lesions with some sporulation. This warrants further exploration and fine mapping of *rpp2* contributed by PI 224270, as well as potentially exploiting it as a resistance gene source in the USA (Walker et al., 2014b).

Hyten et al. (2009) mapped the resistance of PI 462312, known as *Rpp3,* using a combination of bulked segregant analysis and F_2 linkage mapping to a 1.4-cM region of LG-C2 (Chr 6) that is flanked by the SSR markers BARC_Satt460 and BARC_Sat_263. An additional *Rpp* locus known as *Rpp?*(Hyuuga), contributed by PI 506764, that confers RB resistance to some *P. pachyrhizi* isolates has been mapped to and subsequently confirmed to be on Chr 6 between the SSR markers Satt134 and Satt460, the same locus that harbors *Rpp3* from PI 462312 (Monteros et al., 2007). *Rpp?*(Hyuuga) explained 22% of the variation in field severity and 15% of the variation in lesion density in the greenhouse. The presence of *Rpp?*(Hyuuga) was associated with a decrease in number of lesions, lower sporulation, and reduced SBR severity (Monteros et al., 2007). Both *Rpp?*(Hyuuga) and *Rpp3* map to the bottom of Chr 6; however, PI 462312 (*Rpp3*) is susceptible to *P. pachyrhizi* isolates(s) from Brazil and produced TAN lesions, while *Rpp?*(Hyuuga) reacted with a reddish-brown lesion type, which initially suggested that *Rpp*3 and *Rpp?*(Hyuuga) may be different alleles at the same locus (Garcia et al., 2008; Monteros et al., 2007). Monteros et al. (2010) fine-mapped *Rpp?*(Hyuuga) to LG-C2 (chromosome 6), a 371-kb locus containing seven candidate genes between the sequence-tagged sites (STS) STS70887 and STS70923. A high-throughput single nucleotide polymorphism

(SNP) assay was then developed using a SimpleProbe based on melting curve analysis for SNP06-44058. This SNP is tightly linked to *Rpp?*(Hyuuga) and can differentiate between all reported SBR resistance genes, as well as most of the alleles present in elite North American breeding populations at this locus.

Kendrick et al. (2011) screened Hyuuga-derived recombinant inbred lines (RILs) with a set of eight *P. pachyrhizi* isolates collected from various regions around the world and found that Hyuuga carries a natural pyramid of two resistance genes; one on LG-C2 (Chr 6) near *Rpp3* and a second gene on LG-N (Chr 3) near *Rpp5*. These results support that Hyuuga's resistant reaction when challenged by the isolates of *P. pachyrhizi* from Brazil may be caused by the cultivar having an *Rpp* gene at the *Rpp3* and *Rpp5* locus, when compared to the phenotypic reaction of PI 462312 (*Rpp3*). Haplotype analysis of Hyuuga and PI 462312 demonstrated the two PIs have a similar haplotype at the *Rpp3* locus, however Hyuuga has some heterozygous regions of its genome (Harris et al., 2015). Harris et al. (2015) studied the resistance of 75 SBR resistant PIs where approximately 15% of the lines possessed a natural pyramid of two *Rpp* genes. It appears that Hyuuga was the first discovery of a natural phenomenon of *Rpp* gene pyramid (Kendrick et al., 2011).

Garcia et al. (2008) mapped the resistance gene contributed by PI 459025B, known as *Rpp4*, to LG-G (Chr 18). Silva et al. (2008) mapped *Rpp4* within 1.9 cM of Satt288 on LG-G (Chr 18). Meyer et al. (2009) completed the fine mapping work of *Rpp4* and identified a putative candidate gene (*Rpp4C4* from PI 459025B) that is believed to control the *Rpp4*-mediated resistance to SBR. *Rpp4 is* approximately 4.5 Mbp away from *Rpp1* based on the Gmax_275_v2.0.softmasked genome sequence (Harris et al., 2015).

Garcia et al. (2008) mapped dominant *Rpp* genes from three PIs to LG-N (Chr 3): PI 200487, PI 200526, and PI 471904 were designated to have *Rpp5*. A major recessive *Rpp* gene contributed by PI 200456 was designated as *rpp5,* and maps to LG-N (Chr 3) (Calvo et al., 2008; Garcia et al., 2008). Kendrick et al. (2011) compared the *Rpp5* and *rpp5* genotypes to one another using a panel of eight *P. pachyrhizi* isolates, and found that PI 200526 is distinct from PI 471904, PI 200487, and 'Hyuuga,' and that the latter set of genotypes likely share the same natural pyramid of *Rpp* genes at the *Rpp3* and *Rpp5* loci. The resistance of PI 200526 may be a different allele or a resistance gene tightly linked to the *Rpp5* locus that is entirely different than the other sources of *Rpp5* (Kendrick et al., 2011).

The *Rpp6* locus from PI 567102B was mapped between SSR markers Satt324 and Satt394, which is on the top of Chr 18 at a distinctly different location compared to *Rpp1* and *Rpp4* (Li et al., 2012). PI 567102B produced a reddish-brown resistance or immune reaction to SBR in greenhouse assays from 2008 to 2013 and, in 2012 PI567102B had the lowest number of lesions and no detectable sporulation under high disease pressure in a two-replication field experiment in Attapulgus GA, making the *Rpp6* gene promising for a resistance source to be used in the Southeast (Walker et al., 2014a; 2014b). The genetic information associated with fine mapping and haplotype analysis of the soybean germplasm collection allows for both putatively novel *Rpp* sources to be identified as well as the identification of germplasm that has currently characterized *Rpp* genes (Harris et al., 2015; Song et al., 2013).

There are at least nine races of *P. pachyrhizi* that have been described (Burdon and Speer, 1984; Sinclair and Hartman, 1999). Some races of the pathogen have overcome some of these resistant genes (i.e. *Rpp1* and *Rpp3*) (Godoy, 2005). However, if several or all resistance genes at independent loci were pyramided into a cultivar, a broader spectrum of resistance to SBR may

be obtained in the USA (Boerma et al., 2011; Hartman et al., 2005; Lemos et al., 2011). *P. pachyrhizi* has the ability to adapt to what are considered some of the best sources of resistance in the USA, including *Rpp1* and *Rpp6*. Isolates obtained from Florida in 2011 (FL-Q11-1) and 2012 (FL-Q12-1) produced TAN, highly sporulating lesions when used to challenge PI 567102B (*Rpp6*), PI 200492 (*Rpp1*), and L85-2378 (isoline of 'Williams 82' with *Rpp1*) in greenhouse assays (Paul et al., 2013).

Comparison of field, greenhouse, and detached-leaf evaluations of soybean germplasm for resistance to *Phakopsora pachyrhizi*

Purified *P. pachyrhizi* cultures can be obtained by selecting a single urediniospore from a uredinium (Furtado et al., 2008; Smith et al., 2007). The single-spore isolate is then used to inoculate detached leaves from a 3- to 4-week-old susceptible soybean cultivar in a Petri dish. The culture is incubated with a 12-h light cycle at 20 to 23°C to increase inoculum for large-scale infections with a purified single spore derived isolate (Paul and Hartman 2009; Twizeyimana et al., 2007). There are several advantages to using single-spore isolates, including characterizing expressed genes of the pathogen, and ensuring only one *P. pachyrhizi* genotype is present, ensuring pathogenic homogeneity (Tremblay et al., 2008).

For relevance to soybean breeding, the development of successful SBR resistant cultivars will rely on using data on which specific resistance genes confer resistance or immunity to the domestic and international isolate collections of *P. pachyrhizi* (Kendrick et al., 2011; Paul and Hartman, 2009; Pham et al., 2009; Walker et al., 2014a; 2014b). Breeding efforts focused on obtaining SBR resistance use spores collected from either surrounding areas (Calvo et al., 2008; Garcia et al., 2008; Monteros et al., 2007; 2010; Walker et al., 2014a; 2014b) or at a Biosafety

Level 3 Containment Facility at the USDA-ARS Foreign Disease-Weed Science Research Unit located at Fort Detrick, MD (Melching et al., 1983) due to restrictions to avoid pathogen introduction (Bromfield et al., 1980; Bromfield and Hartwig, 1980; Hartwig, 1986; Hartwig and Bromfield, 1983; Kendrick et al., 2011).

Soybean lines that potentially contain unique resistance genes need to be challenged with an international set of distinct *P. pachyrhizi* isolates in order to evaluate the usefulness of a given resistance gene internationally, or identify resistance genes that may be allelic, tightly linked, the same *Rpp* gene, or a natural pyramid at different loci as in the case of PI 506764 ('Hyuuga') (Chakraborty et al., 2009; Harris et al., 2015; Kendrick et al., 2011; Ray et al., 2009). Isolates that exhibit the same pathogenicity are a hindrance to the progress of screening plant introductions for unique resistance (e.g., Harris et al., 2015).

Disadvantages of single-spore isolation include the time and effort to create and maintain the genetic fungal stocks. There is also the potential danger of selecting a less infective/aggressive *P. pachyrhizi* genotype, and/or truncating the genetic diversity of the population that may contain several unique pathenogenicity genes (Paul and Hartman, 2009).

Walker et al. (2014b) reported greenhouse seedling assay and host plant resistance results from 2008 to 2013 that included semi-quantitative ratings including the lesion type, level of urediniospore production from uredinia in rust pustules, and the lesion density or count of lesions per cm^2. These data were compared to the field data of SBR infected research plots in 2008 in Quincy, FL, where a rust index score was generated by combining both rust severity and sporulation, as described by Walker et al. (2014a). The greenhouse seedling assay data collected in 2008, 2009, and 2010 had a 0.52, 0.74, and 0.79 correlation coefficients, respectively, with the 2008 rust index score data from the field in Quincy FL. The seedling rust resistance reactions

were again documented in greenhouse seedling assays from 2011 to 2013, however, only the 2011 greenhouse data were comparable to field data in 2012 at Attapulgus GA. These data had a 0.52 correlation coefficient to field data.

Several factors may account for the lack of correlation between greenhouse and field host plant resistance *P. pachyrhizi* data sets including the use of different isolates over years and slightly different rating scales. Both greenhouse and field host-plant resistance assays to SBR have limitations. Field assays are more limited, as the environment is more variable, there is no control over the pathotypes or aggressiveness of the *P. pachyrhizi* population, other diseases and insects may be potentially infecting, or defoliating, and feeding on the plants, and perhaps most importantly, SBR lesions can change color, often becoming darker over time (Twizeyimana and Harman, 2012; Walker et al., 2014b).

Greenhouse phenotypic data are more rapidly generated and inoculations are more reliable, consistent, and easier to control, as environmental factors such as humidity and temperature can be somewhat regulated. Additionally, researchers have control over the *P. pachyrhizi* isolate(s) used. For this reason infection type is one of the most important phenotypic observations in the greenhouse and has been used extensively for the genetic mapping of *Rpp* genes (e.g., Harris et al., 2015; Hyten et al., 2007; 2010; Kendrick et al., 2011; Li et al., 2012; Monteros et al., 2007). Bulked segregant analysis (Michelmore et al., 1991) with high-density markers (Hyten et al. 2010; Song et al., 2013) has been especially useful to rapidly identify *Rpp* loci for genetic mapping (e.g., Harris et al. 2015; Hyten et al., 2007; 2009), however this method has limitations if the germplasm or plant introduction line has more than one *Rpp* gene (Harris et al., 2015).

Likely, the best method to be employed would be the continued use of greenhouse methods to phenotype and subsequently genetically map *Rpp* genes using known isolates, followed by the simultaneous testing of the resistance source in multi-location field trials in the target growing environments to ensure the *Rpp* genes are efficacious in production areas. The speed and accuracy of greenhouse methods provide reliable phenotypic data in one month (Harris et al., 2015). Additionally, screening given *Rpp* germplasm sources with large panels of diverse *P. pachyrhizi* isolates (e.g. Harris et al., 2015; Kendrick et al., 2011) will further characterize the potential weaknesses and strengths of a given *Rpp* gene or pyramid of *Rpp* genes and could indicate the usefulness of a given *Rpp* gene in diverse geographic areas.

Preservation of *Phakopsora pachyrhizi* urediniospores

P. pachyrhizi inoculum is usually maintained on living hosts in the greenhouse, often on universally susceptible SBR cultivar (e.g. 'Williams 82') (Kendrick et al., 2011; Twizeyimana and Harman, 2012); however the long-term preservation of urediniospores is possible. Specifically, Furtado et al. (2008) preserved urediniospores collected from soybean by dehydrating them using silica gel for 24 hours and subsequently stored the urediniospores at -80°C. Once removed from cold storage, spores were coated on the adaxial leaf surfaces of a susceptible soybean cultivar using a solution of water and Tween-20 (0.05% v/v), which allowed approximately 30 and 100 lesions to form per cm^2 after 8 and 3 months of storage time, respectively (Furtado et al., 2008).

Cultivar development

Strategies for the development of a rust resistant cultivar will likely depend on the use of pyramiding multiple resistance genes in a single cultivar. *P. pachyrhizi* will likely overcome single resistance genes (Godoy, 2005; Paul et al., 2013), therefore a more durable resistance would be derived from using pyramids of the strongest resistance genes for a given location and potentially partial resistance genes (Lemos et al., 2011; Hartman et al., 2005).

> "Go back young man and gather up your weary and defeated genes of the past, take your currently successful genes, find some new ones if you can, and build yourself a pyramid."
> -1978, R.R. Nelson

Lemos et al. (2011) have validated the utility of gene pyramids to achieve SBR resistance. Lines containing a pyramid of three resistance genes were developed from two parents, 'An-76' that harbors *Rpp2* and *Rpp4* and 'Kinoshita,' a source for *Rpp5*. The F_3 lines homozygous for *Rpp2+Rpp4+Rpp5* performed better than any single resistance gene, or the natural pyramid found in An-76 when analyzed for frequency of rust lesions with uredinia, number of uredinia per lesion, frequency of open uredinia, and sporulation level when inoculated with a Brazilian rust population. Thus, additive resistance likely holds many advantages including more durable resistance, and should be one of the key methods for breeding SBR resistant cultivars in the future, especially for Brazil. This is evident as monogenic resistance of *Rpp1* and *Rpp3* was overcome in the state of Mato Grasso, Brazil (Ribeiro et al., 2007). The current focus in the southeastern USA is the development of a high-yielding elite cultivar with a

pyramid of resistance genes *Rpp1* and *Rpp3* which have shown durable RB resistance when challenged with USA rust populations (H.R. Boerma, personal communication, 2012).

Partial resistance and/or tolerance

Studies indicate that all isolates of *P. pachyrhizi* overcome at least one of the known resistance genes (Bonde et al., 2006; Kendrick et al., 2011; Paul et al., 2013; Paul and Hartman 2009; Pham et al., 2009). Therefore, partial resistance as well as single-gene resistance may need to be used together, or pyramids of several *Rpp* genes and partial resistance could be beneficial in breeding soybean cultivars for resistance to *P. pachyrhizi* (Hartman et al., 2005; Lemos et al., 2011; Pham et al., 2009). Partial resistance to SBR has been reported (Wang and Hartman, 1992). When inoculated with a Taiwanese *P. pachyrhizi* strain, PI 200492 (*Rpp1*) showed latent infection, slower uredinial development, and earlier senescence of uredinia (2-4 days) compared to PI 559369 ('Lee 68'), demonstrating that a single dominant gene can also confer partial resistance throughout the growing season compared to susceptible genotypes (Marchetti et al., 1975). The identification and utilization of partial resistance is limited in soybean breeding programs due to time-consuming evaluation methods (Hartman et al., 1991; Hartman et al., 2005), and this goal is still currently under pursuit (Oloka et al., 2009).

Another strategy proposed has been to select for tolerance or yield stability despite a soybean plant or plot being heavily infected with SBR (Hartman et al., 2005). Very little has been reported in the literature on this strategy. The process involves selecting genotypes with high-yield potential and less yield loss in the presence of SBR, which requires larger replicated yield plots, and bringing plants to maturity (Tschanz et al., 1986). Screening for yield stability at the Asian Vegetable Research and Development Center by Hartman et al. (1995) identified some

high-yielding genotypes with lower yield losses under severe SBR infection. These lines were evaluated for tolerance by comparing yields from fungicide treated versus untreated paired plots.

Screening genotypes for yield stability is costly due to the need for replicated plots to measure yield for both fungicide treated and untreated plots, and manual SBR inoculation if naturally occurring SBR is not present (Hartman et al., 2005; Walker et al., 2011). There are many environmental and biological variables that impact SBR development and it can be difficult to achieve a sufficient SBR inoculation and subsequent infection in the field (Hartman et al., 2005). Nevertheless, some groups have successfully bred for yield stability under SBR presence. One example is the lines MNG 2.13, 1.37, and 8.10 that on average yielded approximately 300 kg ha^{-1} more than the check variety Nam 1 in Uganda over 4 years at four locations (Tukamuhabwa et al., 2012).

Current status of SBR resistance in the USA

As of 2012, soybean cultivars with resistance to *P. pachyrhizi* conferred by single *Rpp* gene(s) are not yet commercially available (Li et al., 2012). Recently, substantial efforts have led to the development of near isogenic lines in elite breeding materials for the northern and southern regions of the USA. This is a critical step toward the development of cultivars with rust resistance. Diers et al. (2013) released eight rust resistant near-isogenic lines of MG II and MG IV cultivars LD01-7323 and LD00-3309. LD01-7323 isolines contain the rust resistance genes from *Rpp1* (PI 200492), *Rpp1-b* (PI 594538A), *Rpp?*(Hyuuga) (PI 506764), or *Rpp5* (PI 200456). For the LD00-3309 isolines, the same sources of resistance were used for all *Rpp* genes with the exception of *Rpp5*, whereby PI 471904 was used. These lines had equivalent yield and agronomic characteristics to their elite recurrent parents after four backcrosses and

selection. Additionally, King et al. (submitted to Journal of Plant Registrations) developed four near- isogenic lines of the elite MG VII line G00-3213 that was developed at the University of Georgia. The sources of *Rpp* genes were derived from PI 200492 (*Rpp1*), PI 230970 (*Rpp2*), PI 462312 (*Rpp3*), and PI 459025B (*Rpp4*). Additionally, G01-PR16 (PI 659503) was released. G01-PR16 is a MG VI germplasm line that harbors *Rpp?*(Hyuuga) allele derived from PI 506764 as well as resistance to other southern-related diseases and possesses 90% of the yield potential of the recurrent parent 'Dillon' (Boerma et al., 2011). These germplasm lines will help breeders to eventually deploy cultivars with single *Rpp* genes or pyramids of the most effective *Rpp* genes in the same background.

There have been mixed results in studies of soybean genotypes challenged with isolates of *P. pachyrhizi* collected from the USA. Differential reactions were observed by Pham et al. (2009) when sources of resistance were challenged with three isolates collected in the USA (two collected from Alabama and one from Louisiana) in 2004. However, Paul and Hartman (2009) found no differential response among resistant genotypes when challenged with six isolates collected in the USA (two collected from Florida, and an isolate each from Mississippi, South Carolina, Texas, and Illinois) in 2006 and 2007. Data on the number of *P. pachyrhizi* races present in the USA was recently made available allowing for a better understanding of the spectrum of virulence and diversity of this pathogen (Twizeyimana and Harman, 2012). This resource, coupled with the additional international isolate collection resource, should better enable soybean breeders to more effectively develop cultivars with resistance to race(s) specific to the USA (Kendrick et al., 2011; Twizeyimana and Harman, 2012).

The broad host range of *P. pachyrhizi* is not common among rust species, and increases the likelihood of this particular rust species, to continue to survive and overwinter in the

southern, frost-free regions of the USA (e.g. Florida) as well as in Central America and Mexico. Furthermore, *P. pachyrhizi* may contain genes that contribute to a diverse and multifaceted virulence pattern as a result of this broad host range (Hartman et al., 2005). The likelihood of continuous adaptation through genetic recombination or additional introduction to the continental USA by *P. pachyrhizi* should not be underestimated (Vittal et al., 2012). There is clear evidence of changes in rust virulence over time as mentioned above, with *Rpp1* and *Rpp6* capable of being overcome by some Florida genotypes of the *P. pachyrhizi* (Paul et al., 2013). Therefore, continued breeding efforts are imperative to combat this pathogen, such as the identification, and pyramiding of new SBR resistance genes and/or tolerance genes are vital to the U.S. soybean industry.

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Table 1.1: Soybean sources for resistance genes to *Phakopsora pachyrhizi* (*Rpp*), genomic positions of *Rpp* genes and their reaction types.

Gene designation	*P. pachyrhizi* reaction, sporulation[§#]	Line name	MG#	Province	Country	Reported DNA markers¶¶	SNP markers§§	Genomic of SNPs††	Genomic of SNPs¶	*P. pachyrhizi* isolate(s) for mapping‡	Reference
Rpp1	IM, No	PI200492	VII	Shikoku	Japan	Sct_187, Sat_064	ss715632302, ss715632319	Chr 18 60,460,936 - 60,616,971	Chr 18 56,180,270 - 56,338,144	IN73-1	Hyten et al., 2007
Rpp1-b‡	N/A	PI594538A	IX	Fujian	China	Sat_064, Sat_372#	ss715632302, ss715632389	Chr 18 60,460,936 - 61,096,028	Chr 18 56,180,270 - 56,797,850	ZM01-1	Chakrabort y et al., 2009
Rpp2	RB, Yes	PI230970	VII	Unknown	Japan	BARCSOYSS R_16_0902, BARCSOYSS R_16_0908	ss715624066, ss715624108	Chr 16 28,882,177 - 29,084,869	Chr 16 29,250,025 - 29,451,934	GA08	Yu et al., 2014
rpp2‡‡	RB, Yes	PI224270	VII	Hyogo	Japan	Satt215, Sat_361	ss715624003, ss715624278	Chr 16 28,581,875 - 30,113,002	Chr 16 28,937,079 - 30,480,508	BZ04	Garcia et al., 2008
Rpp3	RB/IM, No	PI462312	VIII	Uttar Pradesh	India	Satt460, Sat_263	ss715594464, ss715594493	Chr 6 43,324,763 - 44,307,623	Chr. 6 44,083,402 – 44,867,962	IN73-1	Hyten et al., 2009
Rpp?(Hyuuga)	RB, Few	PI506764	VII	Kyushu	Japan	Satt460, Satt307	ss715594462, ss715594584	Chr 6 43,290,568 - 46,316,646	Chr 6 44,049,171 - 46,850,451	GA05	Monteros et al., 2007
Rpp4	RB, Yes	PI459025B	VIII	Fujian	China	Satt288, AF162283[##]	ss715631693, ss715631715	Chr 18 55,715,639 - 55,913,511	Chr 18 51,440,452 - 51,640,472	BZ	Meyer et al., 2009
Rpp5	RB/IM, No	PI200487	VIII	Shikoku	Japan	Sat_275, Sat_280	ss715585148, ss715585260	Chr 3 31,901,551 - 34,741,364	Chr 3 29,849,341 - 32,698,446	BZ04	Garcia et al., 2008
Rpp5	TAN, Yes	PI200526	VIII	Shikoku	Japan	Sat_275, Sat_280	ss715585148, ss715585260	Chr 3 31,901,551 - 34,741,364	Chr 3 29,849,341 -	BZ04	Garcia et al., 2008

									32,698,446		
Rpp5	RB/IM, No	PI471904	IX	Java	Indonesia	Sat_275, Sat_280	ss715585148, ss715585260	Chr 3 31,901,551 - 34,741,364	Chr 3 29,849,341 - 32,698,446	BZ04	Garcia et al., 2008
rpp5†	N/A	PI200456	VIII	Shikoku	Japan	Sat_275, Sat_280	ss715585148, ss715585260	Chr 3 31,901,551 - 34,741,364	Chr 3 29,849,341 - 32,698,446	BZ04	Garcia et al., 2008
Rpp6	RB/IM, no	PI567102B	IX	East Java	Indonesia	Satt324, Satt394	ss715632062, ss715633009	Chr 18 5,883,221 - 9,984,276	Chr. 18 5,920,452 - 10,016,762	LA04-1, MS06-1	Li et al., 2012

§A bulk isolate of *P. pachyrhizi* collected from field grown kudzu and soybean in 2012 was tested on some of the PI sources harboring known *Rpp* genes (Walker et al., 2014b)

§§Flanking SNP markers were identified by Harris et al. (2015) for *Rpp1*, *Rpp3*, *Rpp4*, *Rpp5*, *rpp5*, and *Rpp6*; and Yu et al., (2014) identified the flanking markers used for *Rpp2*. Flanking markers were determined for *rpp2* in the same manner as described by Harris et al. (2015).

¶Physical genomic locations were determined by using a BLAST search of genetic marker sequences to select the largest haplotype that correspond to Gmax_275_v2.0.softmasked database sequence and indicate the dbSNP location (e.g. ss715632302) available online at www.soybase.org/dlpages/index.php#snp50k (Song et al., 2013).

¶¶Genetic markers published in the linkage mapping study listed in the references section of this table.

#Soybean reactions when challenged with *P. pachyrhizi*: IM, Immune reaction type; RB, reddish-brown reaction type; TAN, tan colored, susceptible reaction with high sporulation; Few indicates sporulation on only some lesions.

‡Alternative allele at the *Rpp1* locus.

‡‡Recessive allele at *Rpp2* locus.

†Recessive allele at *Rpp5* locus.

††Physical genomic locations were determined by using a BLAST search of genetic marker sequences to select the largest haplotype that correspond to Wm82.a1 sequence and indicate the dbSNP location (e.g. ss715632302) available online at www.soybase.org/dlpages/index.php#snp50k (Song et al., 2013).

⋕Marker interval was determined using 77 F2:3 lines in the PI 564538A x Loda population

ǂResistant germplasm source or plant introduction from the USDA National Genetic Resources Program.

⋕Maturity group.

Table 1.2: *Phakopsora pachyrhizi* isolates used to phenotype segregating populations for the genetic mapping of *Rpp* genes.

Isolate	Country	Location	Year collected	Reference[§]
BZ	Brazil	Unknown	Unknown	Meyer et al., 2009
BZ04	Brazil	Cambé, Brazil	2004	Garcia et al., 2008
GA05	United States	Several[¶]	2005	Monteros et al., 2007
GA08	United States	Several[†]	2008	Yu et al., 2014
IN73-1	India	Pantnagar	1973	Hyten et al., 2007; 2009
LA04-1	United States	Ben Hur, Louisiana	2004	Li et al., 2012
MS06-1	United States	Jefferson County	2006	Li et al., 2012
ZM01-1	Zimbabwe	Narare	2001	Chakraborty et al., 2009

§The reference in which the particular isolate or bulk isolate was used to map an *Rpp* gene or allele (Table 1.1).

†*P. pachyrhizi* bulk isolate GA08 was collected from field-grown soybean and kudzu plants in Georgia during the summer of 2008.

¶*P. pachyrhizi* bulk isolate GA05 was collected from field-grown soybean and kudzu plants near Athens, Attapulgus, Griffin, and Eatonton, GA during the summer of 2005.

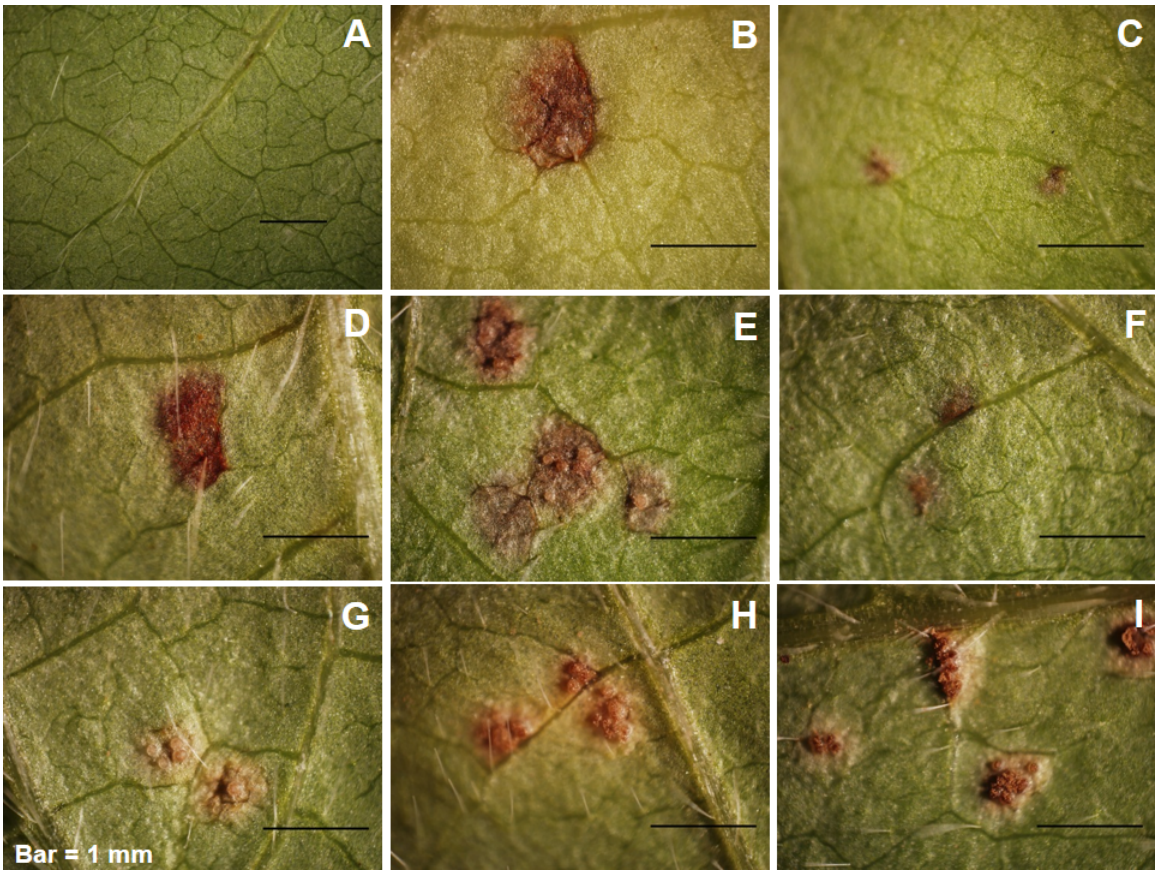

Figure 1.1: Reactions of plant introductions known to harbor *Rpp* genes and two susceptible controls 14 days post infection with *P. pachyrhizi*: (A) PI 200492 (*Rpp1*) shows an immune reaction, and (B) PI 230970 (*Rpp2*), (C) PI 462312 (*Rpp3*), (D) PI 459025A (*Rpp4*), (E) PI506764'Hyuuga'(*Rpp3*+*Rpp5*), (F) PI 567102B (*Rpp6*) show reddish-brown resistant reactions. Lines showing TAN lesions and a susceptible reaction include (G) PI 200526 (*Rpp5*), (H) PI 612157 'Prichard,' and (I) G00-3880. All results occurred when soybean genotypes were challenged with the Georgia 2012 (GA12) *P. pachyrhizi* bulk isolate in a greenhouse experiment conducted in Griffin Georgia. The GA12 bulk isolate and inoculation methods are described by Walker et al. (2014), the abaxial portion of the leaf was photographed. Bar = 1 mm.

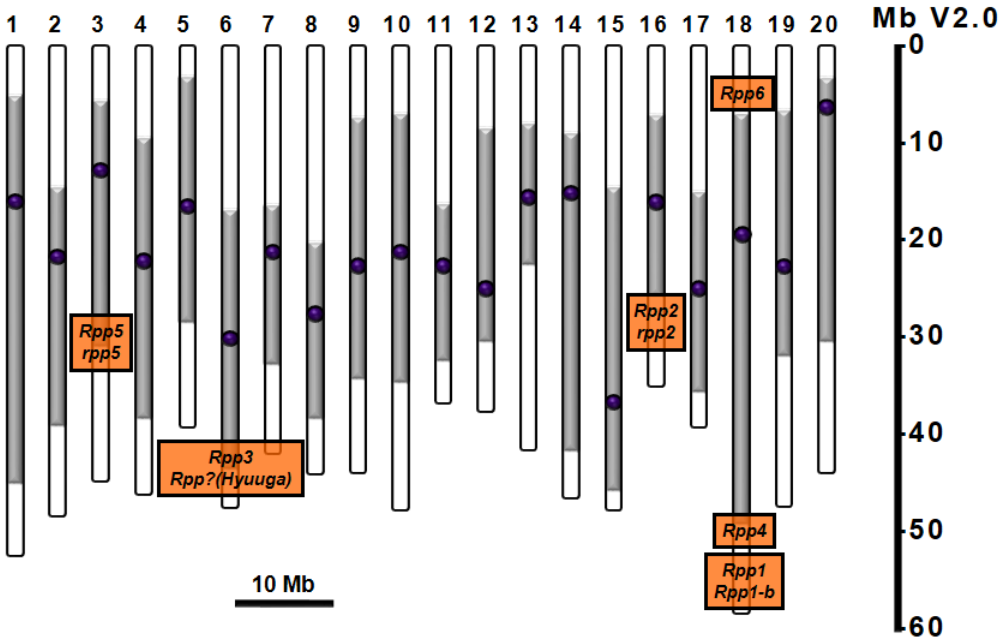

Figure 1.2: A diagram showing the relative genomic positions of described *Rpp* genes. The scale is million DNA basepairs (Mb) based on the Gmax_275_v2.0.softmasked database sequence of the soybean genome available at soybase.org.

CHAPTER 2

A NOVEL *PHAKOPSORA PACHYRHIZI* RESISTANCE ALLELE (*RPP*) CONTRIBUTED BY PI 567068A[1]

[1]King, Z.R., Harris, D.K., Pedley, K.F., Song, Q., Wang, D., Wen, Z., Buck, J.W., Li, Z., and H.R. Boerma. A Novel *Phakopsora pachyrhizi* Resistance Allele (*Rpp*) Contributed by PI 567068A. Submitted to Theoretical and Applied Genetics (2015).

Abstract

Soybean rust (SBR), caused by the obligate, fungal pathogen *Phakopsora pachyrhizi* is an economic threat to soybean production, especially in the Americas. Host plant resistance is an important management strategy for SBR. The most recently described resistance to *P. pachyrhizi* (*Rpp*) gene is *Rpp6* contributed by PI 567102B. *Rpp6* was previously mapped to an interval of over four million base pairs on chromosome 18. PI 567068A was recently demonstrated to possess a resistance gene near the *Rpp6* locus, yet PI 567068A gave a differential isolate reaction to several international isolates of *P. pachyrhizi.* The goals of this research were to fine map the *Rpp6* locus of PI 567102B and PI 567068A and determine whether or not PI 567068A harbors a novel *Rpp6* allele or another allele at a tightly linked resistance locus. Linkage mapping in this study mapped *Rpp6* from 5,916,005 to 5,961,229 bp (LOD score of 58.3) and the resistance from PI 567068A from 5,961,229 to 6,122,387 bp (LOD score of 4.4) (Wm82.a1 genome sequence). The two PIs shared the same haplotype window in these genomic regions. QTL peaks were 67,745 bp apart from one another as determined by the most significant SNPs in QTL mapping. The results of haplotype analysis demonstrated that PI 567102B and PI 567068A share the same haplotype in the resistance locus containing both *Rpp* alleles. Therefore the haplotype was designated as the *Rpp6/Rpp[PI567068A]* haplotype. The *Rpp6/Rpp[PI567068A]* haplotype identified in this study can be used as a tool to rapidly screen other genotypes that possess a *Rpp* gene(s) and detect resistance at the *Rpp6* locus in diverse germplasm.

Key message

The *Rpp6* locus of PI 567102B was mapped from 5,916,005 to 5,961,229 bp (chromosome 18); and a novel allele at the *Rpp6* locus or tightly linked gene *Rpp[PI567068A]* of PI 567068A was mapped from 5,961,229 to 6,122,387 bp.

Keywords

Haplotype analysis; bulked segregant analysis (BSA); resistance to *Phakopsora pachyrhizi* gene (*Rpp*); quantitative trait locus (QTL) mapping; soybean rust; single nucleotide polymorphism (SNP)

Author contribution statement

Zachary King designed SNP markers, phenotyped populations, completed mapping experiments, generated tables and figures, and wrote the manuscript. Donna Harris and James Buck phenotyped populations and reviewed the manuscript. Kerry Pedley tested lines with a panel of *Phakopsora pachyrhizi* isolates and edited the manuscript. Qijian Song provided the sequences of SNPs described here. Dechun Wang and Zixiang Wen ran the SoySNP50K Infinium Chips test. Zenglu Li and Roger Boerma interpreted the results, provided oversight for experiments, and edited the manuscript.

Introduction

Soybean rust (SBR), caused by the obligate, basidiomycete pathogen *Phakopsora pachyrhizi* Syd., is a detrimental disease to soybean (*Glycine max* L. Merill) production. *P. pachyrhizi* has a broad host range and is capable of infecting over 50 genera of plants (Lynch et

al. 2006a; 2006b; Ono et al. 1992; Slaminko et al. 2008a; 2008b). Susceptible soybean genotypes typically develop lesions on the abaxial side of their leaves that form uredinia and subsequently urediniospores that are primarily disseminated to other plants by wind (Goellner et al. 2010). The susceptible lesion type of soybean is referred to as TAN; due to the often tan colored lesion type that is associated with uredinia and high levels of sporulation. Resistant genotypes are defined by a reddish-brown (RB) lesion that can be sporulating or non-sporulating; or immunity (IM), where plants have no visible lesion to the naked eye after being challenged with *P. pachyrhizi* (Miles et al. 2006; Walker et al. 2014a; 2014b; Harris et al. 2015).

Soybean rust was first reported in the continental USA in 2004 in Louisiana, and may have been transported by hurricane Ivan (Isard et al. 2005; Schneider et al., 2005). *P. pachyrhizi* has low to no infectivity after freezing temperatures, therefore SBR is more prevalent in the Southern USA (Jurick et al. 2008). In the USA, it was estimated that Alabama, Arkansas, Georgia, Louisiana, North Carolina, Oklahoma, South Carolina, and Texas had 53.65 million metric tons of yield losses from 2005 to 2007 due to SBR (Wrather and Koenning 2009).

Developing cultivars with host-plant resistance is the preferred means of managing SBR, allowing for minimal reliance on fungicides and fossil fuels (Hartman et al. 2005; Hartman et al. 2011). Six resistance loci to *P. pachyrhizi* (*Rpp*), *Rpp1* to *Rpp6* have been reported that harbor at least 10 described resistance alleles, named in order of discovery: *Rpp1*, *Rpp1-b*, *Rpp2*, *rpp2*, *Rpp3*, *Rpp?*(Hyuuga) , *Rpp4*, *Rpp5*, *rpp5*, and *Rpp6* (Bromfield et al., 1980; Chakraborty et al. 2009; Garcia et al. 2008; Hartwig 1986; Hartwig and Bromfield 1983; Li et al. 2012; McLean and Byth 1980; Monteros et al. 2007).

Mapping work of the *Rpp1* (PI 200492) and *Rpp3* (PI 462312) loci was performed using the *P. pachyrhizi* India 1973 (IN73-1) isolate (Hyten et al. 2007; 2009). *Rpp2* (PI 230970) was

fine mapped using the Georgia 2008 bulk isolate (GA08) (Yu et al. 2014). The *rpp2* (PI 224270), *Rpp5* alleles (sources are PI 200487, PI 200526, and PI 471904), and *rpp5* (PI 200456) were mapped using a Cambé, Brazil 2004 isolate (BZ04) (Garcia et al., 2008). *Rpp?*(Hyuuga) (PI 506764) was mapped using the Georgia 2005 bulk isolate (GA05) (Monteros et al., 2007). *Rpp4* (PI 459025B) was fine mapped using a Brazilian isolate; the area it was collected from within Brazil was not disclosed (Meyer et al. 2009). *Rpp6* (PI 567102B) was mapped using the Louisiana 2004 (LA04-1) and Mississippi 2006 (MS06-1) isolates (Li et al. 2012).

In the Southeast US, *Rpp1*, *Rpp2*, *rpp2*, *Rpp3*, *Rpp?*(Hyuuga), *Rpp4*, and *Rpp6* have been shown to condition varying levels of resistance to SBR (Walker et al. 2014a; 2014b). The source of *Rpp1-b* does not provide effective resistance against field populations of *P. pachyrhizi* in the Southeastern USA (Walker et al. 2014a). PI 200487 and PI 471904 are believed to contain the *Rpp* resistance alleles at the *Rpp3* and *Rpp5*, loci and have effective resistance against soybean rust in the southeastern USA (Kendrick et al. 2011; Walker et al. 2014a; 2014b). PI 200526, which only has a known *Rpp* gene at the *Rpp5* locus, is susceptible to *P. pachyrhizi* in the southeastern USA (Kendrick et al. 2011; Walker et al. 2014b).

Breeding efforts to introgress *Rpp* genes into elite germplasm have been successful. Diers et al. (2013) developed eight elite near isogenic lines (NILs) with *Rpp1* (PI 200492), *Rpp1-b* (PI 594538A), *Rpp?*(Hyuuga) (PI 506764), *Rpp5* (PI 200456), or *Rpp5* PI 471904 alleles. In each NIL, the *Rpp* gene of interest was integrated via marker assisted backcrossing into the elite lines, LD01-7323 and LD00-3309 in maturity group (MG) II and IV, respectively. The NILs with the various *Rpp* genes yielded as well as their recurrent parents (Diers et al. 2013). G01-PR16 (PI 659503) is another example of an elite germplasm line that contains an *Rpp* gene. G01-PR16 was developed as a MG VI germplasm line with the *Rpp?*(Hyuuga) allele contributed

by PI 506764, and demonstrated 90% of the yield of its elite parent 'Dillon' (PI 592756) (Boerma et al. 2011). These examples illustrate that marker assisted breeding can be used successfully to develop useful germplasm for the areas at risk for SBR epidemics. Additionally, NILs possessing different *Rpp* genes could allow breeders to pyramid specific *Rpp* genes in the same genetic background.

Rpp6 from PI 567102B was previously mapped to chromosome (Chr) 18 (Li et al. 2012). *Rpp6* is a single, dominant gene that was mapped using two independent populations, whereby each population was phenotyped with the *P. pachyrhizi* isolates MS06-1, or LA04-1. Linkage mapping in each population placed *Rpp6* on Chr 18 between the simple sequence repeat (SSR) markers Satt324 and Satt394, with an interval of over 4 Mb (Wm82.a1 genome sequence). Resistant progeny from either mapping population developed an IM or RB lesion phenotype when challenged with the MS06-1 or the LA04-1 isolate (Li et al. 2012).

When *Rpp* genes map to the same locus, new potential alleles or tightly linked *Rpp* genes may be differentiated from one another using a panel of diverse *P. pachyrhizi* isolates, or a single *P. pachyrhizi* isolate that is informative. For example, PI 200492, the source of *Rpp1* is susceptible to the Zimbabwe 2001 (ZM01-1) isolate, while PI 594538A, the source of *Rpp1-b* is resistant to the ZM01-1 isolate, which was used to map the *Rpp1-b* allele (Chakraborty et al. 2009). Hyuuga which was originally believed to have a novel allele at the *Rpp3* locus (Monteros et al. 2007). A combination of using recombinant inbred line mapping and a panel of eight geographically diverse *P. pachyrhizi* isolates was used to identify that the cultivar 'Hyuuga' (PI 506764) harbors two *Rpp* genes (*Rpp3* and *Rpp5*) (Kendrick et al. 2011). Therefore differential isolates can differentiate between resistance alleles and identify multiple *Rpp* genes that a single isolate may not be able to identify.

P. pachyrhizi isolates Columbia 2004 (CO04-2), Hawaii 1998 (HW98-1), India 1973 (IN73-1), Louisiana 2004-1 (LA04-1), South Africa 2001 (SA01-1), Taiwan 1972 (TW72-1), Louisiana 2004-3 (LA04-3), Zimbabwe 2001 (ZM01-1), and Australia 1979 (AU79-1) are regularly used by the USDA-ARS Foreign Disease-Weed Science Research Unit (Ft. Detrick, MD) singularly or in multiple tests to differentiate types of resistance or to assist mappping *Rpp* genes and have been described in detail (Chakraborty et al. 2009; Harris et al. 2015; Hyten et al. 2007; 2009; Kendrick et al. 2011; Pham et al. 2009). When Harris et al. (2015) challenged PI 567102B with this panel of isolates, it reacted with RB resistant lesions for all isolates except TW72-1, to which PI 567102B reacted with a mixed reaction of plants that had TAN or RB lesions. Results of bulked segregant analysis (BSA) indicated that the resistance of PI 567068A was located within 5 cM of the *Rpp6* locus, and PI 567068A had RB resistant reactions to HW98-1, LA04-1, and LA04-3; however, it reacted with TAN lesions when challenged with the isolates ZM01-1, AU79-1, SA01-1, and TW72-1. Additionally, PI 567068A did not have haplotype allele matches for the *Rpp1* or *Rpp4* loci defined by Harris et al. (2015) that are also on Chr 18. These data supported that PI 567068A may harbor another allele at the *Rpp6* locus, or may possess a new gene that is linked to the *Rpp6* locus of PI 567102B (Harris et al. 2015).

Recently a SoySNP50K iSelect SNP BeadChip was developed with Illumina and used to genotype *G. max* and *G. soja* (soybean ancestor subspecies) genotypes in the USDA Soybean Germplasm Collection (Song et al. 2013; http://soybase.org/dlpages/index.php#snp50k). This has provided a wealth of genomic information, as the 50K SNPs span primarily euchromatic regions and cover all 20 chromosomes of the *G. max* and *G. soja* genomes. Polymorphisms are now easily located by accessing Soybase data (soybase.org) for mapping regions of interest of the soybean genome. Additionally, Kompetitive Allele Specific PCR (KASP) marker assays can

be developed for reliable and cost efficient genotyping and QTL mapping using the SNP data and sequence surrounding the SNP (Pham et al. 2013). Regions of the soybean genome associated with *Rpp* genes can be translated to the SoySNP50K data. Harris et al. (2015) used SoySNP50K data, in combination with BSA, and diverse panels of *P. pachyrhizi* isolates as tools to rapidly screen PIs with known *Rpp* gene resistance. They were able to identify PIs that likely harbor the same *Rpp* genes in different PI sources, or to identify PIs with putatively novel resistance. This approach allowed Harris et al. (2015) to define haplotype windows using the SoySNP50K data for *Rpp1*, *Rpp3,* and *Rpp4.* Yu et al. (2014) recently fine mapped the *Rpp2* locus and defined the unique haplotype window of this locus. One of the PIs identified by Harris et al. (2015) that putatively contained a novel mode of *Rpp* resistance near the *Rpp6* locus was PI 567068A. The objectives of this study were to: map the *Rpp* gene from PI 567068A and saturate the resistance gene locus from PI 567102B with SNP markers in order to determine if the resistance allele from PI 567102B is allelic to the *Rpp6* allele from PI 567068A.

Materials and methods

Plant material and population development

PI 567068A was selected for mapping because BSA data and differential *P. pachyrhizi* isolate data compared to PI 567102B (*Rpp6*) supported that PI 567068A possessed a putatively novel resistance allele within 5 cM of the *Rpp6* locus (Harris et al. 2015; Li et al. 2012). Genetic mapping populations were created by crossing a susceptible, elite cultivar or breeding line to a plant introduction (PI) with known soybean rust (SBR) resistance. The cross of 'Prichard' (PI 612157) × PI 567068A was made in the summer of 2011 in Athens, GA. The F_1 plants were grown in the winter (2011-2012) in the University of Georgia (UGA) greenhouse located in

Athens, GA. F_2 plants were grown in the summer of 2012 and threshed individually to form the $F_{2:3}$ families. Prichard, a maturity group (MG) VIII cultivar released from UGA with white flowers, gray pubescence, and tan pod walls (Boerma et al. 2001), is susceptible to the Georgia 2012 bulk *P. pachyrhizi* isolate (GA12) (Fig. 2.1; Walker et al. 2014b; Harris et al. 2015).

The cross of G00-3213 × PI 567102B (*Rpp6*) was created to fine map the *Rpp6* locus that was described by Li et al. (2012). G00-3213 is an elite MG VII soybean breeding line developed at UGA, and was derived from a cross of 'N7001' (Carter et al., 2003) × 'Boggs' (Boerma et al., 2000). G00-3213 has white flowers, tawny pubescence, tan pod walls, black hila, and is susceptible to the GA12 isolate of *P. pachyrhizi* (Fig. 2.1). The G00-3213 × PI 567102B cross was made in the 2011-2012 winter greenhouse at UGA located in Athens, GA. The F_1 seeds from the cross were grown in the summer in the UGA greenhouse in 2012. The F_2 seeds were planted in the summer of 2013 at Athens, GA and were advanced using a single-seed descent method. The F_3 and F_4 generations were advanced at the at USDA-ARS station in Isabella, Puerto Rico in winter of 2013-2014 by single-seed descent. The F_5 seed were grown in the summer of 2014 at the UGA Plant Science Farm and at harvest, 184 single plants were pulled and threshed to establish the $F_{5:6}$ recombinant inbred line (RIL) population.

Greenhouse phenotyping assay and phenotypic classification

The Prichard × PI 567068A F_2 population previously described by Harris et al. (2015) was advanced to an $F_{2:3}$ population. The experimental design, including planting, *P. pachyrhizi* inoculation, growing conditions, and disease rating was the same as that described by Harris et al. (2015). Twelve plants were rated for SBR reaction per family. The Prichard × PI 567068A population was rated for SRB reaction using the GA12 bulk isolate. The GA12 isolate has been

used in previous studies and was collected from *P. pachyrhizi*-infected field-grown kudzu and soybean in 2012 throughout the state of Georgia, therefore it is referred to as a bulk isolate (Harris et al. 2015; Walker et al. 2014b). The Prichard x PI 567068A $F_{2:3}$ population was phenotyped for SRB reaction in May 2014.

For the G00-3213 × PI 567102B population, 184 $F_{5:6}$ RILs were rated for SBR reaction in the same manner as the above population, whereby each RIL was planted into half of a plastic tray (2 seeds per pot and 12 plants per RIL) and the parents were placed in the experiment four times each throughout the experiment in the same manner. Each plastic tray contained 15 spots for pots. Plastic pots were 10-cm × 10-cm Kord Presto sheet pots (Griffin Greenhouse Supplies, Inc., Tewksbury, MA). Plants were grown in Fafard® 3B blend potting soil (Sun Gro Horticulture, Agawam, MA). The outside 12 positions of the tray were used for planting and the three spots in each tray were left open to allow for light penetration and to reduce crowding of the seedling. The G00-3213 x PI 567102B population was rated for SBR reaction in January 2015.

All phenotyping work was done at the UGA greenhouse located at the Griffin Campus in Griffin, GA. The GA12 bulk isolate used to inoculate and rate the populations was maintained and propagated on susceptible 'Cobb' plants (Hartwig and Jamison, 1975; Harris et al. 2015). Plants were inoculated approximately 14 days after planting and were rated approximately 14 days after inoculation, when disease symptoms were readily visible. Harris et al. (2015) has described this process in detail. A representative lesion reaction for each mapping population parent is shown in Fig. 2.1.

Due to variable seed germination, 10 to 12 plants from each of $F_{2:3}$ family from Prichard × PI 567068A population and each of RILs derived from G00-3213 × PI 567102B RILs were

rated. The following guidelines were developed to classify each $F_{2:3}$ family or RIL as resistant or susceptible, which is similar to the method that was previously used to by Li et al. (2012) to map the *Rpp6* locus. TAN lesions are a susceptible reaction classified by the presence of uredinia and profuse sporulation; RB (reddish-brown) lesions are classified as a resistance reaction and typically non-sporulating. A single family or RIL was considered homozygous susceptible if over 66% of the plants were rated as TAN (susceptible). If 100% of the plants were RB or IM, the family or RIL was classified as homozygous resistant. All other families or RILs were considered heterozygous or heterogeneous.

Evaluation of plant introductions with different *P. pachyrhizi* isolates

It was previously shown that PI 567102B (*Rpp6*) and PI 567068A produced different isolate × genotype patterns of resistance when challenged with a diverse panel of *P. pachyrhizi* isolates that were collected from South Africa in 2001 (SA01-1); Taiwan in 1972 (TW72-1); Zimbabwe in 2001 (ZM01-1); and Australia in 1979 (AU79-1) (Harris et al. 2015). We wished to test these several of these isolates again to confirm the result.

Isolate reaction experiments were conducted with *P. pachyrhizi* isolates SA01-1, ZM01-1, and AU79-1 at the USDA-ARS Foreign Disease-Weed Science Research Unit located at Ft. Detrick, MD. The experimental design was the same as that reported by Harris et al. (2015). Briefly, four replications were tested per isolate. A replication consisted of three plants of a given genotype in a single pot tested with a specific isolate. All pots inoculated with the same isolate were randomly arranged in trays. After planting, seedlings were allowed to grow for three weeks and were then transferred to a Biological Safety Level-3 Plant pathogen containment facility for inoculation. Approximately 14 days post-inoculation seedlings were rated for their

response to the given *P. pachyrhizi* isolate. Each replicate consisted of five lines: PI 518671 ('Williams 82'), G00-3213, PI 612157 (Prichard), PI 567102B (*Rpp6*), and PI 567068A (Table 2.1). Williams 82 was used as a susceptible control, as it is known to be universally susceptible (TAN lesions) to SBR (Harris et al. 2015; Hyten et al. 2009; Kendrick et al. 2011). The lesion reaction types of the seedlings were scored qualitatively as TAN, RB, or INT in April of 2015.

Fingerprinting and super bulked segregant analysis

For each family or RIL, a minimum of 10 of the 12 plants were sampled, and a newly expanded trifoliolate leaf was collected from every plant. The leaf samples were combined to form a bulk for that respective family or RIL. The tissue sample from each bulk was lyophilized for 36 hr and ground into a fine powder using a GenoGrinder (SPEX US). DNA extractions were performed as per Keim et al. (1988) using the CTAB (Hexadecyltrimethylammonium bromide) method. DNA samples were diluted in water to obtain a final concentration ranging from 10 to 50 ng μL^{-1}.

A modified BSA (Michelmore et al. 1991) method was used to identify the specific region on Chr 18 that harbors the *Rpp* resistance locus contributed by PI 567068A. This technique is referred to as "super bulked segregant analysis" (SBSA), as it includes an informative resistant or susceptible bulk of individuals not previously used in $F_{2:3}$ BSA mapping (Hyten et al. 2009). Briefly, of the 140 families phenotyped, 28 families were 100% homozygous susceptible; and 36 families were 100% homozygous resistant, showing no segregation in any of the families. From the Prichard x PI 567068A population, an equal tissue contribution of leaf powder was taken from each of the 28 susceptible families to create the susceptible super bulk. The resistant bulk was created in the same manner using the 36 resistant

families. The powdered leaf tissue in each bulk was homogenized and used for DNA extraction as described above. DNA was then diluted to a concentration of 75 ng μl^{-1}. The resistant and susceptible DNA bulks from the Prichard × PI 567068A population (one of each) were fingerprinted with the SoySNP50K iSelect SNP BeadChips (Song et al. 2013) at the Soybean Genetics Lab at Michigan State University. Genotypes were called using the program GenomeStudio V2011.1 (Illumina, San Diego, USA). PI 567068A and Prichard were not included in the fingerprinting because SoySNP50K data for both lines are available on Soybase (Song et al. 2013). A putative resistance region from SBSA was determined when the genotypic alleles of PI 567068A matched the alleles of a resistant super bulk (e.g., both TT) and were different from the susceptible parent Prichard and the susceptible super bulk (e.g., both CC), which were also homozygous.

SNP assay design and genotyping

The parents of the mapping populations, Prichard and PI 567068A, and G00-3213 and PI 567102B, were compared to identify the polymorphic SNPs surrounding the BSA-identified genomic regions using the SoySNP50K data (Song et al. 2013) or in our laboratory database. Fifteen KASP (LGC Genomics, Middlesex, UK) assays were then developed from these SNP markers which were used for linkage and QTL mapping for both populations (Table 2.3). To further saturate the genomic region, additional SNPs from the region that are not included in the SoySNP50K Infinium Chips were screened. KASP assays were designed using the criteria established by the KASP User Guide and Manual available online (www.lgcgroup.com/LGCGroup/media/PDFs/Products/Genotyping/KASP-genotyping-chemistry-User-guide.pdf?ext=.pdf). Genotyping of the mapping population(s) using KASP

assays was conducted using the protocol reported by Pham et al. (2013) for the master mix preparation and thermocycling conditions. The endpoint reading was determined using either a Tecan M1000 Pro Infinite Reader (Tecan Group Ltd., Männedorf, Switzerland) or a Roche LightCycler 480 II with LightCycler® Software (Roche Diagnostics Corporation Indianapolis, IN). When the Tecan Reader was used, allele calls were determined with KlusterCaller software. The allele calls that were ambiguous (did not distinctly cluster) were designated as missing for both populations. Some markers behaved as dominant with the KASP system, even though it was expected they would be co-dominant (Table 2.3). For the Prichard × PI 567068A $F_{2:3}$ population, both homozygous and heterozygous genotypes were used to construct the linkage map and perform QTL analysis. However, heterozygous calls for the G00-3213 × PI 567102B RIL population were excluded (Yan et al. 2009).

Linkage and QTL mapping

The comparative linkage maps of the resistance loci of PI 567102B and PI 567068A were created using Kosambi's regression model function with JoinMap 4.1 software (Van Ooijen, 2006). Linkage was established using a LOD score of 3.0 (Figs. 2.2, 2.3, and 2.4). JoinMap was used to calculate Chi-square values for both populations (Table 2.2). Composite interval mapping for the G00-3213 × PI 567102B RIL and Prichard × PI 567068A $F_{2:3}$ populations was accomplished using Windows QTL Cartographer 2.5 (Basten et al. 2002), using the "All Marker Control Model" parameters with a 1-cM or 2-cM walking window, 2,000 permutations, and a 0.001 level of significance (Fig. 2.4).

A diagram was created using Flapjack software (Milne et al. 2010), showed that the physical interval where *Rpp6* and the resistance from PI 567068A, designated as

Rpp[PI567068A], mapped to on Chr 18 (Fig. 2.3). The estimated positions of *Rpp6* and *Rpp[PI567068A]* were determined using linkage mapping (Fig. 2.2). All the physical locations of SNPs correspond to the Wm82.a1 genome sequence.

Haplotype analysis and comparisons at the *Rpp6* locus

After defining the interval containing *Rpp6* and *Rpp[PI567068A]* the haplotypes of PI 567102B and PI 567068A were compared. Haplotype analysis was performed using a panel of genotypes that included 32 soybean ancestors representing 95% of the allelic diversity of North American cultivars from 1947 to 1988; a panel of known PIs harboring resistance alleles at the *Rpp1, Rpp2, Rpp3, Rpp4, Rpp5* and *Rpp6* loci (Gizlice et al. 1994; Monteros et al. 2010; Yu et al. 2014), several elite U.S. cultivars (Williams 82, Prichard, 5601T, and Boggs), and several PIs that possessed SRB resistance genes located at the *Rpp6* locus by Harris et al. (2015). Additionally, Flapjack software (Milne et al. 2010) was used to compare all genotypes listed in Table 2.4 by accessing the SoySNP50K data available for all these genotypes (Song et al, 2013) with the exception of G00-3213, which is in our internal laboratory database. The SoySNP50K data were used in their entirety, except for unanchored scaffold sequences that were removed from the analysis. FlapJack software was used to create a comparative matrix and dendrogram for all lines listed in Table 2.4, which uses a function to create a hierarchical cluster analysis of dissimilarities across all the SoySNP50K SNPs being analyzed. Missing data did not count as a dissimilarity, and heterozygous locus data are treated as a 50% match to homozygous allele calls (Supplementary Fig. 2.1).

Results

Phenotypes of the populations and parental controls

The inoculations of the G00-3213 × PI 567102B RIL and Prichard × PI 567068A $F_{2:3}$ populations and parents with the GA12 bulk isolate were as expected. In each case the susceptible parent controls G00-3213 or Prichard had TAN, susceptible lesion reactions that produced uredinia and were profusely sporulating. The resistant parents PI 567102B (*Rpp6*) and PI 567068A each produced faint RB lesions that were never observed to produce uredinia (Fig. 2.1). No segregation was observed in any of the parental controls.

The parameters for classifying families and RILs here were considered to be realistic based on analysis of data compared to expectations of segregation for each population being a 1:2:1 ratio (resistant:segregating:susceptible) or 1:1 ratio (resistant:susceptible) for the Prichard × PI 567068A and G00-3213 × PI 567102B populations, respectively (Table 2.2). The *Rpp* gene from PI 567068A behaved as a single dominant gene in this study and as did the resistance gene from PI 567102B in previous studies (Harris et al. 2015; Li et al. 2012). The segregation ratios of the Prichard × PI 567068A $F_{2:3}$ and G00-3213 × PI 567102B RIL populations were as expected demonstrating that the resistance conferred by PI 567068A and PI 567102B both behaved as a single gene.

Super bulked segregant analysis, linkage, and QTL mapping

For the Prichard × PI 567068A population, 35 positive SBSA hits fell on Chr 18 between ss715630656 (4,614,748 bp) and ss715629019 (14,689,691 bp); of these 35 positive hits, 34 were from ss715630656 (4,614,748 bp) to ss715632778 (8,403,159 bp) (data not shown; Wm82.a1 genome sequence; www.soybase.org/dlpages/index.php#snp50k). Of the positive

SBSA hits, KASP assays ss715632549 (GSM0357), ss715632566 (GSM0358), ss715632179 (GSM0374), and ss715631635 (GSM0442) were developed. GSM0374 is a flanking marker for both *Rpp6* of PI 567102B and *Rpp[PI567068A]* of PI 567068A (Table 2.3; Fig. 2.3). Therefore, SBSA using the Prichard × PI 567068A $F_{2:3}$ families was able to detect a SNP (GSM0374) that mapped approximately 100 kb away from *Rpp[PI567068A]*. Additional KASP marker assays in the SBSA region were created, including some assays slightly outside of the interval, to ensure saturation of the region containing *Rpp[PI567068A]*.

The *Rpp6* gene contributed by PI 567102B and *Rpp[PI567068A]* contributed by PI 567068A were mapped using a RIL and a $F_{2:3}$ family population, respectively. Once *Rpp6* from the G00-3213 × PI 567102B population was found to be flanked by SNP markers GSM0373 and GSM0374, this region was saturated by markers GSM0435, GSM0438, and GSM0442 to further narrow the *Rpp6* locus of PI 567102B (Table 2.3; Fig. 2.2).

All SNP marker coordinates and physical positions, as well as estimations of *Rpp* gene locations, were defined using the Wm82.a1 sequence (soybase.org). The information on the location of the SNPs used for mapping is reported in Table 2.3. For both populations, none of the markers showed significant segregation distortion from what was expected (data not shown, $p > 0.05$). The *Rpp6* resistance genes contributed by PI 567102B and *Rpp[PI567068A]* from PI 567068A were both mapped to Chr 18. Composite interval mapping was performed on both populations using the "All Marker Control" parameters that controls for genetic background.

Linkage mapping narrowed the *Rpp6* interval of PI 567102B to a 42,224 bp region that is 1.8 cM long, flanked by KASP markers GSM0374 and GSM0427 (Fig. 2.2 and 2.3; Table 2.3). *Rpp6* contributed by PI 567102B mapped from 5,916,005 to 5,961,229 bp (Fig. 2.2; Fig. 2.3; Table 2.3). *Rpp6* had a peak LOD score of 58.3 over SNP GSM0438 (5,893,484 bp; Fig. 2.4).

Information on the genomic configuration of individual RILs from the G00-3213 × PI 567102B RIL population that were homozygous susceptible, homozygous resistant, and had a recombination in the marker interval shown below, or a recombination on either side of the *Rpp6* locus are located in Supplementary Table 2.1. Of the 184 RILs, two had recombinations flanking each side of the *Rpp6* locus (4726 and 4774) (Supplementary Table 2.1).

Rpp[PI567068A] was fine mapped from 5,961,229 to 6,122,387 bp, spanning a 161,158 bp interval (3.9-cM) flanked by KASP markers GSM0427 and GSM0374 (Fig. 2.2 and 2.3; Table 2.3). *Rpp[PI567068A]* had a LOD score of 4.4 over SNP GSM0374 (5,961,229 bp). *Rpp6* and *Rpp[PI567068A]* are both flanked by the SNP marker GSM0374 (Fig. 2.3; Table 2.3).

A diagram showing the physical interval where *Rpp6* and *Rpp[PI567068A]* are located on Chr 18 was created to show the tight linkage between *Rpp6* and *Rpp[PI567068A]* (Fig. 2.3). Based on the SoySNP50K Infinium Chip (Song et al. 2013), PI 567102B and PI 567068A have identical haplotypes from 5,924,556 (ss715632113) to 6,384,548 bp (ss715632525) of the Wm82.a1 sequence. This haplotype is defined by 16 SoySNP50K markers: ss715632113, ss715632123, ss715632129, ss715632179, ss715632196, ss715632280, ss715632362, ss715632369, ss715632399, ss715632451, ss715632467, ss715632499, ss715632517, ss715632521, ss715632523, and ss715632525. Since the haplotype is identical between PI 567102B and PI 567068A, it is referred to as the *Rpp6/Rpp[PI567068A]* haplotype.

Haplotype analysis at the *Rpp6* locus and rust phenotypes of PIs and cultivars to inoculation with *P. pachyrhizi* isolates

The *P. pachyrhizi* isolates SA01, ZM01-1, AU79-1, and GA12 were used to challenge PI 518671 (Williams 82), G00-3213, PI 612157 (Prichard), PI 567102B (*Rpp6*), and PI 567068A

(Table 2.1). Williams 82, which was used as the susceptible control, was susceptible to all isolates tested, and PI 567102B produced RB lesions when challenged with these isolates. PI 567068A produced TAN reactions to SA01 and ZM01-1; a mixture of INT and RB reactions to AU79-1; and a RB reaction to GA12. The mapping population parents G00-3213 and Prichard were susceptible to the GA12 isolate (Fig. 2.1; Table 2.1).

Data were compiled from Harris et al. (2015) using PIs with genes that mapped to the *Rpp6* locus and from the current research on PI 567102B (*Rpp6*) and PI 567068A (Table 2.4). Phenotypically, when tested with a unique panel of *P. pachyrhizi* isolates, PI 476905A showed a unique isolate panel reaction pattern; PI 567076 and PI 567090 were similar to PI 567068A; PI 567129 was not tested with an isolate panel; and PI 567104B reacted as if it had the *Rpp4* and *Rpp6* loci of PI 459025B (*Rpp4*) and PI 567102B *Rpp6* (Table 2.4; Harris et al. 2015).

PI 567102B and PI 567068A have an identical haplotype allele in the interval of ss715630691 (4,713,455 bp) to ss715632534 (6,566,001 bp); a region spanning over 1.85 Mbp. Within that interval, PI 476905A, PI 567068A, PI 567076, PI 567090, PI 567129, PI 567102B, and PI 567104B shared an identical haplotype from SNP markers ss715632113 to ss715632525 (5,924,556 to 6,384,548 bp), spanning 459,992 bp. Interestingly, the 16 SNPs that define the *Rpp6/Rpp[PI567068A]* haplotype window are identical amongst all the PIs that had BSA data or that mapped to the *Rpp6,* locus including PI 476905A, PI 567068A (*Rpp[PI567068A]*), PI 567076, PI 567104B, PI 567129, and PI 567102B (*Rpp6*) (Table 2.4). Other than PI 476905A, which was collected from an unknown province in China in 1983; PI 566956, PI 566984, PI 567068A, PI 567076, PI 567090, PI 567104B, PI 567123A PI 567129, and PI 567102B all were collected from East Java, Indonesia in 1993, which further suggests these genotypes may be closely related (Table 2.3).

Within the haplotype window defined by 16 SNPs that is shared by PI 567102B and PI 567068A, three unique SNPs were identified that create a CAG haplotype (ss715632362, ss715632523, and ss715632525) (Tables 2.3-2.4). PI 476905A, PI 567068A (*Rpp[PI567068A]*), PI 567076, and PI 567102B (*Rpp6*), PI 567104B, and PI 567129 all possess the *Rpp6/Rpp[PI567068A]* haplotype and have data that support they possess an *Rpp* gene near the *Rpp6Rpp[PI567068A]* locus (Table 2.4). None of the 32 North American soybean accessions, SBR susceptible soybean cultivars (Prichard, Boggs, 5601T, and Williams 82), or any other known sources of *Rpp* genes at loci *Rpp1* to *Rpp5* possess this CAG haplotype (Table 2.4), indicating that the CAG haplotype is unique.

The haplotype window identified here to detect an *Rpp* gene at the *Rpp6/Rpp[PI567068A]* locus had three SNPs. The three SNPs would theoretically allow for eight possible haplotypes. Excluding PI 506764 (Hyuuga), which had a heterozygous haplotype at ss715632523, the panel of PIs examined in Table 2.4 had six of the eight possible haplotypes.

The SoySNP50K data with the exception for the SNPs from unanchored scaffold sequences were used to create a comparative matrix and dendrogram for all lines listed in Table 2.4 using FlapJack software (Milne et al. 2010). The dendrogram showed that all genotypes that possessed the *Rpp6/Rpp[PI567068A]* haplotype (PI 567068A, PI 567076, PI 567090, PI 567102B, PI 567104B, and PI 567129) that were collected from East Java, Indonesia in 1993 clustered tightly together, however, PI 476905A (collected from China) did not cluster with the other *Rpp6/Rpp[PI567068A]* haplotype lines (Supplementary Fig. 2.1; Table 2.4).

PI 567102B and PI 567068A clustered together and were 77.4% similar (Supplementary Fig. 2.1; data not shown). PI 476905A, which was collected from an unknown location in China in 1983 and which also that possessed the *Rpp6/Rpp[PI567068A]* haplotype, distinctly clustered

with PI 240664 (collected from the Philippines), PI 548461 (China), PI 548485 (Jiangsu, China), and PI 594538A (Fujian, China). It is not surprising that PI 476905A clustered with other genotypes from China and the Philippines (Table 2.4; Supplementary Fig. 2.1).

Discussion

The resistance gene *Rpp6* contributed by PI 567102B and *Rpp[PI567068A]* from PI 567068A were both mapped using a relatively high density of SNP markers, and each *Rpp* gene is flanked by the GSM0374 SNP identified in this study (Fig. 2.3; Table 2.3). Through linkage mapping, *Rpp6* was mapped from 5,916,005 to 5,961,229 bp; and *Rpp[PI567068A]* was mapped from 5,961,229 to 6,122,387 bp (Fig. 2.3; Table 2.3). Even though the *Rpp6* interval of PI 567102B is less than 50 kb, recombinations on either side of the *Rpp6* locus were observed in two of the 184 RILs, indicating that recombinations are possible in close proximity to the *Rpp6* locus and that none of the SNPs identified in the mapping of *Rpp6* are causative (Supplementary Table 2.1). QTL peaks for *Rpp6* (LOD score of 58.3), and *Rpp[PI567068A]* (LOD score of 4.4) were 67,745 bp apart (Fig. 2.2; Table 2.3). This suggests that *Rpp6* and *Rpp[PI567068A]* are either tightly linked or possibly allelic. An allelism test or further fine mapping may lead to the confirmation of this.

Harris et al. (2015) challenged numerous PIs with a panel of diverse *P. pachyrhizi* isolates. PI 567102B and PI 567068A had differential reactions when challenged with isolates SA01-1, TW72-1, ZM01-1, and AU79-1. Specifically, PI 567068A had a TAN lesion type when challenged by SA01-1, TW72-1, ZM01-1, and AU79-1; and PI 567102B had a RB lesion type to all these isolates with the exception of TW72-1, to which PI 567102B reacted with a mixture plants with RB or TAN lesions.

The *P. pachyrhizi* isolates SA01, ZM01-1, and AU79-1 that gave clean differential reactions for PI 567102B (*Rpp6*) and PI 567068A from Harris et al. (2015); were used in the present study with similar results. Additionally, the susceptible control PI 518671 (Williams 82) was included and was susceptible (TAN) to SA01, ZM01-1, AU79-1, as well as to the GA12 bulk isolate used to map the traits in this study (Table 2.1). The mapping population parents G00-3213 and Prichard were also susceptible (TAN) to the GA12 bulk isolate, as expected (Fig. 2.1; Table 2.1). PI 567102B and PI 567068A were both resistant to the GA12 bulk isolate and produced faint, relatively small RB lesions measuring approximately 1 mm in diameter. The RB lesions of PI 567102B and PI 567068A were never observed to produce uredinia when challenged with GA12 after 14 days (Fig. 2.1; Table 2.1). When PI 567102B and PI 567068A were challenged with SA01-1, and ZM01-1, and AU79-1 again in this study, PI 567102B reacted with RB lesions; PI 567068A reacted with TAN reactions to SA01-1, and ZM01-1; and a mixture of INT and RB reactions to AU79-1. The only discrepancy between the Harris et al. (2015) study and our results is when PI 567068A was challenged with the ZM01-1. Harris et al. (2015) observed a TAN reaction and we observed a mixture of INT and RB plants. This could potentially be due to small variations in the growth conditions between experiments that may have resulted in more or less uredinia production. Additionally, the reaction of PI 567068A to the ZM01-1 isolate was difficult to score. The differential isolate reactions presented here and in Harris et al. (2015) support that PI 567068A *Rpp[PI567068A]* has a different source of *Rpp* resistance from PI 567102B (*Rpp6*).

PI 476905A has the *Rpp6/Rpp[PI567068A]* haplotype, yet has a unique *P. pachyrhizi* isolate pattern from PI 567102B (*Rpp6*) and PI 567068A (*Rpp[PI567068A]*) (Table 2.4; Harris et al. 2015). This indicates that PI 476905A may harbor a novel resistance allele at the

Rpp6/Rpp[PI567068A] locus, or a tightly linked, novel *Rpp* gene. PI 476905A also stands out as the only PI with an *Rpp* gene that mapped to the *Rpp6* locus, but was not collected from East Java, Indonesia (Table 2.4). When a panel of diverse genotypes were compared using the SoySNP50K data, all genotypes from East Java, Indonesia clustered together distinctly from all other genotypes, and PI 476905A clustered with PI 240664, PI 548461, PI 548485, and PI 594538A, all of which were collected from China, other than PI 240664 which was collected from the Philippines (Table 2.4; Supplementary Fig. 2.1).

Several PIs have a natural *Rpp* gene pyramid based on haplotype data. It is estimated as many as 15% of rust resistant PIs harbor more than one *Rpp* gene (Harris et al. 2015; Kendrick et al. 2011). Interestingly, PI 567104B has the *Rpp4* haplotype of PI 459025B and the *Rpp6/Rpp[PI567068A]* haplotype. The resistance of PI 567104B also maps to the *Rpp4* and *Rpp6* loci, and reacted like the PI 567102B (*Rpp6*) and PI 459025B (*Rpp4*) genotypes to the panel of *P. pachyrhizi* isolates used in this study, providing evidence that this PI may contain an *Rpp* gene at both the *Rpp4* and *Rpp6* locus (Table 2.4; Harris et al. 2015).

In field screens in 2008 in Quincy, Florida, PI 567104B had lower field rust severity scores than either PI 567102B (*Rpp6*) and PI 459025B (*Rpp4*), and PI 567068A was not tested (Walker et al. 2014a). Additionally, PI 567104B had a lower lesion density than PI 567102B (*Rpp6*), PI 567068A (*Rpp[PI567068A]*), and PI 459025B (*Rpp4*) when challenged with the GA 2008 (GA08) bulk *P. pachyrhizi* isolate in a greenhouse assay in 2011 (Walker et al. 2014b). These results may indicate the higher resistance of PI 567104B is caused by an additive resistance effect of the *Rpp4* and *Rpp6* loci.

The current study has mapped the *Rpp6* and *Rpp[PI567068A]* SBR resistance genes. Further research is needed to resolve whether or not *Rpp[PI567068A]* is allelic to *Rpp6* or a

tightly linked resistance gene. These findings can be used to incorporate the *Rpp6* or the *Rpp[PI567068A]* resistance allele into elite germplasm. The *Rpp6/Rpp[PI567068A]* haplotype provides soybean researchers with additional genomic resources to identify new, unique sources of SBR resistance.

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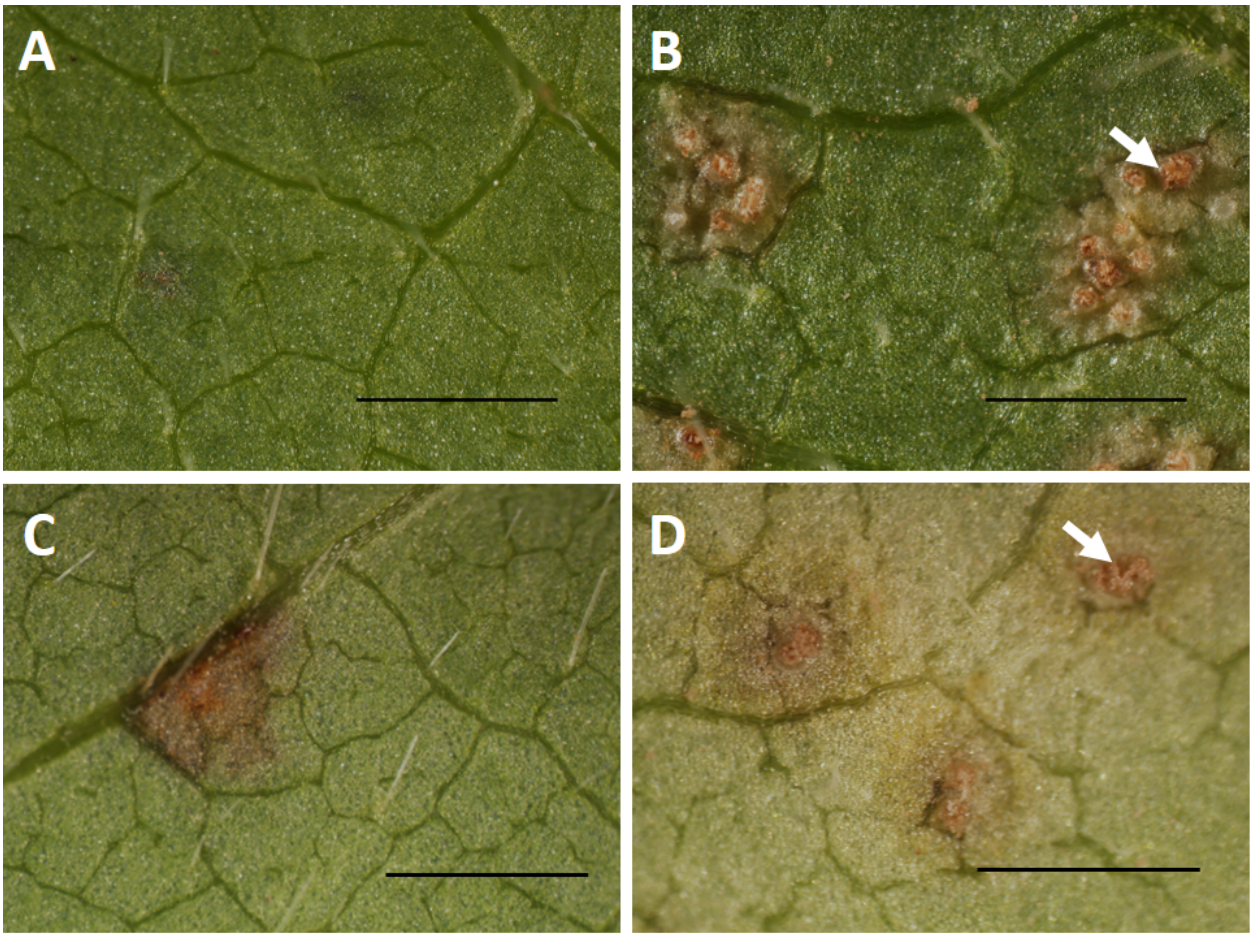


Figure 2.1: The reactions of mapping population parents to the Georgia 2012 (GA12) *P. pachyrhizi* bulk isolate: (A) PI 567102B (*Rpp6*), (B) G00-3213, (C) PI 567068A, and (D) Prichard. G00-3213 and Prichard had TAN, highly sporulating lesions (B and D). PI 567102B (*Rpp6*) and PI 567068A had faint reddish-brown resistant lesions that did not sporulate (A and C). The presence of urediniospores are indicated by the white arrows. Bar = 1 mm.

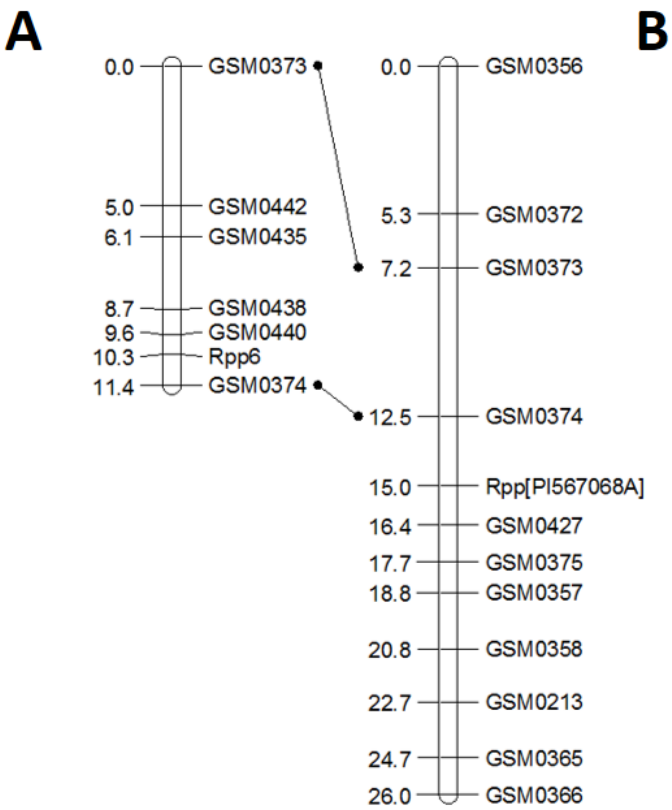


Figure 2.2: Linkage map constructed with SNP markers: (A) Recombinant inbred line of G00-3213 × PI 567102B (*Rpp6*) and (B) $F_{2:3}$ population of Prichard × PI 567068A. The left side of the linkage map displays distance in centiMorgans and the right side shows the KASP SNP assay ID (Table 2.4). Note *Rpp6* and *Rpp[PI567068A]* map to different intervals. Solid lines highlight shared SNP markers used to assay both mapping populations.

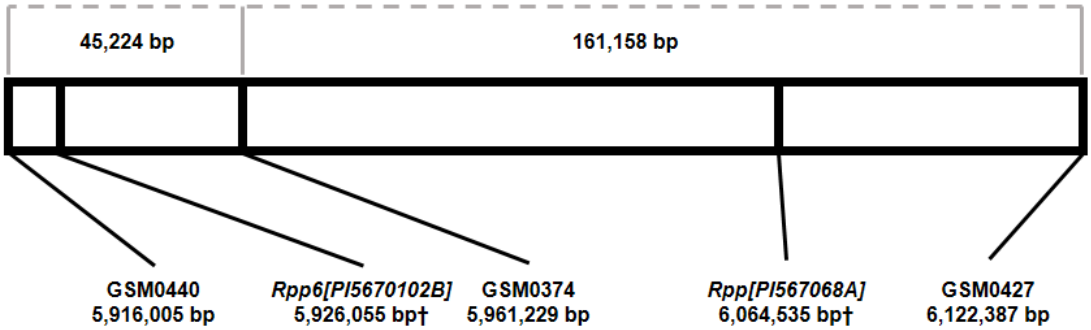


Figure 2.3: Physical interval where *Rpp6* and *Rpp[PI567068A]* mapped to on chromosome 18. The physical locations of GSM markers correspond to SNP positions in the Wm82.a1 genome sequence. The positions of *Rpp6* and *Rpp[PI567068A]* were assigned based on linkage maps generated in this study, and therefore represent estimated positions (†). The solid gray lines represent the physical intervals that harbor *Rpp6* (42,224 bp) and *Rpp[PI567068A]* (161,158 bp) determined by SNP markers. The interval that harbors both *Rpp6* and *Rpp[PI567068A]*, shown with the dotted gray line is 206,382 bp.

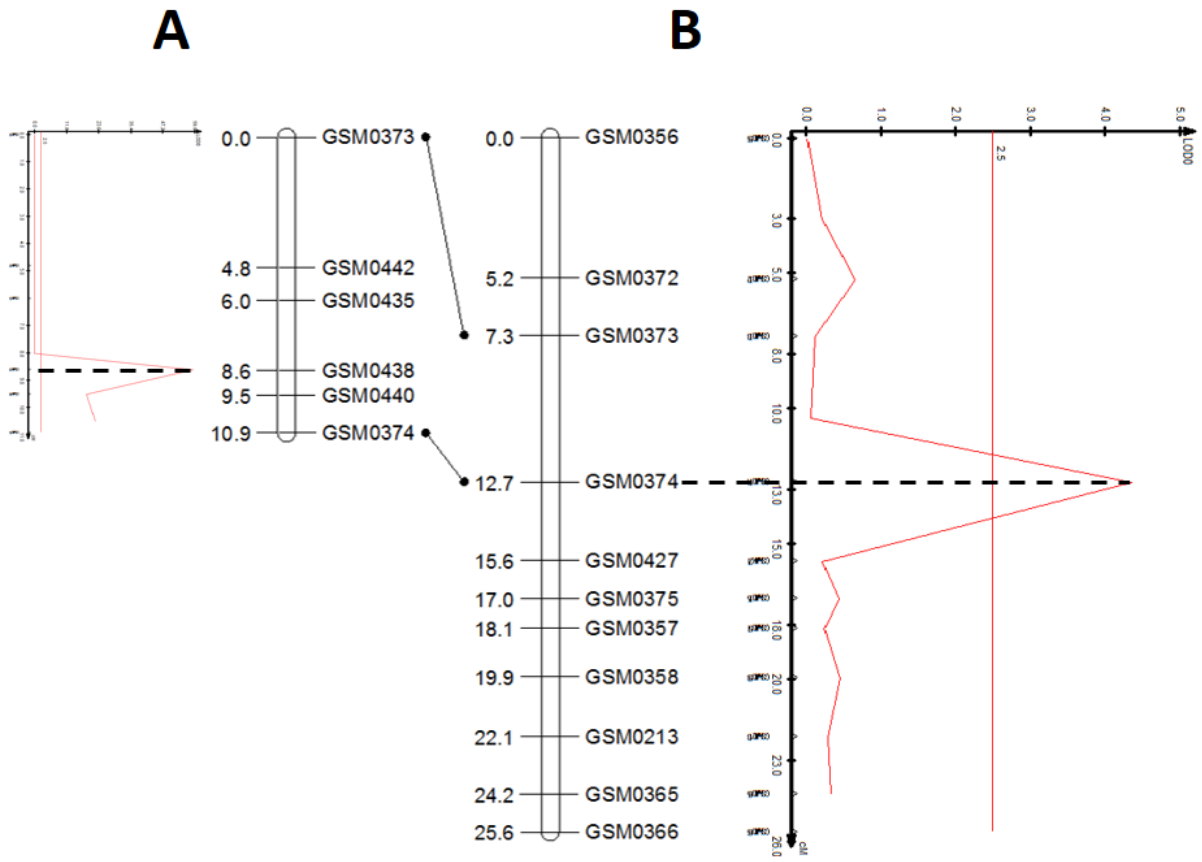


Figure 2.4: The linkage map and QTL likelihood plot for (A) RILs of G00-3213 × PI 567102B (*Rpp6*) and (B) $F_{2:3}$ population of Prichard × PI 567068A. Displayed on the left side of the linkage map is genetic distance in centiMorgans. The right side displays the KASP SNP assay ID (Table 2.4). Linkage maps were created with JoinMap 4.1 and QTL plots were generated with Windows QTL Cartographer. The black dotted line highlights where the QTL peak was determined to be on the physical map in relation to the SNP markers tested. The KASP assay IDs are on the x-axis of the QTL map.

Table 2.1: Reactions of mapping population parents to *Phakopsora pachyrhizi* isolates and sources of resistance

	Phakopsora pachyrhizi isolate and lesion reactions‡‡			
Germplasm	SA01-1	ZM01-1	AU79-1	GA12‡
PI 518671 (Williams 82)	TAN	TAN	TAN	TAN
G00-3213	-	-	-	TAN
PI 612157 (Prichard)	-	-	-	TAN
PI 567102B (*Rpp6*)	RB	RB	RB§§†	RB
PI 567068A	TAN¶	TAN	RB/INT††	RB§

†One TAN plant in replication two.
¶One replication showed an intermediate phenotype.
††Two replications showed intermediate phenotype, two replications showed a reddish-brown phenotype.
§A few lesions showed sporulation.
§§Harris et al. (2015) demonstrated an all TAN reaction of PI 567102B to AU79-1.
‡The Georgia 2012 (GA12) bulk isolate was collected from field-grown kudzu and soybean in 2012.
‡‡TAN, susceptible reaction classified by the presence of uredinia with profuse sporulation, often tan colored lesions; RB, reddish-brown resistance reaction, typically non-sporulating lesion; INT, intermediate reaction had a dark colored lesion similar to the RB type, however lesions are relatively smaller and produced urediniospores.
-: Not tested.

Table 2.2: Population segregation ratios and chi-square analysis of G00-3213 × PI 567102B RILs or Prichard × PI 567068A $F_{2:3}$ families.

Cross	Number of RILs or families[§]					
	R	H	S	Total	Expected segregation	χ^2
G00-3213 × PI 567102B	91	NA†	74	184	1:1	1.75 NS
Prichard × PI 567068A	46	60	34	140	1:2:1	4.91 NS

Each population was phenotyped for rust resistance using the Georgia 2012 bulk *P. pachyrhizi* isolate. The *Rpp* gene contributed by PI 567068A and *Rpp6* gene contributed by PI 567102B was tested under the hypothesis that it was a single dominant gene.
NS: non-significant result when compared to the expected Chi-square value ($p > 0.05$).
§Phenotypic reactions of each family or RIL were determined to be homozygous resistant (R), heterozygous (H), or homozygous susceptible (S).
†Heterozygous genotypes and individuals with missing phenotypic data were considered not applicable (NA) from the chi-square analysis and were excluded.

Table 2.3: KASP assay ID and primer sequences that were used for mapping resistance from PI 567102B (*Rpp6*) and PI 567068A.

Assay ID	dbSNP ID††	SNP ID¶	Resistance source	PI SNP allele†	Forward primer 1 5'-3' (FAM)	Forward primer 2 5'-3' (HEX)	Reverse primer 5'-3'
GSM0213	ss715632657	Gm18_7515726_T_C	PI 567068A	C	GAAGGTGACCAAGTTCATGCTCTGCTCGCTGATGCTGc	GAAGGTCGGAGTCAACGGATTCCTGCTCGCTGATGCTGt	CGCCCCGTATTCTTCAAAAC
GSM0356‡	ss715630605	Gm18_4451304_C_T	PI 567068A	T	GAAGGTGACCAAGTTCATGCTTCTCGAAGGCCAAGTTGTGc	GAAGGTCGGAGTCAACGGATTGATCTCGAAGGCCAAGTTGTGt	AGCATTGGTCGTCGTTGTTC
GSM0357	ss715632549	Gm18_6697094_C_T	PI 567068A	T	GAAGGTGACCAAGTTCATGCTCTCGTCCGGTGCACTGTCc	GAAGGTCGGAGTCAACGGATTCTATCTCGTCCGGTGCACTGTCt	TGAAAATAGGCTGAATAGTGACGAG
GSM0358	ss715632566	Gm18_6840290_T_C	PI 567068A	C	GAAGGTGACCAAGTTCATGCTATTGCCACCACCAGTAAGCAc	GAAGGTCGGAGTCAACGGATTATTGCCACCACCAGTAAGCAt	GGCCTCCCATGCAATTGTTA
GSM0365	ss715632812	Gm18_8777288_A_G	PI 567068A	G	GAAGGTGACCAAGTTCATGCTCGATGGGGAACTGGCAGAa	GAAGGTCGGAGTCAACGGATTCGATGGGGAACTGGCAGAg	CTCGCTTGACGGAAAACTTTGA
GSM0366	ss715632832	Gm18_8928479_A_G	PI 567068A	A	GAAGGTGACCAAGTTCATGCTACACCCTGAAGGCCAGACAa	GAAGGTCGGAGTCAACGGATTACACCCTGAAGGCCAGACAg	GGTTGATTTGCCCAGAATAGGA
GSM0372	ss715630789	Gm18_4784486_C_A_RC	PI 567068A	A	GAAGGTGACCAAGTTCATGCTAGGAGAGTGAATTAATTAGAAGTGACAa	GAAGGTCGGAGTCAACGGATTAGGAGAGTGAATTAATTAGAAGTGACAc	TCCCAACCCAAGAGAAATC
GSM0373	ss715630802	Gm18_4792076_G_A_RC	PI 567068A/PI 567102B	A	GAAGGTGACCAAGTTCATGCTTCAATAATAGAATTCTTTACCCAAATAGa	GAAGGTCGGAGTCAACGGATTCAATAATAGAATTCTTTACCCAAATAGg	GTTGAATTTGTGGTCAATCCAG
GSM0374	ss715632179	Gm18_5961229_C_T_RC	PI 567068A/PI 567102B	C	GAAGGTGACCAAGTTCATGCTGGGGCATTGCTACGTACACc	GAAGGTCGGAGTCAACGGATTTGGGGCATTGCTACGTACACt	ACCTGTGCCCGTTTACCCA
GSM0375	ss715632525‡	Gm18_6384548_A_C_RC	PI 567068A	C	GAAGGTGACCAAGTTCATGCTTCCTTCTTCTTTACCCTACTTGCCAAa	GAAGGTCGGAGTCAACGGATTTCCTTCTTCTTTACCCTACTTGCCAAc	GAGGGACCAAGCCATATCAACG
GSM0427	NA	Gm18_6122387_G_A_RC	PI 567068A	T	GAAGGTGACCAAGTTCATGCTTGGCACTATGACATTTGTTAAGGAAc	GAAGGTCGGAGTCAACGGATTGTGGCACTATGACATTTGTTAAGGAAt	GGATAAGTGTCAAAAAGACCATGGAC
GSM0435	NA	Gm18_5627744_C_A_RC	PI 567102B	A	GAAGGTGACCAAGTTCATGCTCCTAGAGGTGTTATGTGGTATCACAa	GAAGGTCGGAGTCAACGGATTACCTAGAGGTGTTATGTGGTATCACAc	AAAACTTTGTGTCACATGATTCCA
GSM0438	NA	Gm18_5893484_G_A	PI 567102B	G	GAAGGTGACCAAGTTCATGCTGATGATTGTCCCAGCAATAATGTTa	GAAGGTCGGAGTCAACGGATTTGATTGTCCCAGCAATAATGTTg	TTGCTGGTGACTAAAGGGGG
GSM0440	NA	Gm18_5916005_T_A	PI 567102B	T	GAAGGTGACCAAGTTCATGCTCTTATTTCCATCAACACTTGCACa	GAAGGTCGGAGTCAACGGATTTCTTATTTCCATCAACACTTGCACt	TCGCTGTTTTGGACTTTTCTCC
GSM0442	ss715631635	Gm18_5517202_G_A_RC	PI 567102B	A	GAAGGTGACCAAGTTCATGCTGGGCACAGAGATTTGGATCAa	GAAGGTCGGAGTCAACGGATTGGGCACAGAGATTTGGATCAg	CCTTCAACACTCCAACCACCAA

††dbSNP ID found at www.soybase.org/dlpages/index.php#snp50k (Song et al. 2013). Those SNPs listed as not applicable (NA) do not have assigned dbSNP IDs.

¶Physical genomic locations correspond to the Wm82.a1 sequence RC indicates the reverse compliment orientation of the sequence was used to design the KASP assays.

‡Indicates marker was dominant as opposed to co-dominant with the PI 567068A population.

Table 2.4: Haplotypes of the PIs having *Rpp* genes indicated to be at the *Rpp6* locus, soybean ancestors and PIs with known *Rpp* genes. All isolate reaction data is from seedling host plant resistance tests in the greenhouse using the GA12 bulk *P. pachyrhizi* isolate. The CAG haplotype at SNP markers ss715632362, ss715632523, and ss715632525, respectively are associated with the *Rpp6* locus using this panel of genotypes.

§PI	Cultivar	Collection location (Year)	MG ‡‡	*Phakopsora pachyrhizi* reaction††	Maps to known locus	Phenotyped with isolate panel if *Rpp6* locus	Phenotyping results	Known gene	ss715632362 6,089,186¶	ss715632523 6,370,950	ss715632525 6,384,548
PI 567102B	NA	Indonesia, East Java (1993)	IX	R	*Rpp6*	Yes¶	*Rpp6* like¶	*Rpp6*	C	A	G
PI 567129	NA	Indonesia, East Java (1993)	IX	R‡	*Rpp6*¶	No	NA	NA	C	A	G
PI 567104B	NA	Indonesia, East Java (1993)	IX	R‡	*Rpp4*/*Rpp6*¶	Yes¶	*Rpp4* and *Rpp6* like†¶	NA	C	A	G
PI 567090	NA	Indonesia, East Java (1993)	IX	R‡	hits on several chr.¶	Yes¶	Unique pattern¶	NA	C	A	G
PI 567068A	NA	Indonesia, East Java (1993)	VII	R	*Rpp6*	Yes¶	*Rpp[PI 567068A]* like¶	NA	C	A	G
PI 476905A	Nguu mao hong	China, unknown (1983)	V	R‡	*Rpp6*¶	Yes¶	Unique pattern¶	NA	C	A	G
PI 567076	NA	Indonesia, East Java (1993)	VII	R‡	*Rpp6*¶	Yes¶	*Rpp[PI567068A]* like¶	NA	C	A	G
FC031745	NA	unknown, unknown (1948)	VI	S	None	NA	NA	NA	C	A	T
FC033243-1	Anderson	unknown, unknown (1954)	IV	S	None	NA	NA	NA	C	A	T
PI 080837	Mejiro	Japan, unknown (1929)	IV	S	None	NA	NA	NA	C	A	T
PI 180501	Strain No. 18	Germany, unknown (1949)	0	S	None	NA	NA	NA	C	A	T
PI 240664	Bilomi No. 3	Philippines, unknown (1957)	X	S	None	NA	NA	NA	C	A	T
PI 438471	Fiskeby III	Sweden, Ostergotland (1980)	00	S	None	NA	NA	NA	C	A	T
PI 438477	Fiskeby 84NA-7-3	Sweden, Ostergotland (1980)	00	S	None	NA	NA	NA	C	A	T
PI 548298	A.K. Harrow	China, NortheastChina (1939)	III	S	None	NA	NA	NA	C	A	T
PI 548302	Bansei	Japan, Hokkaido (1936)	II	S	None	NA	NA	NA	C	A	T
PI 548311	Capital	China, Northeast China (1944)	0	S	None	NA	NA	NA	C	A	T
PI 548318	Dunfield	China, Jilin (1923)	III	S	None	NA	NA	NA	C	A	T
PI 548325	Flambeau	Russia, unknown (1944)	00	S	None	NA	NA	NA	C	A	T
PI 548348	Illini	China, Heilongjiang (1927)	III	S	None	NA	NA	NA	C	A	T
PI 548352	Jogun	North Korea, Hamgyong Puk (1936)	III	S	None	NA	NA	NA	C	A	T
PI 548356	Kanro	North Korea, Pyongyang (1936)	II	S	None	NA	NA	NA	C	A	T
PI 548360	Korean	North Korea, unknown (1928)	II	S	None	NA	NA	NA	C	A	T
PI 548362	Lincoln	China, unknown (1943)	III	S	None	NA	NA	NA	C	A	T
PI 548379	Mandarin Ottawa	China, Heilongjiang (1934)	0	S	None	NA	NA	NA	C	A	T
PI 548382	Manitoba Brown	unknown, unknown (1939)	00	S	None	NA	NA	NA	C	G	T
PI 548391	Mukden	China, Liaoning (1932)	II	S	None	NA	NA	NA	C	G	T
PI 548402	Peking	China, Beijing (1910)	IV	S	None	NA	NA	NA	C	A	T
PI 548406	Richland	China, Jilin (1938)	II	S	None	NA	NA	NA	C	A	T
PI 548438	Arksoy	North Korea, Pyongyang (1937)	VI	S	None	NA	NA	NA	T	A	T
PI 548445	CNS	China, Jiangsu (1943)	VII	S	None	NA	NA	NA	C	A	T
PI 548456	Haberlandt	North Korea, Pyongyang (1907)	VI	S	None	NA	NA	NA	T	A	G
PI 548461	Improved Pelican	China, unknown (1950)	VIII	S	None	NA	NA	NA	C	A	T
PI 548477	Ogden	unknown, unknown (1940)	VI	S	None	NA	NA	NA	C	G	T
PI 548484	Ralsoy	North Korea, Pyongyang (1940)	VI	S	None	NA	NA	NA	T	A	T
PI 548485	Roanoke	China, Jiangsu (1946)	VII	S	None	NA	NA	NA	C	G	T
PI 548488	S-1NANA	China, Heilongjiang (1945)	V	S	None	NA	NA	NA	C	A	T
PI 548603	Perry	United States, Indiana (1952)	IV	S	None	NA	NA	NA	C	A	T
PI 548657	Jackson	United States, North Carolina (1953)	VII	S	None	NA	NA	NA	C	A	T
PI 518671	Williams 82	United States, Illinois (1981)	III	S	None	NA	NA	NA	C	A	T
PI 602597	Boggs	United States, Georgia (1998)	VI	S	None	NA	NA	NA	C	G	T
PI 612157	Prichard	United States, Georgia (2000)	VIII	S	None	NA	NA	NA	C	A	T
PI 630984	5601T	United States, Tennessee (2002)	V	S	None	NA	NA	NA	C	A	T
NA	G00-3213	United States, Georgia (NA)	VII	S	None	NA	NA	NA	C	G	T
PI 200492	Komata	Japan, Shikoku (1952)	VII	R‡	*Rpp1*	NA	NA	*Rpp1*	C	A	T
PI 594538A	Minhoubaishawan dou	China, Fujian (1996)	IX	NA	*Rpp1*	NA	NA	*Rpp1-b*	C	A	T
PI 230970	NA	Japan, unknown (1956)	VII	R‡	*Rpp2*	NA	NA	*Rpp2*	C	A	T

PI 224270	Howgyoku	Japan, Hyogo (1955)	VII	R‡	*Rpp2*	NA	NA	*rpp2?*	C	G	T
PI 462312	Ankur	India, Uttar Pradesh (1981)	VIII	R‡	*Rpp3*	NA	NA	*Rpp3*	C	A	T
PI 506764	Hyuuga	Japan, Kyushu (1986)	VII	R‡	*Rpp3*	NA	NA	*Rpp?[Hyuuga]*	C	H	T
PI 459025B	Bing nan	China, Fujian (1981)	VIII	R‡	*Rpp4*	NA	NA	*Rpp4*	C	G	G
PI 471904	Orba	Indonesia, Java (1982)	IX	R‡	*Rpp5*	NA	NA	*Rpp5* allele	C	G	T
PI 200526	Shira Nuhi	Japan, Shikoku (1952)	VIII	S‡	*Rpp5*	NA	NA	*Rpp5* allele	C	G	T
PI 200487	Kinoshita	Japan, Shikoku (1952)	VIII	R‡	*Rpp5*	NA	NA	*Rpp5* allele	C	G	T
PI 200456	Awashima Zairai	Japan, Shikoku (1952)	VIII	NA	*Rpp5*	NA	NA	*rpp5?*	C	G	T

§PI, plant introduction ID from the USDA Germplasm Resources Information Network.

‡‡MG, maturity group.

†Only difference between PI 567104B and PI 567102B (*Rpp6*) is a RB or mixed reaction when challenged with TW72-1 *P. pachyrhizi* isolate, respectively.

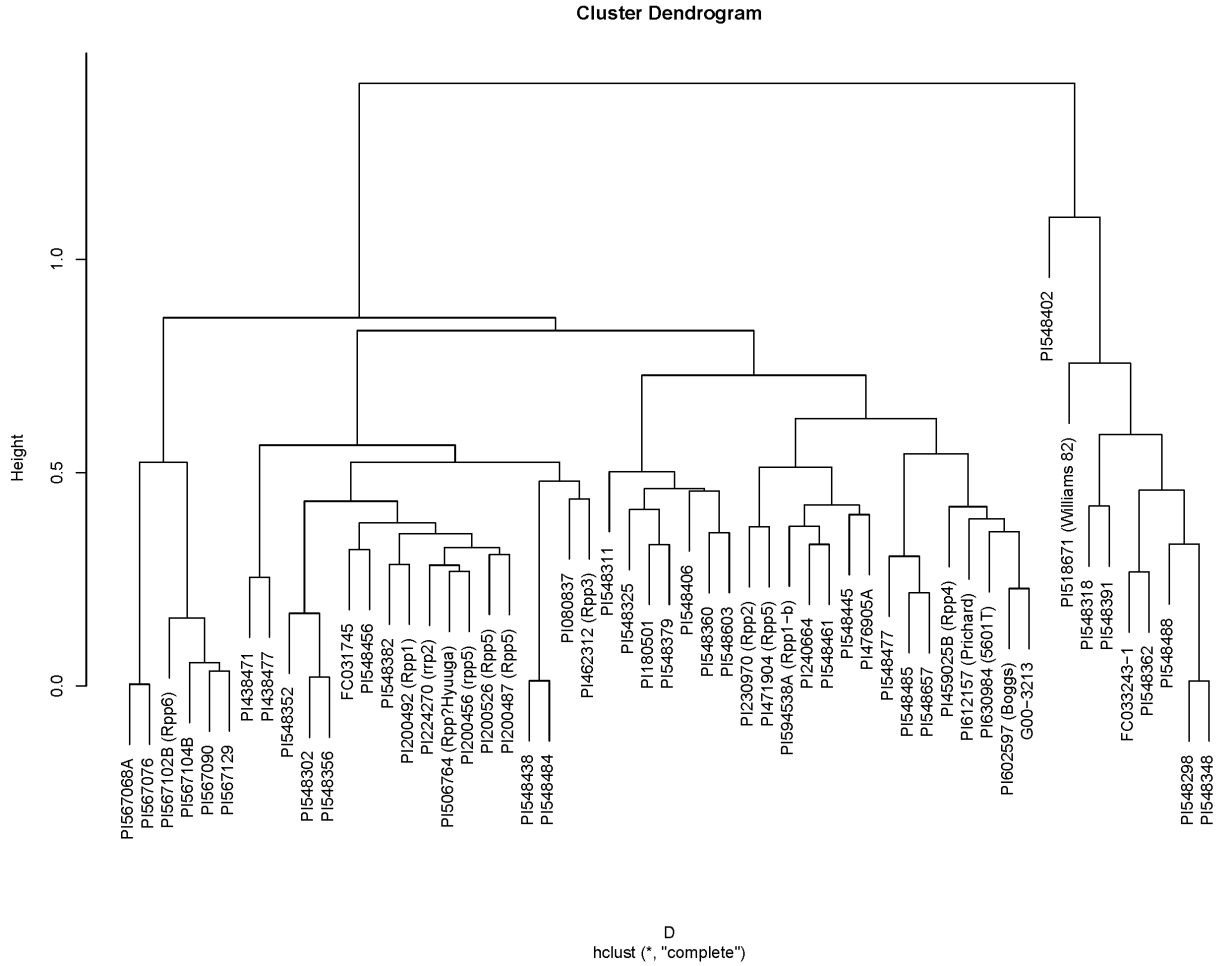
††The *P. pachyrhizi* isolate used to test for host plant resistance was the GA12 bulk isolate. GA12 was collected from field-grown kudzu and soybean in 2012. R indicates a reddish-brown or immune resistance reaction types, and S indicates a susceptible TAN lesion reaction.

¶Data extrapolated from Harris et al. 2015.

‡Data extrapolated from Walker et al. 2014b.

¶The genomic locations are from chromosome 18 of the Wm82.a1 sequence and indicate the dbSNP location (e.g. ss715632525) in bp. These data are available online at www.soybase.org/dlpages/index.php#snp50k (Song et al. 2013). The gray highlights if a haplotype allele is representative of the *Rpp6* locus. H indicates that the SNP call at that locus was heterozygous.

Not applicable (NA), was not tested.



Supplementary Figure 2.1: A comparative dendrogram for all lines listed in Table 2.4 and a hierarchical cluster analysis of dissimilarities across all the SoySNP50K SNPs.

Supplementary Table 2.1: Selected recombinant lines from the population derived from G00-3213 × PI 567102B. Progeny were shown if they were classified as homozygous resistant or susceptible and if they had a recombination in the marker interval shown below or a double recombination around the *Rpp6* locus. Progeny with the same allele or phenotype as PI 567102B (*Rpp6*) are highlighted in gray.

KASP assay SNP position Chr 18 (bp)¶	GSM0373 4,792,076	GSM0442 5,517,202	GSM0435 5,627,744	GSM0438 5,893,484	GSM0440 5,916,005	*Rpp6* locus‡	GSM0374 5,961,229
G00-3213	G	G	C	A	A	S	T
PI 567102B	A	A	A	G	T	**R**	C
Progeny							
4723	A	A	A	G	T	**R**	T
4726	G	G	C	-	A	**R**	T
4750	G	G	C	G	T	**R**	C
4755	G	G	C	G	T	**R**	T
4761	A	A	C	G	T	**R**	T
4776	G	G	A	G	T	**R**	C
4778	G	G	A	G	T	**R**	C
4811	G	G	C	G	T	**R**	C
4812	G	G	C	G	T	**R**	C
4836	G	A	A	G	T	**R**	C
4845	-	G	A	G	T	**R**	C
4855	G	A	-	-	T	**R**	C
4703	A	-	C	A	A	S	T
4705	A	A	A	A	A	S	T
4710	A	G	C	A	A	S	T
4719	A	-	-	G	A	S	T
4727	A	G	C	A	A	S	T
4728	G	G	C	A	A	S	C
4737	A	G	C	A	A	S	-
4741	A	A	A	G	A	S	T
4759	A	G	C	A	A	S	-
4760	A	G	C	A	A	S	T
4767	A	G	C	A	A	S	T
4769	A	-	-	-	A	S	T
4772	A	A	A	A	A	S	T
4773	A	G	C	A	A	S	T
4774	A	A	A	G	T	S	C
4775	A	A	A	A	A	S	T
4790	A	G	C	A	A	S	T
4799	A	A	A	G	A	S	T
4809	A	G	C	A	-	S	-
4832	A	A	A	G	T	S	-

4859	A	G	C	A	A	S	T
4874	G	G	C	A	A	S	C

-: Missing or heterozygous data

¶The genomic locations are from chromosome 18 of the Wm82.a1 sequence and indicate the dbSNP location (e.g. ss715632525) (Song et al. 2013).

ǂWhen challenged with the 2012 *P. pachyrhizi* bulk isolate parents and progeny listed in this table gave either a R homozygous resistant or S, homozygous susceptible response.

CHAPTER 3

REGISTRATION OF FOUR NEAR-ISOGENIC SOYBEAN LINES OF G00-3213 RESISTANT TO ASIAN SOYBEAN RUST[2]

[2]King, Z.R., Harris, D.K., Wood, E.D., Buck, J.W., Boerma, H.R., and Z. Li. Registration of Four Near-isogenic Soybean Lines of G00-3213 Resistant to Asian Soybean Rust. Submitted to the Journal of Plant Registration (2015).

Abstract

Soybean rust (SBR) is caused by an obligate biotrophic basidiomycete fungus, *Phakopsora pachyrhizi.* SBR arrived in the continental USA in 2004, where it has since proven to be detrimental to southern soybean production due to the cost of yield losses and the environmental concerns and expenses caused by reliance on fungicides for control. Resistance to SBR has primarily been developed by introgressing single, dominant resistance gene(s) into an elite soybean cultivar background. Here we describe four near-isogenic lines (NIL) of G00-3213 that have been named G00-3213*Rpp1*, G00-3213*Rpp2*, G00-3213*Rpp3*, and G00-3213*Rpp4*. These NILs were developed by backcrossing rust resistance genes *Rpp1*, *Rpp2*, *Rpp3*, or *Rpp4* into an elite, maturity group VII soybean line, G00-3213. These NILs have tawny pubescence, tan pod walls, and white flowers, and had the same general appearance to the elite, recurrent parent G00-3213 in the field in 2014. Additionally, each NIL exhibits a similar level of resistance to the GA12 *P. pachyrhizi* bulk isolate as the original resistant sources of the *Rpp* genes. These NILs will be useful as parents for public and private plant breeders, as well as for extension agents, crop consultants, and plant pathologists in conducting in-field determinations for *Rpp* gene effectiveness in the southern USA.

Abbreviations

IM, immune resistant reaction; RB, reddish-brown resistant reaction; TAN, tan susceptible reaction; SBR, soybean rust; SSR, simple sequence repeat; SNP, single nucleotide polymorphism.

Introduction

Soybean rust (SBR) is one of the most economically important foliar diseases of soybean (*Glycine max* (L.) Merrill) and is caused by the obligate, basidiomycete pathogen *Phakopsora pachyrhizi* (Sydow and Sydow) (Yorinori et al., 2005; Sydow and Sydow, 1914). *P. pachyrhizi* was first observed in the continental USA in 2004 (Schneider et al., 2005). SBR is a threat to soybean production in much of the world and is capable of causing yield losses from 15 to 70%, resulting in significant economic losses (Hartman et al., 1991; 2011; Livingston et al., 2004; Mueller et al., 2009; Ogle et al., 1979; Sinclair, 1989; Sinclair and Hartman, 1999; Yorinori et al., 2005). SBR-infected soybean plants can have reduced seed quality and quantity, which is a result from poorly filled beans, seed abortion, and lowered oil content of seed (Ogle et al., 1979). Some soybean genotypes have shown tolerance to *P. pachyrhizi* (Hartman et al., 2005; Hartman et al., 1991). SBR can be diagnosed by the presence of small lesions that are generally tan (TAN) in appearance due to uredinia and subsequently the presence of uredinospores on susceptible genotypes. Most genotypes with SBR resistance react by forming reddish-brown (RB) lesions, which may give rise to uredinia with the right environmental conditions and strain of *P. pachyrhizi* (Bromfield et al., 1980; Bromfield and Hartwig, 1980; Miles et al., 2011). Some soybean genotypes show immunity to certain *P. pachyrhizi* isolates, bearing no visible signs of infection on the leaves (Bromfield et al., 1980; Bromfield and Hartwig, 1980; Miles et al., 2011; Walker et al., 2014b).

Although SBR related yield losses have been marginal in the USA compared to the losses experienced in other areas, the southern states, including Georgia, South

Carolina, Alabama, Mississippi, Louisiana, North Carolina, Arkansas, Oklahoma, and Texas, have been the most affected. From 2005 to 2007, approximately 53.65 million metric tons of soybean yields were lost to SBR (Wrather and Koenning, 2009). In controlled studies, fungicide-treated soybean plots in Georgia and Florida yielded from 15 to 55% greater, respectively, than plots not treated with fungicides in the presence of SBR (Mueller et al., 2009). SBR losses have been mitigated through a coordinated monitoring effort of soybean sentinel plots by public and private organizations to establish an early warning system for growers (Isard et al., 2006; Sikora et al., 2014).

Due to the annual threat of SBR related yield losses in the southeastern USA and the current heavy reliance on fungicide-based control, genetic resistance to *P. pachyrhizi* would be valuable to manage this disease, as *Rpp* genes could be deployed in elite cultivars to provide a sustainable solution for farmers. The availability of cultivars with resistance would lead to a reduction in the need for foliar fungicides, and therefore would contribute to a reduction in production costs in areas at risk for SBR, as well as providing a more environmentally sustainable solution for SBR management (Hartman et al., 2005; Lemos et al., 2011).

A total of 16,595 soybean accessions from the USDA Soybean Germplasm Collection were screened for resistance to soybean rust in greenhouse trials (Miles et al., 2006). Additionally, known sources of resistance have been well documented from plant introductions (PIs), with six described resistance loci on four chromosomes (chr.) with more than seven total, single, dominant resistance genes (*Rpp1* (chr. 18; PI 200492), *Rpp1-b* (chr. 18; PI 594538A), *Rpp2* (chr. 16; PI 230970), *Rpp3* (chr. 6; PI 462312), *Rpp4* (chr. 18; PI 459025B), *Rpp5* (chr. 3; PI 200526, and PI 200487, and PI 471904),

and *Rpp6* (chr. 18; PI 567102B), and two loci with recessive resistance genes *rpp2* (chr. 16; PI 224270) and *rpp5* (chr. 3; PI 200456) (Bromfield and Hartwig, 1980; Chakraborty et al., 2009; Garcia et al., 2008; Hartwig, 1986; Hartwig and Bromfield, 1983; Hyten et al., 2007; Hyten et al., 2009; Li et al., 2012; Marchetti et al., 1975; Silva et al., 2008; Yu et al., 2015). *Rpp?*(Hyuuga) (chr. 6; PI 506764) was initially believed to be a novel allele at the *Rpp3* locus (Monteros et al., 2007); however, a subsequent study showed that PI 506764 actually has a natural pyramid of genes at the *Rpp3* and *Rpp5* loci (Kendrick et al., 2011). Additionally, many other resistance sources have been documented representing what appears to be many different plant introductions with resistance genes at the same loci, or novel resistance alleles (Harris et al., 2015).

L85-2378 (PI 547875), a 'Williams 82' near-isoline harboring *Rpp1* from PI 200492, PI 230970 (*Rpp2*), PI 462312 (*Rpp3*), and PI 459025B (*Rpp4*) sources were demonstrated to have varying levels of resistance in multi-year field tests in the southern USA in five locations when challenged with field populations of *P. pachyrhizi* from 2009-2012 (Walker et al., 2014b). L85-2378 was generally highly resistant or immune in most locations and years. PI 230970 (*Rpp2*), PI 462312 (*Rpp3*), and PI 459025B (*Rpp4*) have had more varying levels of resistance, and all had moderately resistance at most locations. The exception was at the Bossier City, LA location both PI 462312 (*Rpp3*), and PI 459025B (*Rpp4*) scored similarly to susceptible checks, L85-2378 (*Rpp1* near isoline) was moderately resistant, and no data was available at this location for PI 230970 (*Rpp2*) demonstrating the increased virulence of the *P. pachyrhizi* field population at this location (Walker et al. 2014b).

These PIs have had RB resistance lesions or immunity in the greenhouse when challenged with a bulk isolate of *P. pachyrhizi* urediniospores collected in 2007 or 2008 (*P. pachyrhizi* was collected from naturally infested kudzu (*Pueraria* spp.) and soybean in Georgia), and in 2012, in which the bulk isolate was collected from soybean only (Walker et al., 2014a).

Effective SBR resistance has been controlled by single *Rpp* genes which are capable of providing effective resistance against certain populations or strains of *P. pachyrhizi*. The goal of this work was to integrate the resistance genes from each of four resistance sources, PI 547875 (*Rpp1*; chr. 18), PI 230970 (*Rpp2*, chr. 16), PI 462312 (*Rpp3*, chr. 6), and PI 459025B (*Rpp4*, chr. 18) into a high-yielding soybean line named G00-3213 (Table 3.1). The most recent DNA markers available to flank each *Rpp* gene interval are Sct_187 and Sat_064 (*Rpp1*), BARCSOYSSR_16_0902 and BARCSOYSSR_16_0908 (*Rpp2*), Satt460 and Sat_263 (*Rpp3*), and Satt288 and AF162283 (*Rpp4*) (Hyten et al., 2007; 2009; Meyer et al., 2009; Yu et al., 2014). PI 200492 was used to derive L85-2378 (PI 547875), which is a 'Williams 82' near-isoline containing the *Rpp1* resistance allele (Bernard et al., 1991; Hyten et al., 2007). G00-3213 is a MG VII soybean that was previously developed at the University of Georgia from the cross of 'N7001' (Carter et al., 2003) and 'Boggs' (Boerma et al., 2000), and which is well adapted to the southeastern USA. N7001 was derived from crossing N77-114 x PI 416937; N77-114 was a derivative from a cross of 'Essex' x N70-2173; and N70-2173 was selected from a 'Hampton' x 'Ransom' cross (Smith and Camper, 1973; Webb et al., 1965; Brim and Elledge, 1973). G00-3213 has tawny pubescence, tan pod walls, black hila, and white flowers, and is highly susceptible to SBR.

Methods

Development of near isogenic lines of G00-3213

The four NILs were developed by making five backcrosses to G00-3213 directly using a plant introduction (PI) with soybean rust resistance, or indirectly using a PI-derived soybean elite line. For the development of the NILs containing *Rpp1* and *Rpp3*, soybean progeny derived from crosses between a PI with rust resistance and a Roundup Ready® soybean cultivar, P97M50 were used as resistance gene donors (Table 3.2). P97M50 was derived from backcrossing a glyphosate-resistant donor plant to G93-2225. The glyphosate resistant donor plant was a F_1 plant of the cross of 'Benning' (4) x ('Resnik'(2)-RR) (McBlain et al., 1990; Boerma et al., 1992). G93-2225 is a MG VIII breeding line developed from the cross of 'Cook' x 'Coker 6727' (Boerma et al., 1997).

Backcrosses of PIs with rust resistance were conducted in the field at the University of Georgia (UGA) Plant Sciences Farm near Bogart, GA or in the greenhouse at UGA located in Athens, GA. Upon each backcross, the heterozygous F_1 plants for each respective rust gene were identified with an SSR marker tightly linked to the resistance gene (data not shown). The BC_1F_1 to BC_5F_1 generations were produced in the same manner by marker-assisted backcrossing of the heterozygous plants carrying the respective resistance allele. No phenotypic selection was used during the backcrossing process.

During the summer of 2013, BC_5F_2 plants were allowed to self-pollinate at the UGA Plant Sciences Farm, and individual plants were selected for good agronomic appearance and were single plant threshed. In 2014, $BC_5F_{2:3}$ lines were grown at the UGA Plant Sciences Farm, and superior individual lines were selected and bulk threshed.

This established a population for each near-isogenic line (NIL) and these populations consisted of G00-3213*Rpp1*, G00-3213*Rpp2*, G00-3213*Rpp3*, and G00-3213*Rpp4* (Table 3.2).

SNP marker assays for NILs

Leaf tissue was harvested in a bulk from at least 12 plants of each NIL. Leaf issue was lyophilized and DNA was extracted using a CTAB (Hexadecyltrimethylammonium bromide) method developed by Keim et al. (1988). At the end of the backcrossing process, single nucleotide polymorphism (SNP) assays were developed to confirm the presence of SNP alleles that are tightly linked to *Rpp1* (originally from PI 200492), *Rpp2* (PI 230970), *Rpp3* (PI 462312), or *Rpp4* (PI 459025B) in these NILs.

Based on the haplotype windows within or closely linked to those established by Harris et al. (2015) for *Rpp1, Rpp3*, and *Rpp4,* and Yu et al. (2015) for *Rpp2*, as well as 50K SoySNP Infinium Chip data (Song et al., 2013), polymorphic SNP markers were identified for each of these genomic regions. Kompetitive Allele Specific PCR (KASP) SNP assays were developed from the selected polymorphic SNPs for these regions using the protocol reported by Pham et al. (2013) (Table 3.4). SNP marker assays were performed per the KASP manual (LGC Genomics, Middlesex, UK), as well as the protocol from Pham et al. (2013). The PCR products were read with standard FRET detection using a Tecan M1000 Pro Infinite Reader (Tecan Group Ltd., Männedorf, Switzerland), whereby allele calls were made using KlusterCaller software (LGC Genomics, Middlesex, UK). Only validated markers with clear clustering were selected

and reported (Table 3.4). In the case of G00-3213*Rpp1* and G00-3213*Rpp3*, the donor pedigrees contain P97M50 which carries the Roundup Ready® gene (Monsanto Co.). To ensure that the Roundup Ready® gene was not present; all NILs were screened using a proprietary DNA marker assay provided by Monsanto Company (Table 3.2).

Soybean rust resistance screening

In March of 2014, each $BC_5F_{2:3}$ family was screened for SBR resistance reaction in the UGA Griffin Campus Greenhouse as described by Harris et al. (2015) with a bulk soybean rust isolate known as GA12. GA12 was collected from SBR-infected soybean plants around the state of Georgia in 2012 and was maintained on susceptible soybean plants in the greenhouse (Walker et al., 2014a). Twelve plants were rated per $BC_5F_{2:3}$ NIL family approximately 14 days post infection. Only the families in which all 12 plants were resistant were selected, indicating that no segregation was observed in the progeny. Remnant seed of $BC_5F_{2:3}$ families with resistance were used to produce the $BC_5F_{2:4}$ lines in the summer of 2014, which were again confirmed to have rust resistance using the GA12 isolate in January 2015 in the UGA Griffin Campus Greenhouse in the same manner as described above (Table 3.2; Fig. 3.1).

Field evaluations of NILs

Based on the SBR resistance, 10 to 12 NILs for each resistance gene that were uniformly resistant in the BC_5F_2 stage were selected and planted in Athens, GA during the 2014 growing season in a single replication. The soil type in this location is an Appling coarse sandy loam (Typic Hapludult, clayey, kaolinitic, therm). Rows were 3.65

m long and were spaced 70.2 cm apart. The elite recurrent parent, G00-3213, was placed in each NIL set and used for agronomic comparison to the NILs. Data were collected on flower color when at least 75% of the plants in a row were flowering. Soybean rust was not detected during the growing season and no notes were taken on the presence of any diseases. Maturity date, plant height, lodging, pubescence color, and pod wall color were recorded at the R8 stage as described by Fehr et al. (1971) when 95% of pods had reached their mature color. Plant height and lodging score were recorded as per Diers et al. (2014). Post-harvest observations of seed weight, seed quality, hilum color, and the 100-seed weight were determined using 100 randomly selected seeds and inspecting all seeds sampled to ensure uniformity.

Characteristics

The final NIL lines selected are designated as G00-3213*Rpp1*, G00-3213*Rpp2*, G00-3213*Rpp3*, and G00-3213*Rpp4*. The NILs developed in this work had the expected level of resistance to the GA12 isolate derived from the corresponding PI parent with resistance; G00-3213*Rpp1* was immune, G00-3213*Rpp2* had RB lesions with some sporulation, G00-3213*Rpp3* had RB lesions with no sporulation, and G00-3213*Rpp4* had RB lesions with some sporulation (Table 3.1; Table 3.2). The recurrent parent, G00-3213, showed TAN, highly sporulating lesions when challenged with GA12, as expected (Table 3.2).

Each NIL has the same haplotype in the region of the resistance locus as the PI with resistance used in the backcrossing process (Table 3.1; Table 3.4). This confirms the integration of the resistance locus in each NIL in relation to the parent with SBR

resistance. These NILs have tawny pubescence, tan pod walls, white flowers, comparable height, maturity, lodging, seed characteristics, and general appearance to G00-3213 in the field (Table 3.3).

Discussion

The release of G00-3213*Rpp1*, G00-3213*Rpp2*, G00-3213*Rpp3*, and G00-3213*Rpp4* was approved by the UGA College of Agricultural and Environmental Sciences Plant Cultivar and Germplasm Release Committee in February, 2015. These germplasm lines are all resistant to the *P. pachyrhizi* bulk isolate GA12 and have agronomic equivalency to the recurrent parent G00-3213 in the field (Table 3.2; Table 3.3). These MG VII germplasm lines will allow private and public breeders to readily make crosses without the negative agronomic traits associated with the respective donor plant introductions, and to develop new soybean cultivars for geographic regions where the soybean crop is at the highest level of risk. Similar work has been completed by Diers et al. (2013) in early maturity soybean germplasm, whereby *Rpp1, Rpp1-b, Rpp?*(Hyuuga*),* and *Rpp5* have been backcrossed into the MG II and MG IV breeding lines LD01-7323 and LD00-3309, respectively. Our work compliments theirs, as our germplasm will perform well agronomically in the southern latitudes in the USA.

Additionally, these NILs will be useful resources for geneticists and plant pathologists studying the underlying genes causing resistance and will help them elucidate the various races of *P. pachyrhizi*. Having each *Rpp* gene in the same genetic background allows researchers to evaluate and compare the robustness of each gene without the confounding of different genetic backgrounds and provides a tool for

extension agents and crop consultants to conduct in-field determinations for *Rpp* gene effectiveness. For this reason, determining races of *P. pachyrhizi* can be most accurately achieved by putting different *Rpp* genes in the same background, as is the case presented here.

Availability

Seed for the USDA–ARS National Plant Germplasm System (NPGS) was developed by growing the selected NILs in a well maintained field in Athens, GA in 2015. Research plots were inspected for purity and the final lines were completed at the $BC_5F_{3:4}$ generation. Seed in the NPGS is available upon request from the date of this publication. Seeds of G00-3213*Rpp1*, G00-3213*Rpp2*, G00-3213*Rpp3*, or G00-3213*Rpp4* can be requested from the corresponding author for up to five years. If G00-3213*Rpp1*, G00-3213*Rpp2*, G00-3213*Rpp3*, or G00-3213*Rpp4* contribute to research or the development of germplasm lines and cultivars, we request an appropriate acknowledgement.

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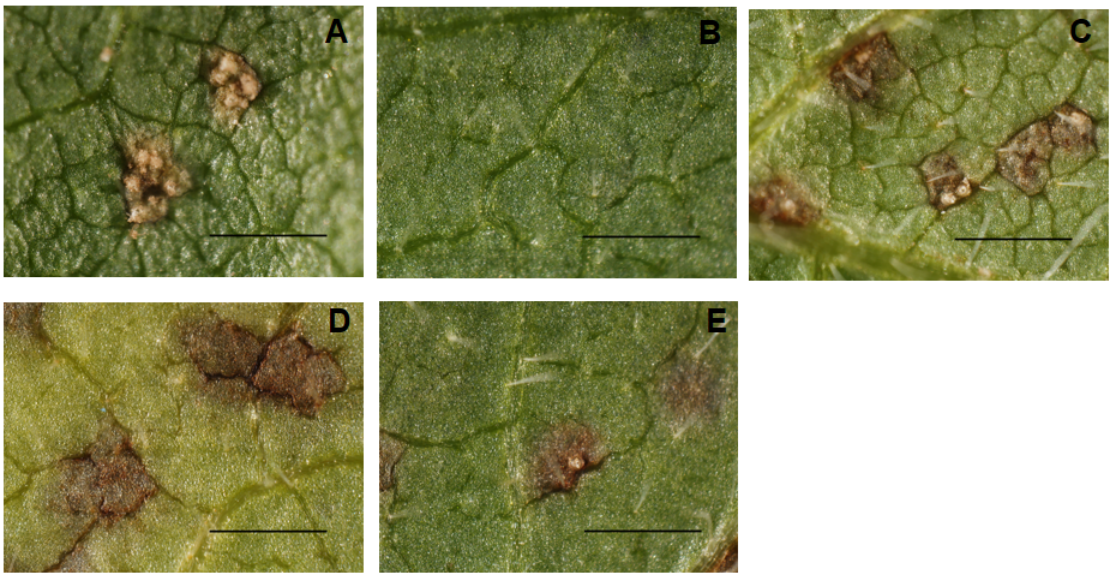

Figure 3.1: The reactions of the recurrent parent, (A) G00-3213 and near isogenic lines (B) G00-3213*Rpp1*, (C) G00-3213*Rpp2*, (D) G00-3213*Rpp3*, and (E) G00-3213*Rpp4* to the GA12 *P. pachyrhizi* bulk isolate. G00-3213 developed TAN, highly sporulating lesions, while the near isogenic lines were immune (B) or developed reddish-brown (RB) lesions associated with resistance (C, D, and E). Bar = 1 mm.

Table 3.1. Soybean rust (SBR) resistance genes, original sources, and reaction types.

Resistant germplasm source	Gene designation	*P. pachyrhizi* reaction[§]	Mapped interval defined by SNPs§§	Physical positions of flanking markers¶	KASP markers developed for each gene (ssID (GSM#))‡
PI 547875 (L85-2378)‡‡	*Rpp1*	Immune, no sporulation	ss715632302, ss715632319	Chr. 18 60,460,936 - 60,616,971	ss715632302 (GSM0419), ss715632313 (GSM0422)
PI 230970	*Rpp2*	Reddish-brown, some sporulation	ss715624066, ss715624108	Chr. 16 28,882,177 – 29,084,869	ss715624122 (GSM0425), ss715624131 (GSM0426)
PI 462312	*Rpp3*	Reddish-brown or immune, no sporulation if RB	ss715594464, ss715594493	Chr. 6 43,324,763 – 44,307,623	ss715594485 (GSM0412), ss715594488 (GSM0415)
PI 459025B	*Rpp4*	Reddish-brown, some sporulation	ss715631693, ss715631715	Chr. 18 55,715,639 – 55,913,511	ss715631686 (GSM0416), ss715631693 (GSM0417), ss715631723 (GSM0418)

§A bulk isolate of *P. pachyrhizi* collected from field-grown kudzu and soybean in 2012 was tested on the PI sources harboring known *Rpp* genes and published by Walker et al. (2014a).
‡‡PI 200492 was used to derive L85-2378 (PI 547875), which is a 'Williams 82' isoline containing the *Rpp1* resistance allele (Hyten et al., 2007).
§§Flanking SNP markers were identified by Harris et al. (2015) for *Rpp1*, *Rpp3,* and *Rpp4*; and Yu et al., (2015) identified the flanking markers used for *Rpp2*.
¶Physical genomic locations correspond to the Wm82.a1 sequence and indicate the dbSNP location (e.g. ss715632302) available online at www.soybase.org/dlpages/index.php#snp50k (Song et al., 2013).
‡KASP markers were developed for each gene and were validated using the assay described above. Sequences of primers are in Table 3.4.

Table 3.2: Pedigrees and phenotypes of G00-3213 NILs that were challenged with *P. pachyrhizi.*

Line name	Pedigree	Generation of release	Reaction to *P. pachyrhizi*§	Presence of Roundup Ready Gene	Lesion Reaction	Sporulation‡
G00-3213 (Recurrent Parent)	N7001 x Boggs	Breeding line	Susceptible	No	TAN‡‡	Highly sporulating
G00-3213*Rpp1*	G00-3213(6) x [P97M50(3) x L85-2378(*Rpp1*)]F2	$BC_5F_{3:5}$	Resistant	No	Immune#	No sporulation like L85-2378
G00-3213*Rpp2*	G00-3213(6) x PI230970(*Rpp2*)	$BC_5F_{3:5}$	Resistant	No	RB†	Some sporulation like PI230970
G00-3213*Rpp3*	G00-3213(6) x [P97M50 x PI462312(*Rpp3*)]	$BC_5F_{3:5}$	Resistant	No	RB†	No sporulation like PI462312
G00-3213*Rpp4*	G00-3213(6) x PI459025B(*Rpp4*)	$BC_5F_{2:4}$	Resistant	No	RB†	Some sporulation like PI459025B

§A bulk isolate of *P. pachyrhizi* collected from field grown kudzu and soybean in 2012 (GA12) was used to inoculate 12 plants per NIL or the recurrent parent.
‡NILs were screened using a proprietary DNA marker assay provided by Monsanto Company to ensure the RoundUp Ready gene was not present.
‡‡Lesion reactions are TAN (susceptible and highly sporulating).
†Reddish-brown (RB) lesions (resistance reaction).
#Immune response (Immune), which is a resistant reaction whereby no lesions are visible.
ǂIndicates whether or not the lesions produced uredinia and subsequently the presence of sporulation and were compared to the source of the resistance in the same test.

Table 3.3: Phenotype of G00-3213 and NILs in Athens, Georgia in 2014.

Germplasm line	Maturity date#	Plant height (cm)	Lodging score‡	Seed weight (mg seed^{-1})	Seed quality ‡‡	Flower color	Hilum color	Pubescence color	Pod wall color
G00-3213	10/25†	53-74††	1	163	1	White	Black	Tawny	Tan
G00-3213*Rpp1*	10/28	71	1	170	1	White	Black	Tawny	Tan
G00-3213*Rpp2*	10/25	69	1	179	1	White	Black	Tawny	Tan
G00-3213*Rpp3*	10/24	66	1	170	1	White	Black	Tawny	Tan
G00-3213*Rpp4*	10/28	69	1	178	1	White	Black	Tawny	Tan

#Date of the R8 stage when 95% of the pods reached their mature color (R8; Fehr et al., 1971).
†G00-3213 maturity based on the average of six, single-row plots within the experiment in Athens, GA during the 2014 growing season.
††G00-3213 height range based on the average of six, single-row plots within the experiment in Athens, GA during the 2014 growing season.
‡Lodging score, where 1 = erect and 5 = prostrate.
‡‡Seed quality rating, where 1 = very good and 5 = very poor.

Table 3.4: Validated KASP primers developed for marker assisted selection of *Rpp1, Rpp2, Rpp3,* or *Rpp4.*

Assay ID	dbSNP ID††	SNP ID¶	Gene	PI SNP allele†	Gene source	Forward primer 1 5'-3' (FAM)	Forward primer 2 5'-3' (HEX)	Reverse primer 5'-3'
GSM0419	ss715632302	Gm18_60460936_T_C	*Rpp1*	T	PI200492 (*Rpp1*)	GAAGGTGACCAAGTTCATGCTCAGCTCATTATAACTCGGGACCc	GAAGGTCGGAGTCAACGGATTCCAGCTCATTATAACTCGGGACCt	GGCAAAACTAGGTATCCATCCCA
GSM0422	ss715632313	Gm18_60590718_C_T	*Rpp1*	T	PI200492 (*Rpp1*)	GAAGGTGACCAAGTTCATGCTCATTGGAGAGACTTCATTATGCCAc	GAAGGTCGGAGTCAACGGATTCATTGGAGAGACTTCATTATGCCAt	GCTCATGTACCTTGTAAGACACCG
GSM0425	ss715624122	Gm16_29128926_T_G	*Rpp2*	T	PI230970 (*Rpp2*)	GAAGGTGACCAAGTTCATGCTAGTCATCTTGTTTTCAGCAAGCg	GAAGGTCGGAGTCAACGGATTGAGTAGTCATCTTGTTTTCAGCAAGCt	TGTCATCTCAGCAACCACTTTTG
GSM0426	ss715624131	Gm16_29153474_C_T	*Rpp2*	C	PI230970 (*Rpp2*)	GAAGGTGACCAAGTTCATGCTACAGTGTTTGAACAGTACAGATCTCc	GAAGGTCGGAGTCAACGGATTCACAGTGTTTGAACAGTACAGATCTCt	CGTGTGGTTTGAATCGGAAC
GSM0412	ss715594485	Gm06_44068533_T_C	*Rpp3*	C	PI462312 (*Rpp3*)	GAAGGTGACCAAGTTCATGCTTGACCGACAAGATGGCTTCAAc	GAAGGTCGGAGTCAACGGATTTGACCGACAAGATGGCTTCAAt	GGCCTTCACACCCTCCACT
GSM0415	ss715594488	Gm06_44185370_G_T	*Rpp3*	A	PI462312 (*Rpp3*)	GAAGGTGACCAAGTTCATGCTTATGGACCAAAGACCCTTCCa	GAAGGTCGGAGTCAACGGATTTATGGACCAAAGACCCTTCCc	CCCTCTGTAGTTTCCACACGCT
GSM0416	ss715631686	Gm18_55672211_A_G	*Rpp4*	G	PI459025B (*Rpp4*)	GAAGGTGACCAAGTTCATGCTGGCACGAGAACTCGCTGCTa	GAAGGTCGGAGTCAACGGATTGCACGAGAACTCGCTGCTg	CGATCATAGCAGGACCTCCA

GSM0417	ss715631693	Gm18_55715639_C_T	*Rpp4*	C	PI459025B (*Rpp4*)	GAAGGTGACCAAGTTCATGC TAGATGCTTTGGCCGAGGc	GAAGGTCGGAGTCAACGG ATTTTAAGATGCTTTGGCC GAGGt	GTCAGAGTAATC GCACCTTGGC
GSM0418	ss715631723	Gm18_55986655_A_C	*Rpp4*	A	PI459025B (*Rpp4*)	GAAGGTGACCAAGTTCATGC TAGTCAAGTGTACTTTATAAA CACCCTCa	GAAGGTCGGAGTCAACGG ATTGTCAAGTGTACTTTAT AAACACCCTCc	CACTTTGCCAGG CTAACAGATAA

††dbSNP ID from www.soybase.org/dlpages/index.php#snp50k.
¶Physical genomic locations correspond to the Wm82.a1 sequence of the dbSNP location (e.g. ss715632302) available online at www.soybase.org/dlpages/index.php#snp50k (Song et al., 2013).
†Indicates the SNP allele linked to the given *Rpp* gene resistance and PI source.

CHAPTER 4

NON-TOXIC AND EFFICIENT DNA EXTRACTIONS FOR SOYBEAN LEAF AND SEED CHIPS FOR HIGH-THROUGHPUT AND LARGE-SCALE GENOTYPING[3]

[3]King, Z., Serrano, J., Boerma, H.R., and Z. Li. Non-toxic and efficient DNA extractions for soybean leaf and seed chips for high-throughput and large-scale genotyping. Reprinted here with permission of publisher. Biotechnology Letters (2014) 36:1875–1879.

Abstract

In applied soybean (*Glycine max* L.) breeding programs, marker-assisted selection has become advantageous to select value-added quantitative trait loci. The goal of this work was to improve marker-assisted selection workflow by developing a reliable, inexpensive, high-throughput DNA extraction protocol for soybean seed and leaf samples that does not generate hazardous waste. The DNA extraction protocol developed allows for the leverage of robust SNP genotyping platforms such as the SimpleProbe Assay and KASPar v4.0 SNP Genotyping System to genotype thousands of seeds nondestructively, or leaves in a single day with a 95% success rate. This methodology makes it possible to run up to 150 SNP markers on the DNA extracted from a single seed chip or leaf sample.

Keywords

DNA extraction; *Glycine max* L.; marker-assisted selection; single nucleotide polymorphism

Introduction

Marker-assisted selection is advantageous in applied plant breeding programs to select for desired traits in major crops such as soybean, which are difficult or costly to select in the field (e.g. seed quality traits), or require the presence of serious disease or pest that injure the crop. Additionally, traits controlled by more than one gene are difficult, or nearly impossible to pyramid in a single cultivar with phenotypic selection (Yamanaka et al. 2013).

Prior to the last decade, DNA based marker assays were costly and not efficiently used in applied plant breeding programs (Moose and Mumm 2008). Currently, innovative marker

detection chemistry coupled with sophisticated equipment has made molecular marker assays significantly lower in cost per data point, (Ha and Boerma 2008) and very informative, which has allowed large-scale marker-assisted selection and trait mapping to become possible (Hyten et al. 2009; Song et al. 2013; Ha et al. 2010; Hyten et al. 2010).

To continue improving the genetic gain of crops, breeding programs need to screen more individuals than ever before in order to find unique progeny with all the desired traits possible in a single seed or plant (Barone et al. 2009). To select for traits of interest with DNA markers, plant breeders need to plant seeds from segregating progeny, and then sample leaf tissues of the plants in order to eventually conduct a genetic fingerprint. Afterwards, unique plants that contain the desired traits can be selected from the large pool of individuals that were originally planted in a field or greenhouse to continue the breeding process. Alternatively, breeders can use seed chipping coupled with DNA marker genotyping to select the seed with traits of interest for planting in the field or greenhouse.

Most published procedures for DNA extractions from leaf tissue to date do not lend themselves to automation or analysis of thousands of samples in a high-throughput manner (Edwards et al. 1991; Weigel and Glazebrook 2009; Xin and Chen 2012). Hazardous compounds requiring the use of fume hoods are involved in the extraction of DNA (e.g. chloroform, β-mercaptoethanol, and phenol) (Keim et al. 1988; Hyten et al. 2007) and reduce the throughput capabilities of DNA extractions. These chemicals pose potential risks to laboratory personnel, and additional time and expense to dispose of hazardous wastes that accumulate on site in satellite accumulation areas (Keim et al. 1988; Hyten et al. 2007). The objective of this study was to develop a rapid, reliable DNA extraction protocol to routinely genotype both soybean seed, and leaf tissue using SNP markers.

Materials and methods

Plant materials

In this study, leaf tissue was collected from two populations, Population 1: an F_2 population of G00-3213 x FAM94-41 and its parents, 'G00-3213' (University of Georgia) and 'FAM94-41' (Zhang et al. 2008); and Population 2: an F_2 population derived from 'Benning' (PI 595645) x 'Hyuuga' (PI 506764). Seed samples consisted of F_5 derived recombinant inbred lines (RILs) of PI 96354 x Bossier (PI 567789) and its parents (Population 3), as well a 'Dillon' (PI 592756) x Hyuuga F_2 population and its parents (Population 4).

Tissue sample preparation, DNA extraction, and quantification, and SNP genotyping

For leaf tissue preparation, penny-sized samples were collected from young, expanded trifoliate leaves into a Matrix Latch Rack (Thermo Scientific). Tissue was dried overnight at 50^0C. One ball bearing was added to each well, and sample plates were sealed using a rounded 96-well CapMat (Thermo Scientific). Tissue was pulverized into a fine powder using a GenoGrinder (SPEX US). For seed sample preparation, seeds were hydrated overnight to ~13% relative humidity and a razor blade was used to collect one-tenth of a seed into separate wells of a 96-well plate.

DNA was extracted by adding 400 µl of the combined NaCl and extraction buffer (40% v/v 5M NaCl and 60% v/v extraction buffer (200 mM Tris-HCl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS)) (Edwards et al.1991) to each well. Sample plates were placed in a 60^0C incubator for 30 minutes. Plates were then centrifuged for 5 minutes at 2500 *g* and 270 µl of the supernatant was transferred to a new 96-round deep-well plate (VWR) containing 270 µl of 100% isopropanol. The plate was sealed with a clean rounded CapMat as before, and rotated

upside down gently several times to mix. The isopropanol plate was then placed at -20^{0}C for 15 minutes. The plate was then centrifuged for 5 minutes at 2500 *g*, and the isopropanol was carefully decanted by turning the plate upside down. Add 200 µl of 70% ethanol to each of the wells and centrifuge for 5 minutes at 2500 *g*. Ethanol was decanted as described before and each plate was placed in a 60^{0}C incubator for 40 minutes until no remnant ethanol remained. DNA pellets were re-suspended in 300 µl of water; alternatively, Tris-EDTA buffer can be used (10 mM Tris, 1 mM EDTA). Plates were incubated for 30 minutes at 60^{0}C, covered with a new rounded cap mat, and rotated upside down gently several times to mix. DNA concentrations were measured using 2 µl of the total sample volume (300 µl) and read with the TECAN infinite M200 and the NanoQuant plate (Tecan US), which uses an endpoint absorbance assay. The range of DNA concentrations, and average was calculated using 24 random samples from each DNA extraction method.

The project utilized four PCR-based SNP genotyping assays to assess the effectiveness of the DNA extraction method. Each assay was carried out in 384-well plates with either the KASP (Kompetitive Allele Specific PCR; LGC Genomics, Middlesex, United Kingdom) or SimpleProbe (Roche Applied Science, Indianapolis, IN, USA) genotyping assay.

DNA sample concentrations were diluted with water to 20 ng µl^{-1} for each assay. In routine genotyping a 10-fold DNA dilution is used. Diluted DNA from the 96-well plates were transferred to 384-well plates (Roche) and then master-mix of either KASP or SimpleProbe was added for PCR. The KASP assayed were run in 4 µl reactions which consisted of 2 µl DNA and 2 µl master mix, while the SimpleProbe assay was run in 3 µl reactions on 384-well plates with 1 µl DNA in each well (Monteros et al. 2010).

PCR plates consisting of KASP assays were read with a TECAN infinite M200 microplate reader (Tecan US) and marker alleles were called using Klustercaller software (LGC Genomics). A Roche LightCycler 480 II with LightCycler® Software (Roche Diagnostics Corporation) was used for obtaining the SimpleProbe genotyping data. The success of each assay was calculated by dividing the total number of samples with reactions that yielded callable alleles. The accuracy of allele calling was calculated by dividing the number of successful reactions by the number of reactions with expected allele calls.

Results

When extracting DNA from a single seed chip using this method, 8-304 ng μl^{-1} DNA were obtained in a total volume of 300 µl of water (average concentration 89 ng μl^{-1}). DNA obtained from leaf tissue ranged from 30-852 ng μl^{-1} with an average of 347 ng μl^{-1}. In comparison with the relatively same amount of tissue, the standard CTAB chloroform method for soybean (Keim et al. 1988) yielded between 0.62 to127 ng μl^{-1} of DNA for seed chip samples (24 samples) and , 6 to 1,434 ng μl^{-1} DNA for leaf samples.

Figure 4.1 illustrates the graphical data for SNP marker assays with both leaf tissue and seed chips. Assay 1 (Fig. 4.1A) was comprised of leaf tissue from the parent lines Benning and Hyuuga, and the F_2 population (Benning x Hyuuga). Assay 2 was comprised of DNA extracted from seed chips (Fig. 4.1B) from parents Dillon and Hyuuga and the F_2 population (Dillon x Hyuuga). In both assay 1 and 2, the DNA samples were genotyped using a SimpleProbe assay and the SNP06-44058 marker (Fig 4.1A; 4.1B) for the detection of soybean rust (*Phakopsora pachyrhizi*) resistance (*Rpp3*) (Monteros et al. 2010) on *Gm06* (LG-C2). The plants containing the Hyuuga allele yielded melting peaks near 60.4 °C, while the plants containing the other allele

(Benning and Dillon) yielded melting peaks around 53.5 °C. The samples carrying both alleles generated the peaks at both these temperatures. In these assays, Hyuuga exhibits the resistant allele to soybean rust [T], while Benning and Dillon exhibit the susceptible allele SNP [G].

Assay 3 shows the KASP genotypic results for leaf tissue from the F_2 population (G00-3213 x FAM94-41) and their parents G00-3213 and FAM94-41 (Fig. 4.2A). The leaf samples in assay 3 were genotyped with KASP assays using a marker (SACPD-C) for stearoyl-ACP desaturase (Zhang et al. 2008) to detect the mutant allele from FAM94-41 for elevated 18:0 content in the soybean on *Gm14* (LG-B2) (Fig. 4.2A). Assay 4 (Fig. 4.2B) was comprised of seed chip DNA extracted from RILs and their parents, PI96354 (resistant) and Bossier (susceptible). A KASP SNP assay with the SNP GSM0040 (Pham et al. 2013), which differentiated PI96354 allele [T] from the Bossier allele [C] located on *Gm10* (LG-O). The success rate of each marker and tissue type combination was calculated as the number of samples, which had successful PCR amplification after DNA extraction over the total number of samples (Fig. 4.3).

Discussion

The objective of this project was to streamline the workflow of genotyping using a soybean model as well as eliminate the use of hazardous chemicals. The extraction protocol proved to be robust enough to obtain DNA from leaf and seed tissue and worked with four sets of diverse germplasm, with three markers at a success rate of >95% for obtaining genotyping results from 1,536 samples in four 384-well plates (Fig. 4.3). The four assays reported confirm that DNA can be efficiently extracted without the use of hazardous chemicals in two hours while reducing the number of steps in previous CTAB/chloroform extraction method (Keim et al.

1988). The SDS extraction protocol provided here gave average DNA concentrations of 89 and 347 ng μl^{-1} in 300 µl of water from a seed chip or leaf tissue, respectively. Comparatively, the SDS extraction method provided approximately four times more DNA for seed chips and 2-fold less DNA than the CTAB/chloroform method, respectively. Either method is amendable for SNP marker based assays shown here. Additionally, we also tested the SDS based DNA extraction method described here using leaf tissue of both alfalfa (*Medicago sativa* L.) and red clover (*Trifolium pratense* L.), yielding on average 237 ng μl^{-1} and 361 ng μl^{-1} of DNA, respectively in 300 µl of water (24 samples each, data not shown). This method is likely amendable for DNA isolation with other plant species as well.

In addition, these 3 to 4 µl PCR reaction volumes dramatically reduced genotyping costs for breeding. The extraction method provides the ability to genotype up to 150 SNP markers at a cost of approximately 8 cents per extraction.

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Competing interests

The authors declare that they have no competing interests.

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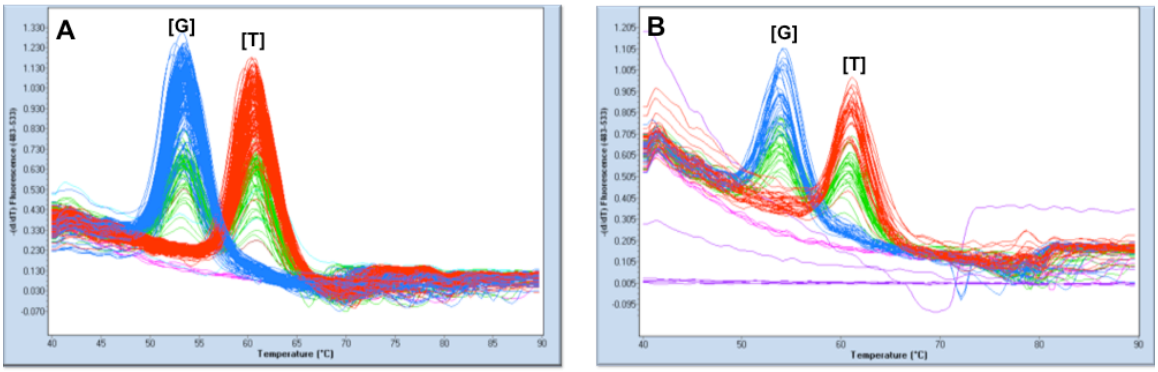

Fig. 4.1: SNP genotyping results using the high-throughput DNA extraction method for marker SNP06-44058. The SNP06-44058 SNP marker for the detection of *Rpp3* was used to genotype DNA extracted from both leaf tissue (**A**) and seed chips (**B**). 384 PCR reactions were run per DNA marker and tissue type.

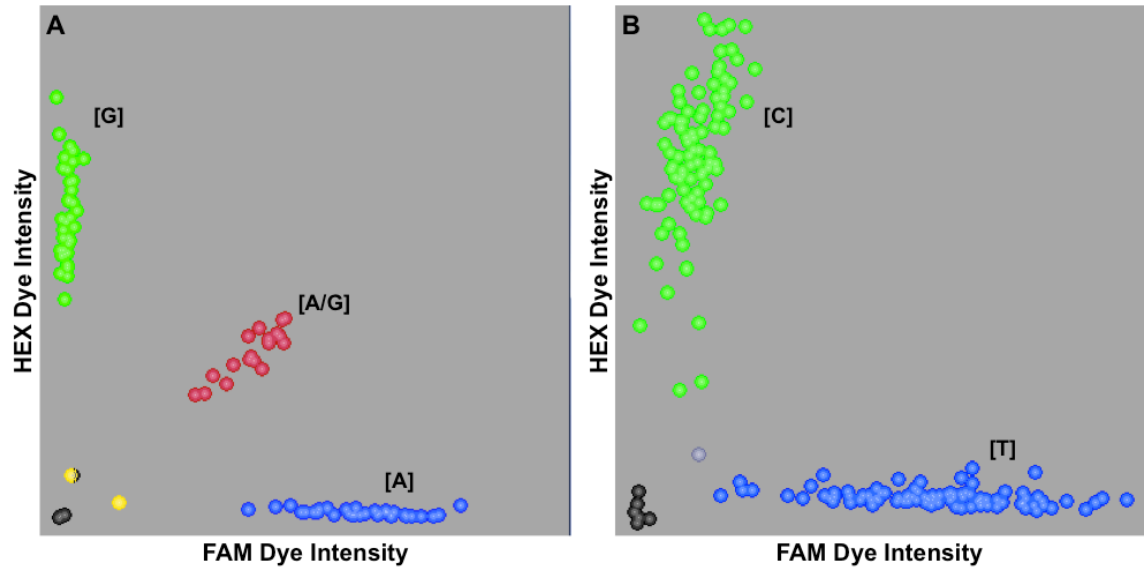

Fig. 4.2: SNP genotyping results using the high-throughput DNA extraction method for markers SAPCD-C and GSM0040. Shown are marker results from SAPCD-C (**A**) and GSM0040 (**B**), the tissue sources for DNA were leaf and seed chips, respectively. 384 PCR reactions were run per DNA marker. Key: yellow=failed; black= No template control (NTC); and gray=ambiguous.

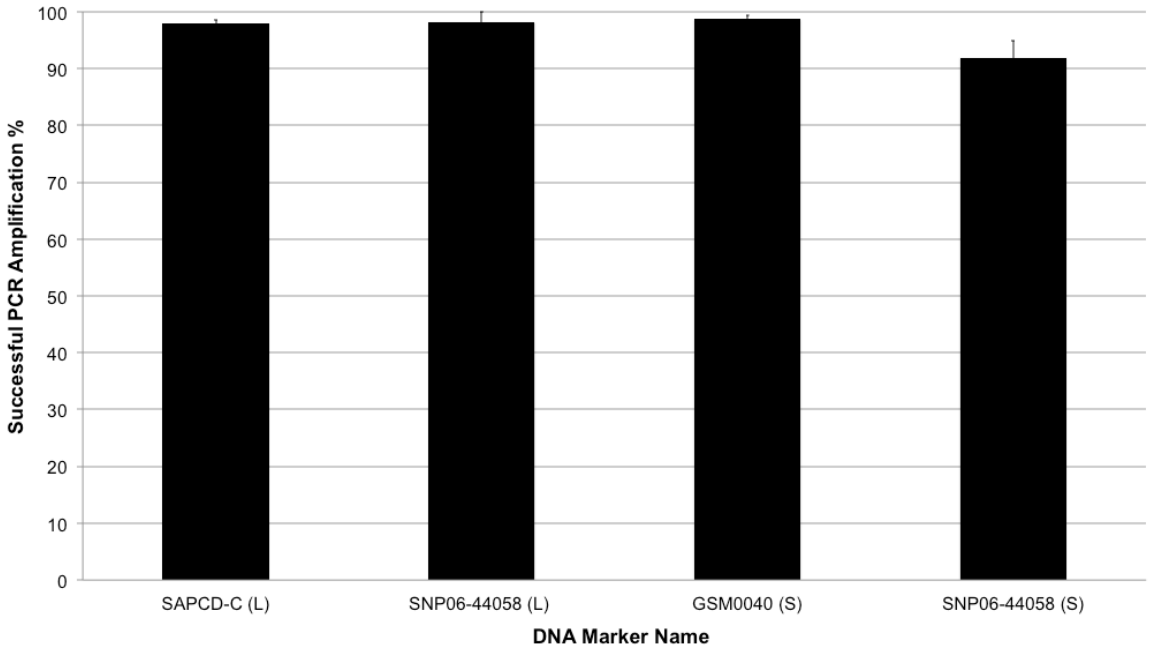

Fig. 4.3: Average SNP marker success rate. Each average was calculated using the success rates from 384 PCR amplifications using three different SNP markers: SAPCD-C (KASP), SNP06-44058 (SimpleProbe), or GSM0040 (KASP). Error bars are standard error and were calculated using the success rates of four 96-well plates per marker per tissue type. (L) and (S) indicate the tissue types, leaf tissue or seed chips that DNA was extracted from, respectively.

CHAPTER 5

SUMMARY AND THE IDENTIFICATION OF PUTATIVE UNIQUE SOURCES OF *RPP* GENES

This dissertation work had three major goals to further the knowledge and efficiency of soybean breeding and genetics: i) to discover and report novel *Rpp* genes and/or alleles; ii) to integrate existing *Rpp* genes into an elite soybean genetic background to develop agronomically elite lines with resistance to SBR; and iii) to provide a rapid, efficient, genotyping system for applied soybean breeding programs.

The first, a goal of this work was to discover and report novel *Rpp* genes and/or alleles. One-hundred and ten crosses were made between an elite, soybean rust (SBR) susceptible soybean line and a plant introduction (PI) containing a putative, unique source of resistance to *P. pachyrhizi* (*Rpp*) at the University of Georgia since 2006 (Harris et al., 2015). Several populations were identified to contain either a putatively novel allele or gene, which have been summarized below (Table 5.1, 5.2, and 5.3).

Using bulked segregant analysis (BSA), the candidate genomic region of many PIs has been identified (Harris et al., 2015). Generally the use of large bulks with at least 25 to 35 individuals or families per bulk and the Infinium chips platform has improved the accuracy of the intervals containing *Rpp* genes (data not shown).

After BSA identified candidate regions of interest for populations involving PI 567068A and PI 423972, KASP SNP assays were developed and used to fine map the novel sources of resistance using 140 $F_{2:3}$ families for each mapping population. The resistance of PI 423972 and

PI 567068A was determined to be novel primarily due to the differential *P. pachyrhizi* isolate reactions, and for PI 423972 a novel haplotype at the *Rpp4* locus (Table 5.1 and 5.3). The interval of the PI 423972 resistance gene overlaps with the *Rpp4* locus corresponding to the SNP markers GSM0389 (ss715631712), GSM0391 (ss715631733), and GSM0392 (ss715631745); with a significant LOD score of 4.9 over the GSM0391 marker. This interval spans from 55,873,296 to 56,226,616 (Wm82.a1 genome sequence) on chromosome (Chr) 18 and has been designated as *Rpp[PI423972]*. As *Rpp[PI423972]* overlaps with the fine mapped PI 459025B (*Rpp4*) interval located from 55,715,639 to 55,913,511 bp, it may be allelic or tightly linked to *Rpp4* (data not shown for mapping; Table 5.1, 5.2 and 5.3). PI 423972 and PI 459025B have different haplotypes at the *Rpp4* locus and are phenotypically different when tested with a unique panel of *P. pachyrhizi* isolates (Table 5.1, 5.2, and 5.3). The *Rpp4* haplotype was previously defined by Harris et al. (2015). PI 423972 was collected from Japan, whereas PI 459025B was collected from Vietnam, representing geographically distanced regions. An allelism test or sequencing the regions of PI 459025B and additional fine mapping of *Rpp[PI423972]* could discern this. Other PIs harboring the unique PI 423972 haplotype at the *Rpp4* locus include PI 566984, PI 567188, and PI 605791A (Tables 5.2-5.3). PI 605791A had a unique isolate pattern when compared to PI 423972 (*Rpp[PI423972]*) and PI 459025B (*Rpp4*) and therefore may harbor a novel resistance allele or tightly-linked gene at the *Rpp4* locus. PI 566984 and PI 567188 have not been tested with a panel of isolates, however, PI 566984 also has the *Rpp6/Rpp[PI567068A]* haplotype and may harbor two resistance genes (see details below) (Tables 5.2-5.3). The *Rpp6/Rpp[PI567068A]* haplotype was defined as a unique CAG haplotype defined by SNP markers ss715632362, ss715632523, and ss715632525, at the *Rpp6/Rpp[PI567068A]* locus (Table 4.2).

Additionally, the resistance *Rpp6* alleles contributed by PI 567102B and *Rpp[PI567068A]* from PI 567068A were fine mapped using a recombinant inbred line and a $F_{2:3}$ family population, respectively. *Rpp6* was mapped to a 42,224 bp interval between SNP markers GSM0374 and GSM0427, and *Rpp[PI567068A]* was mapped to a 161,158 bp interval determined by SNP markers GSM0440 and GSM0374 using the Wm82.a1 reference sequence (Fig. 4.3). A diagram showing the physical interval where *Rpp6* and *Rpp[PI567068A]* map to on Chr 18 was created to show how tightly linked the two sources of resistance appear to be, and both share the marker GSM0374 (Fig. 4.3). The *Rpp6* gene contributed by PI 567102B maps from 5,916,005 to 5,961,229 bp and *Rpp[PI567068A]* from PI 567068A maps from 5,961,229 to 6,122,387 bp using the Wm82.a1 reference sequence (Fig. 4.3). Using the 50K SoySNP Infinium Chip data of Song et al. (2013), PI 567102B and PI 567068A have identical haplotypes from 5,924,556 (ss715632113) to 6,384,548 bp (ss715632525) of the Wm82.a1 sequence. This haplotype is defined by 16 SNP markers: ss715632113, ss715632123, ss715632129, ss715632179, ss715632196, ss715632280, ss715632362, ss715632369, ss715632399, ss715632451, ss715632467, ss715632499, ss715632517, ss715632521, ss715632523, and ss715632525. Since the haplotype is not unique between these PIs, it is referred to the *Rpp6/Rpp[PI567068A]* haplotype, which is also identical amongst all the PIs that have resistance that maps to the *Rpp6* locus including: PI 476905A, PI 567068A (*Rpp[PI 567068A]*), PI 567076, PI 567104B, PI 567129, and PI 567102B (*Rpp6*) (Table 5.2). Other than PI 476905A which was collected from an unknown province in China in 1983; PI 566956, PI 566984, PI 567068A, PI 567076, PI 567090, PI 567104B, PI 567123A PI 567129, and PI 567102B all were collected from East Java, Indonesia in 1993, which further suggests these genotypes may be closely related (Table 5.3).

Because the genotypes mapping to the *Rpp6* locus share an identical haplotype, the region was further investigated by comparing a panel of susceptible soybean ancestors and PIs with known *Rpp* genes. A unique CAG haplotype formed by SNP markers ss715632362, ss715632523, and ss715632525 can discern all PIs that map to the *Rpp6/Rpp[PI567068A]* locus from all other genotypes tested (Table 4.5). Phenotypically, when tested with a panel of *P. pachyrhizi* isolates, PI 476905A showed a unique pattern; PI 567068A, PI 567076, and PI 567090 had a phenotype like PI 567068A; and PI 567104B had a phenotype like *Rpp4* and *Rpp6* genotypes (Tables 5.1-5.3). PI 566956, PI 566984, PI 567123A, and PI 567129 have not been tested with a panel of isolates. It may be that several PIs have a natural *Rpp* gene pyramid based on haplotype data. It is not uncommon for rust resistant PIs to harbor more than one *Rpp* gene (Harris et al., 2015; Kendrick et al., 2011). Interestingly, PI 567104B has the *Rpp4* haplotype of PI 459025B and the *Rpp6/Rpp[PI567068A]* haplotype, BSA data supports this as positive hits are located within 5 cM of the *Rpp4* and *Rpp6* loci (Harris et al. 2015; Table 5.2), and phenotypes like the PI 567102B (*Rpp6*) and PI 459025B (*Rpp4*) genotypes, providing evidence that this PI may contain a *Rpp* gene at both the *Rpp4* and *Rpp6* locus. Additionally, in a field experiment in 2008 at Quincy Florida, PI 567104B had lower field rust severity scores than either PI 567102B (*Rpp6*) and PI 459025B (*Rpp4*). PI 567104B had a lower lesion density than PI 567102B (*Rpp6*) and PI 459025B (*Rpp4*) when challenged with the GA 2008 (GA08) bulk *P. pachyrhizi* isolate in a greenhouse assay in 2011 (Walker et al., 2014). PI 566984 may harbor a natural *Rpp* gene pyramid as it has both the *Rpp6/Rpp[PI567068A]* and *Rpp[PI423972]* haplotypes.

Interestingly, the source of *rpp2* (PI 224270) was found recently to behave as a dominant gene in a segregating population phenotyped in a greenhouse-screening assay when challenged

with the Georgia 2012 (GA12) *P. pachyrhizi* bulk isolate (data not shown). PI 224270 and resistant progeny produced an immune or non-sporulating reddish-brown lesion phenotype to the Georgia 2012 (GA12) *P. pachyrhizi* isolate. Given that *rpp2* (which behaves as *Rpp2-b*) is on Chr 16 (the only known rust locus there), the resistance locus may work well in a gene pyramid in a soybean cultivar. Additionally, haplotype data suggests PI 224270, PI 417125, PI 417126, and PI 417129B may all possess the *rpp2* gene (Table 5.2). PI417125 has been confirmed to have an *Rpp* gene at the *Rpp2* locus through an allelism test with PI 230970 (*Rpp2*) (Yamanaka et al., 2010). The *rpp2* haplotype window of PI 224270 is defined by 174 SNPs and represents the physical distance between simple sequence repeat markers Satt215 and Sat_361 used by Garcia et al. (2008) to map *rpp2*. The haplotype window is defined by 174 SNPs as was determined as per Harris et al. (2015) (Table 5.4). PI 224270, PI 417125, PI 417126, and PI 417129B share over 95% similarity in this haplotype that spans 1,531,127 bp (Wm82.a1 sequence) on Chr 16 (Table 5.4). Additionally, PI 417125 and PI 224270 both harbor a single dominant resistance gene, and mapped to the *rpp2/Rpp2* locus.

PI 224270 (*rpp2*) was collected from Hyogo Japan in 1955; and PI 417125, PI 417126, and PI 417129B were collected from the Kyushu Province of Japan in 1977 and bear similar cultivar names of 'Kyushu 31,' 'Kyushu 32,' and 'Kyushu 40,' respectively. For these reasons, it may be worth fine mapping the *rpp2* locus.

Lastly, PI 594796 and PI 605823 are top priorities for novel *Rpp* gene discovery. PI 605823 maps to Chr 19 with the majority of BSA hits between ss715634424 and ss715634997 corresponding to 35,903,089 to 40,255,847 bp (Wm82.a1 sequence) and PI 605823 matched no known SBR resistance haplotype (Tables 5.2-5.3). PI 594796 has proven to be challenging to phenotype using Georgia *P. pachyrhizi* bulk isolates. Depending on the year PI 594796 was

challenged with *P. pachyrhizi* in greenhouse assays in 2010, 2011, 2012, or 2013 and produced TAN, RB, TAN, and mixed lesions, respectively, which is uncommon (Walker et al., 2014). This suggests that PI 594796 may be segregating within the seed lot for the *Rpp* gene, or that the *Rpp* gene of this PI may be environmentally sensitive. In greenhouse assays from 2010 to 2012 the Georgia 2008 (GA08) bulk *P. pachyrhizi* isolate was used to challenge PI 594796. Therefore the GA08 bulk isolate is not likely the reason for the discrepancies between years as other PIs behaved similarly from year to year (Walker et al., 2014). The best way to ensure the putatively novel *Rpp* gene is identified in PI 594796 is to test the seed lot for purity to rule out segregation as the cause of error. PI 594796 matches no known SBR resistant haplotypes (Table 5.2 and 5.3).

In conclusion, there are several priority populations for *Rpp* gene mapping. Two population include PI 594796 and PI 605823, where PI 605823 will likely be *Rpp7* (Table 5.2 and 5.3) as no *Rpp* genes currently map to Chr 19. Additionally, PI 476905A has the *Rpp6/Rpp[PI567068A]* haplotype, yet has a unique isolate pattern from PI 567102B (*Rpp6*) and PI 567068A (*Rpp[PI567068A]*) indicating that this may be another allele at the same locus, or a tightly linked novel *Rpp* gene. PI 605791A maps to the *Rpp4* locus, and matches the PI 423972 (*Rpp[PI423972]*) haplotype, however, this PI has a strikingly different isolate reaction pattern compared to both PI 459025B (*Rpp4*) and PI 423972, which indicates this is likely another allele at the *Rpp4* locus or a tightly linked, novel *Rpp* gene.

Based on the Griffin greenhouse screening results of new PIs, six new crosses were made in the summer of 2014 between the susceptible elite line, G00-3213 and each of the six new SBR resistant PIs (PI 566956, PI 566984, PI 567072A, PI 567073A, PI 567089A, and PI 567132C). PI 566956 and PI 566984 are discussed in detail above (Tables 5.2-5.3). PI 567072A, PI

567073A, PI 567089A, PI 567132C and PI 567191 were not listed in the tables above. The cross of G00-3213 x PI 567191 was remade in the summer of 2014, as previous attempts to make this cross were unsuccessful. F_1 seed of the other five crosses was harvested in October of 2014 and depending on seed amounts the F1 seed was grown at the winter nursery in Puerto Rico or the University of Georgia winter greenhouse for a generation advancement to create F_2 mapping populations (Athens, GA). The PIs and their haplotypes (exact match) are as follows: PI 566984 (*Rpp[PI423972]* and *Rpp6/Rpp[PI567068A]*), PI567072A (*Rpp4* and *Rpp6/Rpp[PI567068A]*), PI 567089A (*Rpp4* and *Rpp6/Rpp[PI567068A]*), PI 567132C (*Rpp4* and *Rpp6/Rpp[PI567068A]*), PI 567191 (*Rpp4* and *Rpp6/Rpp[PI567068A]*), PI 566956 (*Rpp6/Rpp[PI567068A]*), and PI 567073A (*Rpp6/Rpp[PI567068A]*). All these PIs were collected from Indonesia in 1993 except for PI 567191, which was collected from Vietnam in 1992. These are not likely worth pursuing as the natural pyramids and *Rpp* gene haplotype matches may not bear novel gene discovery. These haplotype data support that natural pyramids of rust genes may be occur relatively frequently, especially the appearance of the *Rpp4* and *Rpp6* haplotypes together, which are both located on the opposite ends of Chr 18.

A second goal of this work was to introgress existing *Rpp* genes into an elite soybean genetic background to develop breeding lines with resistance to SBR. This has been a long-term goal since the invasion of *P. pachyrhizi* into the USA. The need for SBR resistance is especially warranted in the southeastern soybean production regions where yield losses and the use of pesticides have been greater than other regions of the USA, causing both a reliance on fungicides as well as environmental concerns due to fungicide use. For this reason the goal was to introgress well characterized single, dominant *P. pachyrhizi* resistance genes into the elite soybean cultivar G00-3213. G00-3213 is a MG VII soybean breeding line that was developed at

the University of Georgia and is well adapted agronomically to the southeastern USA and possesses high-yield potential. Four near-isogenic lines (NIL) of G00-3213 were developed and are referred to as G00-3213*Rpp1*, G00-3213*Rpp2*, G00-3213*Rpp3*, and G00-3213*Rpp4*. These germplasm lines were developed by backcrossing the rust resistance genes *Rpp1* (PI 547875 (L85-2378); a 'Williams 82' isoline containing *Rpp1* contributed by PI 200492), *Rpp2* (PI 230970), *Rpp3* (PI 462312), or *Rpp4* (PI 459025B) into G00-3213.

The NILs have tawny pubescence, tan pod walls, and white flowers, and the general appearance of G00-3213 in the field in 2014. Additionally, each NIL exhibits a similar level of resistance to the Georgia 2012 (GA12) *P. pachyrhizi* bulk isolate as the original resistant sources.

At least two KASP SNP marker assays were developed for marker assisted selection for each *Rpp* locus. These markers confirmed the integration and homozygosity of each respective *Rpp* gene haplotype in the backcrossing process. Seed of G00-3213*Rpp1*, G00-3213*Rpp2*, G00-3213*Rpp3*, or G00-3213*Rpp4* will be available through the USDA–ARS National Plant Germplasm System.

The last goal of this research was to provide a rapid, efficient, genotyping system for applied soybean breeding programs. The project was aimed to improve the marker-assisted selection workflow by developing and utilizing a reliable, robust, inexpensive, and high-throughput DNA extraction protocol for plant DNA from soybean seed chips or leaflet samples that did not generate hazardous waste. This was accomplished by modifying the procedures of Edwards et al. (1991) resulting in a DNA extraction buffer that consisted of 40% v/v 5M NaCl and 60% v/v extraction buffer 200 mM Tris-HCl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS. The DNA extraction protocol proved to be rapid and highly compatible with next generation SNP genotyping platforms including the SimpleProbe Assay and KASPar v4.0 SNP

Genotyping System (Chapter 2). It is now possible to routinely genotype thousands of seeds nondestructively using seed chips, or using small portions of a leaf in a single day at a success rate of approximately 95%. This methodology makes it possible to run up to 150 SNP markers on the DNA extracted from a single seed chip or leaf sample making it a robust technique for breeding programs. This technique proved successful in extracting DNA from alfalfa (*Medicago sativa* L.) and red clover (*Trifolium pratense* L.), and may be of use to other crops.

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Table 5.1: Ft. Detrick *P. pachyrhizi* isolate response data from Harris et al. (2015) updated with novel information from the mapping of new alleles from PI 423972 and PI 567068A.

		P. pachyrhizi reactions#									
PI§	Phenotyping results¶	CO04-2	HW98-1	IN73-1	LA04-1	SA01-1	TW72-1	ZM01-1	AU79-1	LA04-3	VT05-1
PI 423972	*Rpp[PI423972]* like	RB	RB	TAN§§	RB	RB	Mixed	RB	RB	RB	-
PI 476905A	Unique pattern	INT	RB	RB	RB	TAN	TAN	RB	TAN	RB	-
PI 567068A	*Rpp[PI567068A]* like	-	RB	-	RB	TAN	TAN‡	TAN	TAN	RB	-
PI 567090	*Rpp[PI567068A]* like††	TAN	RB	RB	Mixed	TAN	TAN	TAN	-	-	TAN
PI 567104B	*Rpp4* and *Rpp6* like†	RB	RB	RB	RB	RB	RB	RB	RB	RB	-
PI 567076	*Rpp[PI567068A]* like	INT	RB	-	RB	TAN	TAN	TAN	TAN	RB	-
PI 605791A	Unique pattern	TAN	TAN	-	RB	-	TAN	TAN	-	TAN	-
PI 200492	*Rpp1* like	TAN	IM	RB	IM	TAN	TAN	TAN	-	IM	TAN
PI 594538A	*Rpp1b* like	-	-	-	TAN	RB	RB	RB	RB	RB	-
PI 462312	*Rpp3* like	TAN	RB	RB	RB	RB	TAN	TAN	-	-	TAN
PI 506764	*Hyuuga* like	RB	RB	RB	RB	RB	TAN	RB	-	-	RB
PI 459025B	*Rpp4* like	RB	RB	RB	RB	RB	RB	RB§	RB	RB	-
PI 567102B	*Rpp6* like	RB	RB	RB	RB	RB	Mixed	RB	RB	RB	-

§PI, plant introduction ID from the USDA Germplasm Resources Information Network that has rust resistance.
†Only difference between PI 567104B and PI 567102B (*Rpp6*) is a RB or mixed reaction when challenged with TW72-1 *P. pachyrhizi* isolate, respectively.
††Only difference between PI 567068A and PI 567090 is a RB or mixed reaction when challenged with LA04-1 P. pachyrhizi isolate, respectively.
§§Only two plants were rated.
§One TAN plant in the first replication.
¶Data extrapolated from Harris et al. 2015.

#INT, intermediate lesion type characterized by a dark reddish-brown lesion type accompanied by smaller lesions with profuse sporulation; Mixed, the mixed lesion type was a mixture of both TAN and RB lesions on the same plant; RB, reddish-brown resistant lesions; TAN, tan susceptible lesions with profuse sporulation; and IM, immune, showing no visible signs of infection.

Table 5.2: Eighteen resistant plant introductions of the highest priority for gene or allele discovery are summarized below. Bulked segregant analysis assay (BSA) results and haplotype analysis at the *Rpp1*, *Rpp2, rpp2, Rpp3, Rpp4,* and *Rpp6* haplotypes was conducted at defined genomic locations. For haplotype analysis, missing data were ignored) and heterozygous SNP loci were scored as 50% for the given SNP to calculate the similarity.

						Haplotype similarity with respect to a known *Rpp* harboring PI‡						
PI§	Generation bulks created	Assay for BSA¶	BSA hits within 5cM of known genes	Phenotyped with a panel of isolates¶	Phenotyping results¶	PI 200492 (*Rpp1*)	PI 230970 (*Rpp2*)	PI 224270 (*rpp2*)	PI 462312 (*Rpp3*)	PI 459025B (*Rpp4*)	PI 423972 (*Rpp4[PI423 972]*)	PI 567102B (*Rpp6*) and PI *567068A Rpp[PI567068 A])*
PI 224270	F_2	Infinium	*Rpp2*	No	NA	0.61	0.82	1.00	0.58	0.20	0.40	0.33
PI 417125	F_2	Infinium	*Rpp2*	No	NA	0.50	0.81	0.95	0.58	0.20	0.40	0.33
PI 417126	F_2	Infinium	hits on several chr.	No	NA	0.50	0.81	0.98	0.33	0.20	0.40	0.67
PI 417129B	F_2	Infinium	hits on several chr.	No	NA	0.56	0.83	0.99	0.33	0.25	0.58	0.50
PI 423972	$F_{2:3}$ super bulk and population	Infinium	*Rpp4*	Yes	*Rpp[PI423972]* like	0.33	0.83	0.59	0.50	0.50	1.00	0.33
PI 476905A	$F_{2:3}$	GoldenGate	*Rpp6*	Yes	Unique pattern	0.44	0.72	0.75	0.50	0.67	0.50	1.00
PI 566956	No cross made	NA	NA	No	NA	0.44	0.92	0.77	0.54	0.50	0.33	1.00
PI 566984	No cross made	NA	NA	No	NA	0.33	0.89	0.67	0.50	0.50	1.00	1.00
PI 567068A	$F_{2:3}$ super bulk and population	Infinium	*Rpp6*	Yes	*Rpp[PI567068 A]* like	0.44	0.94	0.78	0.58	0.50	0.33	1.00
PI 567076	F_2	GoldenGate	*Rpp6*	Yes	*Rpp[PI567068 A]* like	0.44	0.88	0.77	0.58	0.50	0.33	1.00
PI 567090	F_2	GoldenGate	hits on several chr.	Yes	*Rpp[PI567068 A]* like††	0.56	0.89	0.68	0.58	0.50	0.33	1.00
PI 567104B	$F_{2:3}$	GoldenGate and Infinium	*Rpp4*/*Rpp6*	Yes	*Rpp4* and *Rpp6* like†	0.44	0.89	0.68	0.58	1.00	0.50	1.00
PI 567123A	F_2	Infinium	hits on several chr	No	NA	0.56	0.89	0.73	0.58	0.67	0.50	1.00

PI 567129	$F_{2:3}$ super bulk	Infinium	*Rpp6*	No	NA	0.56	0.89	0.68	0.58	0.50	0.33	1.00
PI 567188	No cross made	NA	NA	No	NA	0.56	0.83	0.90	0.50	0.50	1.00	0.33
PI 567189A‡	F_2	Infinium	*Rpp3*	No	NA	0.44	0.62	0.72	0.83	1.00	0.50	0.67
PI 594796	F_2	GoldenGate	Recessive gene? No hits	No	NA	0.33	0.88	0.71	0.33	0.20	0.40	0.67
PI 605791A	F_2	Infinium	*Rpp4*	Yes	Unique pattern	0.61	0.83	0.90	0.50	0.60	1.00	0.33
PI 605823	F_2	Infinium	*Rpp7*	No	NA	0.56	0.61	0.75	0.67	0.92	0.42	0.67
PI 200492	*Rpp1*	NA	Check	Yes	*Rpp1* like	1.00	0.83	0.81	0.50	0.20	0.40	0.67
PI 594538A	*Rpp1-b*	NA	Check	Yes	*Rpp1b* like	0.44	0.78	0.81	0.50	1.00	0.60	0.67
PI 230970	*Rpp2*	NA	Check	No	NA	0.56	1.00	0.90	0.58	0.67	0.50	0.67
PI 224270	*rpp2?*	NA	Check	No	NA	0.61	0.82	1.00	0.58	0.20	0.40	0.33
PI 462312	*Rpp3*	NA	Check	Yes	*Rpp3* like	0.22	0.67	0.52	1.00	0.40	0.40	0.67
PI 506764	*Rpp?[Hyuuga]*	NA	Check	Yes	*Hyuuga* like	0.42	0.64	0.87	0.83	0.42	0.42	0.50
PI 459025B	*Rpp4*	NA	Check	Yes	*Rpp4* like	0.78	0.39	0.41	0.50	1.00	0.50	0.67
PI 471904	*Rpp5*	NA	Check	No	NA	0.33	0.83	0.90	1.00	0.40	0.20	0.33
PI 200526	*Rpp5*	NA	Check	No	NA	0.33	0.44	0.61	0.42	0.40	0.20	0.33
PI 200487	*Rpp5*	NA	Check	No	NA	0.33	0.67	0.70	0.83	0.20	0.40	0.33
PI 200456	*rpp5?*	NA	Check	No	NA	0.44	0.94	0.91	0.42	0.33	0.50	0.33
PI 567102B	*Rpp6*	NA	Check	Yes	*Rpp6* like	0.56	0.89	0.73	0.58	0.50	0.33	1.00

§PI, plant introduction ID from the USDA Germplasm Resources Information Network that has rust resistance.
‡Exception for this PI where data does not come from Harris et al., 2015.
†Only difference between PI 567104B and PI 567102B (*Rpp6*) is a RB or mixed reaction when challenged with TW72-1 *P. pachyrhizi* isolate, respectively.
††Only difference between PI 567068A and PI 567090 is a RB or mixed reaction when challenged with LA04-1 *P. pachyrhizi* isolate, respectively.
¶Data extrapolated from Harris et al. 2015.
ǂData from USDA Germplasm Resource Information Network.

Not applicable (NA), was not tested or unknown.
#PIs with ≥0.95 haplotype similarity are highlighted in gray. Haplotype windows (SNP markers) were identified by Harris et al. (2015) for *Rpp1*, *Rpp3*, and *Rpp4*; and Yu et al., (2015) identified the flanking markers used for *Rpp2*. The *rpp2* haplotype of PI 224270 was established using the data of Garcia et al. (2008) as established by Harris et al. (2015). The *Rpp6/Rpp[PI567068A]* was established in this study.
¶Physical genomic locations correspond to the Wm82.a1 sequence and indicate the dbSNP location (e.g. ss715632302) available online at www.soybase.org/dlpages/index.php#snp50k (Song et al., 2013).

Table 5.3: Summary table of the 18 resistant plant introductions of the highest priority including bulked segregant analysis data, USDA Germplasm Resources Information Network data, phenotyping and haplotype results and maturity group.

PI§	BSA hits within 5cM of known genes	Phenotyping results¶	Haplotype similarity#	Cultivar‡	MG‡‡	Province‡	Country‡	Year‡	Comment
PI 224270	*Rpp2*	NA	*rpp2*	Howgyoku	VII	Hyogo	Japan	1955	This is the *rpp2* check, could pursue fine mapping for a publication
PI 417125	*Rpp2*	NA	*rpp2*	Kyushu 31	VIII	Kyushu	Japan	1977	Haplotype likeness of PI 224270 (*rpp2*), Kyushu Province
PI 417126	hits on several chr.	NA	*rpp2*	Kyushu 32	VIII	Kyushu	Japan	1977	Two hits flank *Rpp3* on chr. 6, haplotype likeness of PI 224270 (*rpp2*), Kyushu Province
PI 417129B	hits on several chr.	NA	*rpp2*	(Kyushu 40)	IX	Kyushu	Japan	1977	This could be a new gene on chr. 15, or a new allele near *Rpp3* on chr. 6, Haplotype likeness of PI 224270 (*rpp2*), Kyushu Province
PI 423972	*Rpp4*	*Rpp[PI423972]* like	*Rpp4[PI423972]*	Takema	IX	Kumamoto	Japan	1978	Novel *Rpp[PI423972]* allele
PI 476905A	*Rpp6*	Unique pattern	*Rpp6/Rpp[PI567068A]*	Nguu mao hong	V	unknown	China	1983	Three BSA hits are near or within the *Rpp6* interval, *Rpp6/Rpp[PI567068A]* haplotype, unique phenotypic pattern
PI 566956	NA	NA	*Rpp6/Rpp[PI567068A]*	NA	IX	East Java	Indonesia	1993	*Rpp6/Rpp[PI567068A]* haplotype, no cross made
PI 566984	NA	NA	*Rpp6/Rpp[PI567068A]* and *Rpp4[PI423972]*	NA	VI	unknown	Indonesia	1993	May be a natural pyramid of *Rpp6/Rpp[PI567068A]* and *Rpp4[PI423972]*
PI 567068A	*Rpp6*	*Rpp[PI567068A]* like	*Rpp6/Rpp[PI567068A]*	NA	VII	East Java	Indonesia	1993	New *Rpp[PI567068A]* allele at the *Rpp6* locus
PI 567076	*Rpp6*	*Rpp[PI567068A]* like	*Rpp6/Rpp[PI567068A]*	NA	VII	East Java	Indonesia	1993	*Rpp6/Rpp[PI567068A]* haplotype and *Rpp[PI567068A]* like for phenotyping
PI 567090	hits on several chr.	*Rpp[PI567068A]* like††	*Rpp6/Rpp[PI567068A]*	NA	IX	East Java	Indonesia	1993	Two hits on Gm18 over 44.053, 55.603 cM and has the *Rpp6/Rpp[PI567068A]* haplotype, phenotypes like PI567068A††

PI 567104B	*Rpp4*/*Rpp6*	*Rpp4* and *Rpp6* like†	*Rpp6/Rpp[PI567068A]* and *Rpp4*	NA	IX	East Java	Indonesia	1993	May be a natural pyramid of *Rpp6/Rpp[PI567068A]* and *Rpp4*; phenotypes like *Rpp4* and *Rpp6*†
PI 567123A	hits on several chr.	NA	*Rpp6/Rpp[PI567068A]*	NA	VIII	East Java	Indonesia	1993	BSA data needs to be re-analyzed, has the *Rpp6/Rpp[PI567068A]* haplotype
PI 567129	*Rpp6*	NA	*Rpp6/Rpp[PI567068A]*	NA	IX	East Java	Indonesia	1993	Mapped to chr. 18 5,510,449-11,964,908 bp, this is a *Rpp6/Rpp[PI567068A]* line
PI 567188	NA	NA	*Rpp4[PI423972]*	VX 9-3	VI	unknown	Vietnam	1992	Haplotype likeness of *Rpp[PI423972]*, has not been mapped
PIPI 567189A‡	*Rpp3*	NA	*Rpp4*	Ekhabac	IV	unknown	Vietnam	1992	Has the *Rpp4* like haplotype, yet maps to *Rpp3*
PI 594796	Recessive gene? No hits	NA	Unknown	Xi bai dou	VIII	Yunnan	China	1996	Likely a new gene or allele
PI 605791A	*Rpp4*	Unique pattern	*Rpp4[PI423972]*	NA	VI	Cao bang	Vietnam	1998	Same haplotype as PI 423972 at *Rpp4,* yet has a unique phenotpyic reaction
PI 605823	*Rpp7*	NA	Unknown	NA	IX	Ha giang	Vietnam	1998	This resistance maps to ch. 19 and therefore is a new gene entirely
PI 200492	Check	*Rpp1* like	*Rpp1*	Komata	VII	Shikoku	Japan	1952	NA
PI 594538A	Check	*Rpp1b* like	NA/*Rpp4*	Min hou bai sha wan dou	IX	Fujian	China	1996	NA
PI 230970	Check	NA	NA	NA	VII	unknown	Japan	1956	NA
PI 224270	Check	NA	*rpp2*	Howgyoku	VII	Hyogo	Japan	1955	NA
PI 462312	Check	*Rpp3* like	*Rpp3*	Ankur	VIII	Uttar Pradesh	India	1981	NA
PI 506764	Check	*Hyuuga* like	NA	Hyuuga	VII	Kyushu	Japan	1986	NA
PI 459025B	Check	*Rpp4* like	*Rpp4*	(Bing nan)	VIII	Fujian	China	1981	NA
PI 471904	Check	NA	*Rpp3*	Orba	IX	Java	Indonesia	1982	NA
PI 200526	Check	NA	NA	Shira Nuhi	VIII	Shikoku	Japan	1952	NA
PI 200487	Check	NA	NA	Kinoshita	VIII	Shikoku	Japan	1952	NA
PI 200456	Check	NA	NA	Awashima Zairai	VIII	Shikoku	Japan	1952	NA

PI 567102B	Check	*Rpp6* like	*Rpp6/Rpp[PI567068A]*	NA	IX	East Java	Indonesia	1993	NA

§PI, plant introduction ID from the USDA Germplasm Resources Information Network that has rust resistance.
‡Exception for this PI where data does not come from Harris et al., 2015.
‡‡MG, maturity group.
†Only difference between PI 567104B and PI 567102B (Rpp6) is a RB or mixed reaction when challenged with TW72-1 *P. pachyrhizi* isolate, respectively.
††Only difference between PI 567068A and PI 567090 is a RB or mixed reaction when challenged with LA04-1 *P. pachyrhizi* isolate, respectively.
¶Data extrapolated from Harris et al. 2015.
#INT, intermediate lesion type characterized by a dark reddish-brown lesion type accompanied by smaller lesions with profuse sporulation; Mixed, the mixed lesion type was a mixture of both TAN and RB lesions on the same plant; RB, reddish-brown resistant lesions; TAN, tan susceptible lesions with profuse sporulation.
ǂData from USDA Germplasm Resource Information Network.
Not applicable (NA), was not tested or unknown.

Table 5.4: The haplotype of the *rpp2* locus of PI224270 is defined by 174 SNPs and represents the physical distance between simple sequence repeat markers Satt215 and Sat_361 used by Garcia et al. (2008) to map *rpp2*.

dbSNP ID at Chr 16†	SNP position¶	SNP Type	PI230970 (*Rpp2*)‡	PI224270 (*rrp2*)
ss715624003	28,581,875	T/C	C	C
ss715624004	28,613,278	C/T	T	T
ss715624005	28,615,587	C/T	T	T
ss715624007	28,628,824	T/C	T	T
ss715624008	28,640,139	G/A	G	G/A
ss715624011	28,647,125	T/G	T	T
ss715624012	28,649,867	C/T	C	-
ss715624013	28,657,775	A/C	A	A
ss715624015	28,676,425	T/C	C	C
ss715624016	28,684,601	G/A	G	G
ss715624017	28,685,379	A/G	A	A
ss715624018	28,696,077	T/C	T	T
ss715624021	28,706,800	A/G	G	G
ss715624022	28,711,579	C/A	C	C
ss715624024	28,719,941	C/T	T	T
ss715624025	28,722,092	T/C	T	T
ss715624026	28,725,647	T/C	C	C
ss715624027	28,726,638	T/C	C	C
ss715624028	28,729,744	G/A	A	A
ss715624030	28,734,825	G/T	G	T
ss715624031	28,740,712	A/G	A	G
ss715624033	28,743,248	C/T	C	C
ss715624034	28,748,225	A/G	G	G
ss715624035	28,751,580	T/C	T	T
ss715624036	28,752,556	T/C	T	T
ss715624037	28,753,577	A/G	A	A
ss715624038	28,763,451	G/A	G	G
ss715624039	28,764,192	G/T	G	T
ss715624040	28,766,686	A/G	A	G
ss715624041	28,767,867	A/G	A	A
ss715624042	28,792,833	T/C	T	T
ss715624043	28,801,593	T/C	C	T
ss715624049	28,821,455	T/C	T	T
ss715624050	28,824,975	A/G	G	A
ss715624051	28,826,442	G/A	G	G
ss715624053	28,835,949	C/T	T	C

ss715624054	28,837,216	T/C	C	T
ss715624055	28,845,819	A/G	A	A
ss715624056	28,851,123	G/A	G	G
ss715624057	28,853,826	C/T	C	C
ss715624058	28,856,157	T/C	T	T
ss715624059	28,860,419	G/T	G	G
ss715624060	28,867,390	T/C	T	T
ss715624061	28,868,599	A/G	A	A
ss715624063	28,869,761	T/G	T	T
ss715624064	28,873,484	G/A	G	G
ss715624066	28,882,177	T/G	T	T
ss715624068	28,888,316	T/C	T	T
ss715624069	28,889,131	G/A	G	G
ss715624073	28,901,653	G/A	G	G
ss715624074	28,918,158	C/A	C	C
ss715624075	28,920,227	T/C	C	T
ss715624078	28,938,128	G/A	G	G
ss715624080	28,941,919	A/G	G	A
ss715624081	28,946,006	C/T	T	C
ss715624083	28,954,377	G/A	G	G
ss715624087	28,961,127	T/G	T	T
ss715624089	28,977,024	A/G	A	A
ss715624091	28,983,507	C/T	C	C
ss715624099	29,044,334	G/A	G	G
ss715624103	29,058,163	C/A	A	A
ss715624104	29,060,367	G/A	A	A
ss715624106	29,065,923	A/G	G	G
ss715624108	29,084,869	A/G	G	-
ss715624109	29,086,073	C/T	T	T
ss715624110	29,087,815	A/G	G	G
ss715624112	29,090,967	G/A	A	A
ss715624113	29,097,420	T/C	T	T
ss715624114	29,104,488	G/A	G	G
ss715624116	29,116,854	G/T	T	T
ss715624117	29,118,839	C/T	C	C
ss715624120	29,124,881	T/C	T	T
ss715624122	29,128,926	T/G	T	T
ss715624123	29,130,014	A/C	C	A/C
ss715624124	29,132,435	T/C	T	T
ss715624125	29,135,922	C/T	C	-
ss715624127	29,144,428	G/A	A	A

ss715624129	29,148,488	G/A	G	-
ss715624130	29,150,479	A/G	A	A
ss715624131	29,153,474	C/T	C	C
ss715624133	29,157,749	T/C	T	T
ss715624134	29,161,177	C/T	C	C
ss715624135	29,164,813	G/A	G	G
ss715624136	29,171,255	A/G	A	A
ss715624140	29,181,015	G/T	G	G
ss715624141	29,184,867	C/T	C	C
ss715624142	29,188,738	T/C	T	T
ss715624144	29,194,555	A/G	A	A
ss715624145	29,197,284	C/T	C	C
ss715624146	29,198,889	A/C	C	C
ss715624147	29,204,603	A/G	A	A
ss715624148	29,209,054	C/T	T	C
ss715624149	29,211,869	G/A	A	A
ss715624151	29,215,338	C/T	C	C
ss715624152	29,217,057	A/C	A	A
ss715624153	29,218,412	T/C	T	T
ss715624156	29,238,255	A/G	G	G
ss715624157	29,240,286	C/T	C	C
ss715624158	29,242,023	A/C	A	A
ss715624159	29,247,106	C/A	C	C
ss715624162	29,252,696	A/G	A	A
ss715624163	29,253,523	G/A	A	A
ss715624165	29,261,393	C/A	C	C
ss715624166	29,266,226	A/G	A	A
ss715624172	29,304,708	A/C	C	C
ss715624173	29,307,050	G/T	T	T
ss715624174	29,308,494	C/A	C	C
ss715624175	29,313,914	G/A	A	A
ss715624177	29,322,493	T/C	T	T
ss715624178	29,340,661	C/T	T	T
ss715624180	29,354,204	A/G	A	A
ss715624181	29,356,240	G/A	A	A
ss715624183	29,371,179	T/C	C	C
ss715624185	29,387,262	T/C	C	C
ss715624188	29,466,150	A/G	G	G
ss715624191	29,518,026	C/T	C	C
ss715624192	29,528,259	T/C	C	C
ss715624196	29,583,934	T/C	T	T

ss715624197	29,590,462	C/T	C	C
ss715624198	29,595,845	G/A	G	G
ss715624199	29,597,918	A/G	G	G
ss715624201	29,666,971	T/C	C	C
ss715624202	29,679,156	G/A	G	G
ss715624203	29,681,065	T/G	G	G
ss715624206	29,715,733	C/A	C	C
ss715624207	29,716,764	A/G	G	G
ss715624208	29,717,973	A/G	G	G
ss715624209	29,718,880	C/T	C	C
ss715624210	29,720,274	A/G	G	G
ss715624211	29,735,297	G/A	A	A
ss715624212	29,776,694	G/A	G	G
ss715624216	29,839,656	A/G	A	A
ss715624217	29,842,937	T/C	C	C
ss715624218	29,867,049	A/G	G	G
ss715624219	29,871,768	C/T	T	C
ss715624223	29,895,378	T/G	G	G
ss715624225	29,905,411	C/T	T	T
ss715624226	29,907,252	T/C	C	C
ss715624227	29,922,186	A/G	A	G
ss715624228	29,930,067	A/G	A	A
ss715624229	29,937,298	A/G	A	A
ss715624230	29,939,737	C/T	C	C
ss715624232	29,942,185	T/G	T	T
ss715624233	29,951,731	G/A	G	G
ss715624235	29,961,485	A/G	G	G
ss715624236	29,964,819	A/G	G	G
ss715624238	29,970,167	C/T	C	C
ss715624241	29,985,920	A/G	A	A
ss715624244	29,994,726	G/A	G	G
ss715624245	29,995,455	C/T	C	C
ss715624248	30,002,009	C/T	C	C
ss715624250	30,005,122	A/G	G	A/G
ss715624251	30,008,893	A/G	G	G
ss715624252	30,011,505	G/T	T	T
ss715624253	30,012,908	C/T	C	C
ss715624254	30,018,533	T/C	T	T
ss715624256	30,022,654	C/T	T	T
ss715624257	30,024,746	T/C	T	T
ss715624259	30,032,344	T/G	G	G

ss715624260	30,033,613	C/T	T	T
ss715624261	30,036,170	A/G	A	A
ss715624262	30,038,668	G/T	T	G
ss715624264	30,041,222	T/C	T/C	C
ss715624265	30,042,058	A/G	G	G
ss715624266	30,043,271	A/G	G	-
ss715624267	30,065,532	G/T	T	T
ss715624269	30,069,110	G/T	T	T
ss715624270	30,075,422	T/C	T	T
ss715624271	30,080,668	C/T	C	C
ss715624272	30,089,800	A/G	G	G
ss715624273	30,096,338	T/C	C	C
ss715624274	30,097,921	A/C	C	C
ss715624277	30,108,889	C/A	C	C
ss715624278	30,113,002	G/T	G	G

-: Missing data.
†dbSNP ID of chromosome 16 are available at www.soybase.org/dlpages/index.php#snp50k (Song et al. 2013).
¶Physical genomic locations correspond to the Wm82.a1 sequence of the SNP available online at www.soybase.org/dlpages/index.php#snp50k.
‡Shared haplotype alleles between PI230970 (*Rpp2*).