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A new soybean rust resistance allele from PI 423972 at the *Rpp4* locus

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Abstract *Phakopsora pachyrhizi* is a fungal pathogen and the cause of Asian soybean rust. *P. pachyrhizi* was first detected in the continental USA in 2004 and has since been a threat to the soybean industry. There are six described loci that harbor resistance to *P. pachyrhizi* (*Rpp*) genes. The resistance of PI 423972 was previously shown to be within 5 cM of the *Rpp4* locus of PI 459025B, yet had differential reactions when challenged with *P. pachyrhizi* isolates India 1973 and Taiwan 1972. In this study, the resistance of PI 423972 was mapped to a 187.5 kb interval between the SNP markers GSM0543 and GSM0387 on chromosome 18 (51,397,064 to 51,584,617 bp, Glyma.Wm82.a2) that overlaps the interval for *Rpp4* and is designated as *Rpp4-b*. A unique haplotype is described for PI 423972 that separates it from PI 459025B, 32 North American soybean ancestors, and all described sources of *Rpp* gene resistance.

Keywords Bulked segregant analysis (BSA) · Haplotype analysis · Linkage mapping · Resistance to *Phakopsora pachyrhizi* (*Rpp*) gene · Single-nucleotide polymorphism (SNP) · Soybean rust (SBR)

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Introduction

Asian soybean rust (SBR) is caused by *Phakopsora pachyrhizi* Syd. & P. Syd. *P. pachyrhizi* is an obligate, fungal pathogen that was first detected in the continental USA in 2004 (Schneider et al. 2005). This pathogen has the potential to reduce yield by 30 to 75% (Bromfield 1984; Kumudini et al. 2008; Yorinori et al. 2005) and infection can result in reduced numbers of pods, lower oil content, and higher rates of seed abortion (Bromfield 1984). In Brazil, costs of fungicide applications and yield losses from SBR have averaged US $1.98 billion/year from 2004 to 2014 (Godoy et al. 2016). In the USA, SBR is a problem in the southern states of Mississippi, Arkansas, Alabama, and Georgia (SBR.IPMPIPE.ORG), but yield losses have been limited, primarily due to unfavorable environmental conditions in many growing seasons for the spread and reproduction of *P. pachyrhizi* (Rosa et al. 2015). Despite this, fungicide usage for soybean has greatly increased since the arrival of SBR in North America, and annual fungicide costs for SBR control have averaged US

$2.22 million (2005–2013) in Georgia alone (Langston 2009; Martinez-Espinoza 2006, 2007, 2008, 2015; Woodward 2010, 2012, 2013, 2015).

In order to manage soybean rust, host plant resistance to *P. pachyrhizi* (*Rpp* genes) is a useful tool. *Rpp* genes interact with specific pathotypes of *P. pachyrhizi* and provide either an immune response (IM, no visible sign of infection) or a reddish-brown resistant-type lesion (RB, non-sporulating or reduced sporulation, depending on pathotype virulence and environmental conditions) as compared to the TAN reaction (susceptible with many uredinia and copious amounts of urediniospores) produced by plants with no *Rpp* genes (Bromfield 1984).

There are six described *Rpp* loci to date. *Rpp1* and *Rpp1-b* were discovered in PI 200492 and PI 594538A, respectively, on chromosome (Chr) 18 (McLean and Byth 1980; Chakraborty et al. 2009; Hyten et al. 2007). *Rpp2* was identified in PI 230970 on Chr 16 (Hartwig and Bromfield 1983; Silva et al. 2008; Yu et al. 2015). A recessive source of resistance, *rpp2*, that produced a different reaction to a panel of *P. pachyrhizi* isolates compared to *Rpp2* was found in PI 224270 (Garcia et al. 2008; Yamanaka et al. 2015). *Rpp3* from PI 462312 was mapped to Chr 6 (Hartwig and Bromfield 1983; Hyten et al. 2009). *Rpp4* was discovered in PI 459025B, mapping approximately 26 cM from *Rpp1* on Chr 18 (Garcia et al. 2008; Hartwig 1986; Silva et al. 2008). *Rpp5* was identified in PI 200526, PI 200487, and PI 471904 and a recessive allele *rpp5* in PI 200456 on Chr 3 (Garcia et al. 2008; Pierozzi et al. 2008). *Rpp6* was mapped to Chr 18 in PI 567102B, approximately 40 cM from the *Rpp4* locus (Li et al. 2012), and a different allele or tightly linked gene, *Rpp*[*PI567068A*], was mapped in PI 567068A to the same locus (King et al. 2015).

No single *Rpp* gene has been shown to provide resistance to all known *P. pachyrhizi* pathotypes (Bonde et al. 2006; Paul and Hartman 2009; Pham et al. 2009). In addition, *P. pachyrhizi* populations vary widely by location and can overcome resistance genes over time (Hartman et al. 2005; Paul et al. 2015). In South America, the resistance of *Rpp1* and *Rpp3* were quickly overcome and the resistance of *Rpp2* and *Rpp4* is only effective against about one-third of rust pathotypes but *Rpp1-b* and *Rpp5* continue to provide good resistance (Akamatsu et al. 2012; TMG 2016; Yamanaka et al. 2010, 2016). In the USA, *Rpp1*, *Rpp2*, *Rpp3*, *Rpp4*, and *Rpp6* each have provided good resistance to field populations of SBR in most years (Walker et al. 2011, 2014). However, additional *Rpp* genes or alleles are needed to provide new sources of resistance and be introgressed into elite cultivars (Harris et al. 2015).

The recent advancements in soybean sequencing and the release of SoySNP50K iSelect BeadChips have provided soybean researchers with a wealth of tools for genomic analysis (Schmutz et al. 2010; Song et al. 2013). The SoySNP50K chips allow for the rapid comparison of polymorphisms between genotypes as well as relatedness comparisons based on the 50 K SNPs across all 20 chromosomes. Haplotype analysis of disease resistance loci enables the prediction of the alleles present in an accession based on its shared ancestry with known sources of resistance (Harris et al. 2015).

P. pachyrhizi populations display considerable variation among locations, possibly due to local selection pressures and the rapid evolution of the species from hyphal anastomosis which leads to heterokaryosis, nuclear fusion, and genetic recombination (Paul et al. 2015; Vittal et al. 2011). In addition, *P. pachyrhizi* urediniospores are spread by wind currents and can travel hundreds of miles, possibly introducing new pathotypes to existing populations (Rocha et al. 2015; Twizeyimana and Hartman 2012). SBR isolates, which represent a *P. pachyrhizi* collection at a specific location and year, have been collected internationally and maintained by the United States Department of Agriculture Agricultural Research Service (USDA-ARS) Foreign Disease–Weed Science Research Unit (FDWSRU) in a Biosafety Level 3 (BSL-3) plant pathogen containment facility at Ft. Detrick, MD since 1972 (Melching et al. 1983). Screening soybean accessions with a panel of these isolates provides differential reactions that have been used to discover new resistance alleles (Harris et al. 2015; Kendrick et al. 2011; King et al. 2015).

The *Rpp4* gene contributed by PI 459025B has been fine mapped to a 55.3 kb region on Chr 18 (51,511,484–51,566,780 bp, Glyma.Wm82.a2) and a candidate gene, *Rpp4C4*, has been identified in PI 459025B (Meyer et al. 2009). We report the discovery of a new allele at the *Rpp4* locus in PI 423972.

Materials and methods

Population development

The SBR resistance gene in PI 423972 was selected for genetic mapping based on the previous work of Harris

et al. (2015), which demonstrated using bulk segregant analysis that the resistance of PI 423972 was within 5 cM of the *Rpp4* locus of PI 459025B. PI 423972 had two differential reactions to the *P. pachyrhizi* isolates IN73-1 (Pantnagar, India, 1973) and TW72-1 (Taipei, Taiwan, 1972) when compared with PI 459025B. Pham et al. (2009) also reported differential reactions between PI 459025B and PI 423972 to *P. pachyrhizi* isolates AL04-3 (Baldwin Co., Alabama, USA, 2004) and BZ01-1 (Parana, Brazil, 2001). Additionally, PI 423972 shares only 50% similarity to the *Rpp4* haplotype (Harris et al. 2015).

A cross was made between Prichard and PI 423972 to create a genetic mapping population. PI 423972 (Takema) is a maturity group (MG) IX soybean landrace that was collected from Kumamoto, Japan in 1976 (ARS-GRIN.GOV) and produces a RB-resistant response to the Georgia 2012 (GA12) *P. pachyrhizi* bulk isolate. Prichard is a MG VIII cultivar with white flowers and gray pubescence (Boerma et al. 2001) and is susceptible to the GA12 *P. pachyrhizi* bulk isolate (Fig. 1). The cross was made in the summer of 2009 in Athens, GA at the University of Georgia (UGA) Plant Sciences Farm. The F_1 seed was grown in the UGA greenhouse in Athens, GA in the winter (2010–2011) to create the F_2 population. The F_2 population was advanced at the Plant Sciences Farm in the summer of 2011. Each F_2 plant was single plant threshed to generate 140 $F_{2:3}$ families for genetic mapping of resistance.

Phenotyping for rust resistance

The rust phenotyping method of $F_{2:3}$ populations has been described in detail by Harris et al. (2015). Briefly, for the Prichard × PI 423972 $F_{2:3}$ population, one family was planted into a black plastic 15-cell tray, of which only the outside 12 cells had pots (Griffin Greenhouse Supplies, Inc., Tewksbury, MA) placed in them and the center three cells were left open for light penetration to reduce crowding. Farfard® 3B potting mix (Sun Gro Horticulture, Agawam, MA, USA) was used to fill each pot and three seeds were planted per pot, with a total of six pots planted per family as well as per each of the parental checks. Plants were later thinned to two plants per pot, but due to variable germination, 8 to 18 plants per family were available for rating. Each parental check was replicated in the experiment for a total of four times.

Seedlings were grown in the greenhouse for 2 weeks and then inoculated with the GA12 bulk *P. pachyrhizi* isolate. The GA12 bulk isolate that has been described by Harris et al. (2015) was collected from soybean and kudzu leaves with natural SBR infection in 2012 and has been maintained since on susceptible check 'Cobb' plants in a greenhouse (Hartwig and Jamison 1975; Harris et al. 2015). Bulk *P. pachyrhizi* isolates derived from the field have been used to previously map rust genes effectively (Garcia et al. 2008 [*rpp5* and *Rpp5*]; King et al. 2015 [*Rpp*(*PI567068A*)]; Monteros et al. 2007 [*Rpp?*(*Hyuuga*)]; Silva et al. 2008 [*Rpp2* and *Rpp4*]). A randomized complete block experiment with three replications was conducted to verify that the 2012 isolate had consistent results with previous isolates collected in Georgia using the PI sources of *Rpp1* (PI 200492), *Rpp2* (PI 230970), *Rpp3* (PI 462312), *Rpp4* (PI 459025B), *Rpp5* (PI 200526), *Rpp*?(*Hyuuga*) (PI 506764), and the susceptible check, G00-3880. Each time the assay was conducted, the results showed that each PI responded with the expected reaction pattern as compared to previous isolates (Harris et al. 2015). The GA12 bulk isolate is tested yearly to ensure that the bulk isolate has not changed.

Inoculations were performed as per Harris et al. (2015). Plants were grown for an additional 2 weeks post GA12 inoculation to develop disease symptoms and all controls and parental checks were evaluated for their SBR reaction phenotypes. Families were scored as susceptible (TAN) or resistant (RB) based on the reaction of the 8 to 18 plants per family. To classify each $F_{2:3}$ family, a method similar to King et al. (2015) was used. A family was designated as susceptible if at least 80% of the individuals were rated as susceptible (TAN). If 100% of the individuals were resistant (RB) in a family, that family was designated as homozygous resistant. All other families were designated as segregating or heterozygous. The appropriateness of this classification was verified using the expected 1:2:1 segregation ratio for the F_2 generation of a single gene (Table 1). In the case of scoring individual plants as RB (resistant) or TAN (susceptible), we observed discrete differences between these reactions illustrated by the PI parents in Fig. 1, whereby little to no sporulation was ever observed on RB resistant progeny. This is similar to the results reported by King et al. (2015) and Harris et al. (2015) using bulk *P. pachyrhizi* isolates collected in Georgia. Six families were later excluded due to an unexpected phenotype, as they were designated as heterozygous but were expected to be susceptible based on the genotyping scores. The few plants that appeared as resistant are

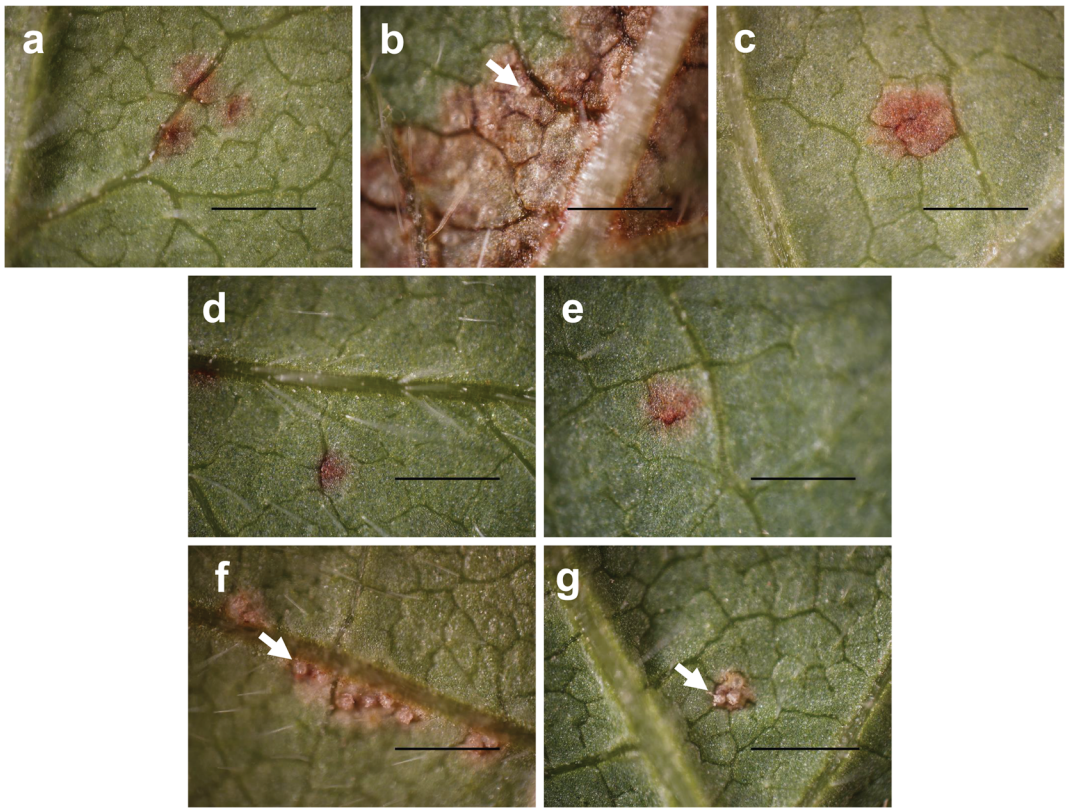

Fig. 1 Phenotypic reactions of selected lines challenged by the GA12 *P. pachyrhizi* bulk isolate. **a** PI 423972, **b** PI 459025B (*Rpp4*), **c** PI 605791A, **d** PI 566984, **e** PI 567188, **f** Prichard, and **g** G00-3213. PI 459025B (**b**) produced RB lesions that occasionally sporulated. The G00-3213 and Prichard genotypes produced TAN, highly sporulating lesions (**f, g**). PI 423972 (**a**), PI 605791A (**c**), PI 566984 (**d**), and PI 567188 (**e**) all produced similar RB resistant lesions that did not produce urediniospores. The presence of *P. pachyrhizi* urediniospores are indicated by *white arrows*. *Bar* = 1 mm

thought to be the escapes of the controlled inoculation of GA12.

Evaluation of a panel of lines

The GA12 *P. pachyrhizi* bulk isolate was used to challenge PI 459025B (*Rpp4*), PI 423972, susceptible controls Prichard and G00-3213, and resistant checks PI 200492 (*Rpp1*) and PI 594538A (*Rpp1-b*). PI 605791A was included in the evaluation as Harris et al. (2015) reported that it has an *Rpp* gene within 5 cM of the *Rpp4* locus. PI 567188 and PI 566984 were also included as they have an unknown resistance locus but share a similar haplotype to PI 423972. The planting, growth conditions, and inoculation were performed as above where each accession was planted into six pots total in half of a tray. The lines were evaluated for SBR reaction phenotypes in May 2015 (Fig. 1; Table 2).

Evaluation of accessions with a panel of international *P. pachyrhizi* isolates

In order to determine if PI 423972 harbored a different *Rpp* allele from PI 459025B (*Rpp4*), a panel of international isolates was used to test the PIs for their SBR reaction phenotypes. Additionally, PI 200492 (*Rpp1*) and PI 594538A (*Rpp1-b*) were tested since the *Rpp1* locus is near the *Rpp4* locus on Chr 18. Williams 82 (PI 518671) was included as a susceptible control. Harris et al. (2015) previously screened these lines with nine international isolates and found differential reactions between PI 459025B and PI 423972 for the isolates TW72-1 and IN73-1. The other isolates used produced an RB reaction for both PI 459025B and PI 423972. The isolates TW72-1 and IN73-1 were retested in this study to verify the differential reaction that was previously observed. Pham et al. (2009) also found differential reactions between PI 459025B and PI 423972 for the

Table 1 Chi-square analysis of 134 $F_{2:3}$ families of the Prichard × PI 423972 population. Plants were phenotyped for resistance after being challenged with the GA12 bulk *P. pachyrhizi* isolate. The *Rpp* gene contributed by PI 423972 was expected to segregate as a single dominant gene

Prichard × PI 423972 $F_{2:3}$ χ^2 analysis						
	Number of plants					
Generation	R	H	S	Total	Expected segregation	χ^2
$F_{2:3}$[a]	35	66	33	134 families	1:2:1	0.09 NS
F_3[b]	604	–	207	811 plants	3:1	0.12 NS

[a] Phenotypic reactions of each family were considered homozygous resistant (R), heterozygous (H), or susceptible (S)

[b] F_3 plants were from segregating F_2 families, as deduced using $F_{2:3}$ progeny testing. Homozygous and heterozygous resistant plants were indistinguishable and were denoted as (R)

isolates AL04-3 and BZ01-1, but these isolates were not retested in this study.

Isolate reaction tests for TW72-1 and IN73-1 were performed at the USDA-ARS FDWSRU in April, 2014. Experimental details have been described by Harris et al. (2015). In short, each line was tested with TW72-1 and IN73-1 with a total of four biological replicates. Each isolate was tested separately using a randomized complete block design. A biological replicate consisted of two seedlings of a given line in a single pot, and pots were randomized in trays. Seedlings were inoculated with isolates after plants were grown for 3 weeks in the greenhouse. At that time, all plants were transferred to a BSL-3 plant pathogen containment facility for inoculation and inoculated with TW72-1 and IN73-1 as previously described by Harris et al. (2015). Two weeks after inoculation, seedlings were scored as TAN, RB, IM, or INT (intermediate, reddish-brown but relatively smaller with uredinia and urediniospores present) (Table 2).

Bulked segregant analysis

Once phenotyped, a leaflet was collected from each plant of an $F_{2:3}$ family from the cross of Prichard × PI 423972, for a total of 8 to 18 plants per family. Leaflets were combined into family bulks, lyophilized for 36 h, and subsequently ground into a fine powder using a GenoGrinder (SPEX, NJ, USA).

DNA was extracted from leaf powder as per the CTAB protocol of Keim et al. (1988). DNA samples for genotyping were diluted to 10 to 20 ng μL^{-1}. For

Table 2 *Phakopsora pachyrhizi* isolate reactions of sources of resistance that map near the *Rpp4* locus and susceptible checks

	Phakopsora pachyrhizi isolate reactions[a]				
	AL04-3[b]	BZ01-1[b]	GA12[c]	IN73-1	TW72-1
PI 518671 (Williams 82)	TAN	TAN	TAN	TAN	TAN
PI 200492 (*Rpp1*)	TAN	TAN	IM	RB/IM[d]	TAN
PI 594538A (*Rpp1-b*)	--	--	MIX	RB/IM[d]	RB
PI 459025B (*Rpp4*)	RB	RB	RB	RB	RB
PI 423972 (*Rpp4-b*)	TAN	TAN	RB	TAN	MIX
PI 612157 (Prichard)	--	--	TAN	--	--

[a] Reaction types are as follows: TAN, susceptible reaction with profuse sporulation; RB, reddish-brown resistance reaction; IM, immune no visible sign of infection; MIX, both RB and TAN lesions present; − (not tested). The gray highlights the isolate reactions similar to *Rpp4*

[b] Reaction data taken from Pham et al. (2009)

[c] The GA12 bulk isolate was collected from field-grown kudzu and soybean in 2012

[d] Plants had either immune or reddish-brown lesions

bulked segregant analysis (BSA; Michelmore et al. 1991), only families showing no segregation were selected; in this way, 35 homozygous resistant and 22 homozygous susceptible families were selected. For each respective bulk, equal amounts of tissue from each family were pooled and homogenized to create two bulks. DNA was extracted from each bulk separately in the same manner as described earlier, and diluted to 75 ng μL^{-1}. Resistant and susceptible bulks were genotyped in the Soybean Genetics Lab at Michigan State University in East Lansing, MI using the SoySNP50K iSelect SNP BeadChips (Song et al. 2013). GenomeStudio V2011.1 software was used to call the genotypes (Illumina, San Diego, USA). SoySNP50K data of Prichard and PI 423972 were obtained from SOYBASE.ORG (Song et al. 2013). Positive BSA hits were scored when the resistant parent (PI 423972) had the same SNP genotype as the resistant bulk (e.g., both AA) and the susceptible parent (Prichard) genotype was the same as the susceptible bulk (e.g., both GG).

SNP assay design and genotyping

Polymorphic SNPs were identified between Prichard and PI 423972 within the interval identified with BSA. Ten KASP (LGC Genomics, Middlesex, UK) markers were developed using the criteria established by the KASP user guide and manual (LGC Genomics 2013) (Supplementary Table 1). Genotyping was performed using the protocol reported by Pham et al. (2013) for master mix preparation and thermocycling conditions. Endpoint genotyping was completed using a Roche LightCycler 480 II with LightCycler® Software (Roche Diagnostics Corporation, Indianapolis, IN) or a Tecan M1000 Pro Infinite Reader (Tecan Group Ltd., Männedorf, Switzerland) with KlusterCaller software (KBiosciences, Hoddesdon, UK). Allele calls that appeared as ambiguous were called as missing data.

Linkage mapping

The 134 $F_{2:3}$ families of the Prichard × PI 423972 population were genotyped using the KASP SNP markers developed in this study (Supplementary Table 1). MAPMAKER software (Lander et al. 1987) was used to analyze linkage of the trait and markers and the results were verified using JoinMap 4.1 software (Van Ooijen 2006) (data not shown). MAPMAKER was used to calculate recombination distances, as it was more robust to inflation of genetic distances, and was executed with error detection turned on and using the commands *order* and *try*. Map distances were calculated using Kosombi's mapping function (Fig. 2a). In addition, MapChart (Voorrips 2002) was used to create a map of the physical position of the genetic markers in this study and those used in previous studies, using the marker GSM0376 as the starting point. The approximate positions of *Rpp4*/*Rpp4-b* are based on the genetic distances between flanking markers sc21_3360 and sc21_3420 (Meyer et al. 2009) and GSM0543 and GSM0387 (current study) and all physical positions were taken from SOYBASE.ORG (Song et al. 2013) (Fig. 2b).

Haplotype analysis at the *Rpp4* locus

The SoySNP50K haplotype of PI 423972 at the *Rpp4* locus is defined by five SNPs: ss715631686, ss715631689, ss715631702, ss715631707, and ss715631709 (Supplementary Table 1). This haplotype was examined in Williams 82; Prichard and PI 423972 (mapping parents); PI 459025B (*Rpp4*); PI 605791A (previously mentioned as having BSA hits within 5 cM of the *Rpp4* locus); PI 567188 and PI 566984 (with unknown resistance loci); known sources of rust resistance; and the 32 significant North American soybean ancestors (Gizlice et al. 1994; Harris et al. 2015; Monteros et al. 2010) (Table 3).

Additionally, FlapJack (Milne et al. 2010) software was used to cluster the lines listed in Table 3 using the SoySNP50K genotypic data across the whole genome (Song et al. 2013). Genotypic data associated with scaffold sequences were removed. The dendrogram was created using hierarchical cluster analysis that takes into account the dissimilarities across the SNP data. If data were missing for a given SNP, it did not count as a mismatch and heterozygous genotype calls were scored as a 50% match to homozygous score (Supplementary Fig. 1).

Results

Resistance reactions

The parents of the mapping population reacted as expected when inoculated with the GA12 bulk *P. pachyrhizi* isolate. Prichard had TAN, highly sporulating lesions and PI

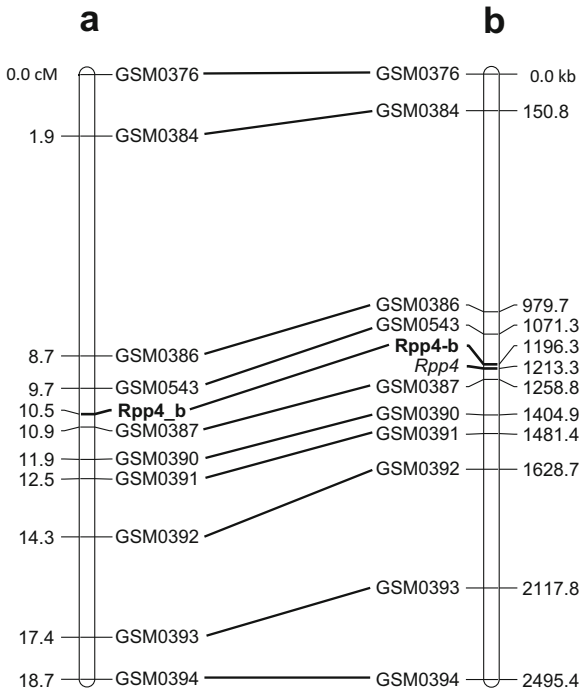

Fig. 2 **a** The genetic map of the Prichard × PI 423972 population. The units on the *left side* of the chromosome map are in centiMorgans (cM) and the KASP marker names are displayed on the right side of the linkage map (Supplementary Table 1). **b** The physical map of the *Rpp4*/*Rpp4-b* locus on chromosome 18. The units on the *right side* of the chromosome are in kilobases (kb) and the physical position of the genetic markers in this study and those used in previous studies was taken from SOYBASE.ORG (Glyma.Wm82.a2). The approximate positions of *Rpp4*/*Rpp4-b* are based on the genetic distances between flanking markers sc21_3360 and sc21_3420 (Meyer et al. 2009) and GSM0543 and GSM0387 (current study)

423972 produced RB lesions that were typically less than 0.5 mm in diameter (Fig. 1a, f). PI 423972 and Prichard were replicated in the experiment four times and showed no segregation in any of the plants observed. The 134 $F_{2:3}$ families were expected to segregate according to a 1:2:1 ratio (resistant:segregating:susceptible) (Table 1). The chi-square value was not significant, indicating the resistance of PI 423972 when challenged by the GA12 isolate is controlled by a single gene. The gene action of this disease resistance gene is dominant, as indicated by the segregation ratios of 811 F_3 plants within heterozygous F_2 families that fit the 3:1 (resistant:susceptible) ratio expected for a completely dominant gene ($p > 0.05$, Table 1).

The GA12 bulk isolate was used to challenge PI 459025B (*Rpp4*), PI 423972, PI 566984, PI 567188, and PI 605791A; the SBR susceptible controls Prichard and G00-3213; and the resistant checks PI 200492 (*Rpp1*) and PI 594538A (*Rpp1-b*). PI 423972, PI 566984, PI 567188, and PI 605791A all produced similar RB-resistant lesions that did not produce uredinia or urediniospores and were generally <1 mm in diameter. PI 459025B (*Rpp4*) also produced RB lesions; however, the RB lesions were often >1 mm in diameter, and would occasionally coalesce. Additionally, uredinia were observed on every plant of PI 459025B, and often associated with relatively low amounts of urediniospores compared to the susceptible control, although some lesions of PI 459025B were RB with no sporulation. G00-3213 and Prichard had TAN lesions with uredinia and high levels of sporulation (Fig. 1).

Bulked segregant analysis and linkage mapping

A total of 17 positive BSA hits were observed from 50,325,784 to 52,979,027 bp (Wm82.a2 genome sequence). Based on the BSA interval, ten KASP markers were developed from polymorphic SoySNP50K SNPs (Supplementary Table 1). The KASP markers GSM0387 and GSM0390 behaved as dominant SNP markers but all other SNP markers behaved co-dominantly as expected (Supplementary Table 1).

For the Prichard × PI 423972 population, none of the SNP markers showed significant segregation distortion from what was expected (data not shown, $p > 0.05$). Linkage mapping using MAPMAKER created a 18.7 cM map distance on Chr 18 that included 10 SNP markers and spanned from 50,325,784 bp (GSM0376) to 52,821,191 bp (GSM0394) (Glyma.Wm82.a2) (Fig. 2; Supplementary Table 1). The PI 423972 *Rpp* resistance gene was mapped to a 187,553-bp region between the markers GSM0543 (51,397,064) and GSM0387 (51,584,617). This interval overlaps with the fine-mapped *Rpp4* locus of PI 459025B (*Rpp4*) defined by sc21_3360 (51,511,484) and sc21_3420 (51,566,780) (Meyer et al. 2009) (Fig. 2).

Haplotype analysis at the *Rpp4* locus using the SoySNP50K Infinium Chip data

The *Rpp4-b* haplotype of PI 423972 within the mapped resistance region is defined by five SoySNP50K SNPs: ss715631686, ss715631689, ss715631702, ss715631707, and ss715631709 (Supplementary Table 1). Of these SNPs, ss715631702 and ss715631707 had been used previously by Harris et al. (2015) to define the *Rpp4* haplotype. However, the *Rpp4-b* haplotype proved to be

Table 3 A unique *Rpp4-b* haplotype that maps to the *Rpp4* locus. This haplotype was determined by comparing a panel of susceptible soybean ancestors, the mapping population parents, and PIs with established *Rpp* genes. Isolate reaction data are from seedling host-plant resistance assays completed in the greenhouse using the Georgia 2012 (GA12) bulk *P. pachyrhizi* isolate

								Gm18_Glyma.Wm82.a2[e]				
								(51,397,064)	(51,421,382)	(51,550,715)	(51,584,617)	(51,591,189)
PI[a]	Cultivar	Year[b]	Country, Region	MG[c]	*P. pachyrhizi* reaction[d]	Maps to known locus	Known gene	ss715631686 (GSM0543)	ss715631689	ss715631702	ss715631707 (GSM0387)	ss715631709
PI 423972	Takema	1978	Japan, Kumamoto	IX	R	*Rpp4*	NA[f]	C	A	T	C	T
PI 459025B	(Bing nan)	1981	China, Fujian	VIII	R	*Rpp4*	*Rpp4*	C	A	C	C	C
PI 566984	NA	1993	Indonesia, unknown	VI	R	None	NA	C	A	T	C	T
PI 567188	VX 9-3	1992	Vietnam, unknown	VI	R	None	NA	C	A	T	C	T
PI 605791A	NA	1998	Vietnam, Cao bang	VI	R	*Rpp4*[g]	NA	C	A	T	C	T
FC031745	NA	1948	unknown, unknown	VI	S	None	NA	C	A	T	T	C
FC033243-1	(Anderson)	1954	unknown, unknown	IV	S	None	NA	T	G	C	T	C
PI 080837	Mejiro	1929	Japan, unknown	IV	S	None	NA	C	A	T	T	C
PI 180501	Strain No.18	1949	Germany, unknown	0	S	None	NA	C	A	T	T	C
PI 240664	Bilomi No. 3	1957	Philippines, unknown	X	S	None	NA	C	A	C	C	C
PI 438471	Fiskeby III	1980	Sweden, Ostergotland	00	S	None	NA	C	-	C	T	T
PI 438477	Fiskeby 840-7-3	1980	Sweden, Ostergotland	00	S	None	NA	C	A	C	T	-
PI 548298	A.K. (Harrow)	1939	China, NE China	III	S	None	NA	T	G	C	T	C
PI 548302	Bansei	1936	Japan, Hokkaido	II	S	None	NA	C	A	T	T	C
PI 548311	Capital	1944	China, NE China	0	S	None	NA	T	G	C	T	C
PI 548318	Dunfield	1923	China, Jilin	III	S	None	NA	T	G	C	T	C
PI 548325	Flambeau	1944	Russia, unknown	00	S	None	NA	C	A	C	T	T
PI 548348	Illini	1927	China, Heilongjiang	III	S	None	NA	T	G	C	T	-
PI 548352	Jogun	1936	Korea, Hamgyong Puk	III	S	None	NA	C	A	T	T	C
PI 548356	Kanro	1936	N. Korea, Pyongyang	II	S	None	NA	C	A	T	T	C
PI 548360	Korean	1928	North Korea, unknown	II	S	None	NA	C	A	T	T	C
PI 548362	Lincoln	1943	China, unknown	III	S	None	NA	T	G	C	T	C
PI 548379	Mandarin (Ottawa)	1934	China, Heilongjiang	0	S	None	NA	T	G/A	C	T	C
PI 548382	Manitoba Brown	1939	unknown, unknown	00	S	None	NA	C	A	T	T	C
PI 548391	Mukden	1932	China, Liaoning	II	S	None	NA	C	A	T	T	C
PI 548402	Peking	1910	China, Beijing	IV	S	None	NA	T	G	C	C	T
PI 548406	Richland	1938	China, Jilin	II	S	None	NA	T	G	C	T	C
PI 548438	Arksoy	1937	N. Korea, Pyongyang	VI	S	None	NA	C	A	C	T	T
PI 548445	CNS	1943	China, Jiangsu	VII	S	None	NA	T	A	T	T	-
PI 548456	Haberlandt	1907	N. Korea, Pyongyang	VI	S	None	NA	C	A	T	T	C
PI 548461	Imp. Pelican	1950	China, unknown	VIII	S	None	NA	C	A	C	T	C
PI 548477	Ogden	1940	unknown, unknown	VI	S	None	NA	C	A	C	T	T
PI 548484	Ralsoy	1940	N. Korea, Pyongyang	VI	S	None	NA	C	A	C	T	T
PI 548485	Roanoke	1946	China, Jiangsu	VII	S	None	NA	C	A	T	T	C
PI 548488	S-100	1945	China, Heilongjiang	V	S	None	NA	T	G	C	T	C
PI 548603	Perry	1952	USA, Indiana	IV	S	None	NA	T	G	C	T	C
PI 548657	Jackson	1953	USA, North Carolina	VII	S	None	NA	T	G	C	C	C
PI 612157	Prichard	2000	USA, Georgia	VIII	S	None	NA	T	A	T	T	C
PI 518671	Williams 82	1981	USA, Illinois	III	S	None	NA	T	G	C	T	C

Table 3 (continued)

PI 200492	Komata	1952	Japan, Shikoku	VII	R	*Rpp1*	*Rpp1*	C	A	C	T	T
PI 594538A	Min hou bai sha wan dou	1996	China, Fujian	IX	M	*Rpp1*	*Rpp1-b*	C	A	C	C	C
PI 230970	NA	1956	Japan, unknown	VII	R[h]	*Rpp2*	*Rpp2*	C	G	C	T	C
PI 224270	Howgyoku	1955	Japan, Hyogo	VII	R[h]	*Rpp2*	*rpp2*	C	A	C	T	T
PI 462312	Ankur	1981	India, Uttar Pradesh	VIII	R[h]	*Rpp3*	*Rpp3*	C	A	C	C	T
PI 506764	Hyuuga	1986	Japan, Kyushu	VII	R[h]	*Rpp3/Rpp5*	*Rpp?[Hyuuga]*	C	A	C	T	C/T
PI 471904	Orba	1982	Indonesia, Java	IX	R[h]	*Rpp5*	*Rpp5*	C	A	C	T	T
PI 200526	Shira Nuhi	1952	Japan, Shikoku	VIII	S[h]	*Rpp5*	*Rpp5*	C	A	C	T	T
PI 200487	Kinoshita	1952	Japan, Shikoku	VIII	R[h]	*Rpp5*	*Rpp5*	C	A	C	T	T
PI 200456	Awashima Zairai	1952	Japan, Shikoku	VIII	NA	*Rpp5*	*rpp5*	C	A	T	T	C
PI 567102B	NA	1993	Indonesia, East Java	IX	R[h]	*Rpp6*	*Rpp6*	-	G	C	C	C

[a]PI, plant introduction ID from the USDA Germplasm Resources Information Network

[b]Year the plant introduction was deposited in the USDA Germplasm Resource Information Network

[c]MG, maturity group

[d]The *P. pachyrhizi* isolate used to test for host plant resistance was the Georgia 2012 (GA12) bulk isolate unless specified. GA12 was collected from field-grown kudzu and soybean in 2012. R indicates an RB or IM resistance reaction types, M indicates a mixed RB and TAN response, and S indicates a susceptible TAN lesion reaction

[e]The genomic locations are from chromosome 18 of the Glyma.Wm82.a2 sequence and indicate the dbSNP location (e.g., ss715632525). These data are available online at www.soybase.org/dlpages/index.php#snp50k (Song et al., 2013). The gray highlights the relatively rare haplotype allele representative of PI 423972

[f]Not applicable (NA), was not tested or is unknown

[g]Preliminary results from bulked segregant analysis

[h]Data extrapolated from Walker et al. 2014

unique to the lines PI 423972, PI 605791A, PI 566984, and PI 567188. All other genotypes tested including lines with known *Rpp* genes and the 32 susceptible soybean ancestors, Prichard, and Williams 82 did not have this haplotype.

The SoySNP50K data were used to create a dendrogram of relatedness across the whole genome to see how closely related the genotypes were that harbor an *Rpp* gene within 5 cM of the *Rpp4* locus. PI 566984, PI 567188, and PI 605791A all may have an *Rpp* gene at the *Rpp4* locus and clustered together (Supplementary Fig. 1). PI 566984 was collected from Indonesia and PI 567188 and PI 605791A were collected from Vietnam, respectively (Table 3). PI 423972 and PI 471904 (*Rpp5*) clustered tightly together (Supplementary Fig. 1); however, they were from different countries, Japan and Indonesia, respectively (Table 3).

Discussion

The resistance of PI 423972 was mapped using 134 $F_{2:3}$ families. The *Rpp* gene from PI 423972 was flanked by GSM0543 (51,397,064) and GSM0387 (51,584,617) and a 187,553-bp interval overlaps with the *Rpp4* interval of PI 459025B (51,511,484–51,566,780) (Fig. 2; Supplementary Table 1). 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, as well as the GA12 bulk isolate (Tables 2 and 3). When challenged with IN73-1, PI 423972 produced TAN lesions and PI 459025B produced RB lesions, and when challenged with TW72-1, PI 423972 produced mostly TAN lesions and a few RB lesions and PI 459025B produced only RB lesions. In this study, we confirmed the same differential reaction of PI 423972 and PI 459025B when challenged with IN73-1 observed by Harris et al. (2015) and Pham et al. (2009). The mostly TAN reaction of PI 423972 to TW72-1 in this study was also similar to the mixed reaction observed by Harris et al. (2015) and the TAN reaction observed by Pham et al. (2009). PI 459025B reacted with RB lesions that occasionally sporulated when challenged with GA12, and PI 423972 produced relatively smaller RB lesions that did not sporulate (Table 2).

Field resistance to SBR in Paraguay showed some difference between PI 423972 and PI 459025B (RB

reaction in three of the four replications for PI 423972 with 5.2% severity, and TAN reaction for PI 459025B with 3.0% severity) (Miles et al. 2008). In the southeastern USA, PI 423972 had a lower average rust severity compared to PI 459025B (1.2 points on a 1 to 5 scale) in three locations in 2008 and a lower rust index (RI; 0.68 points on a 1 to 5 scale) between 2009 to 2012 in all locations tested (except Blackville, SC, 2009, which had a 0.35 point higher RI) (Walker et al. 2011, 2014). In addition, PI 423972 had 33% fewer uredinia per lesion compared to PI 459025B when inoculated with the isolates AL04-1 (Mobile Co., Alabama, USA, 2004), LA04-1 (Ben Hur, Louisiana, USA, 2004), TH01-1 (Chaingmai, Thailand, 2001), and TW72-1 (Taipei, Taiwan, 1972) (Pham et al. 2009). PI 423972 was collected from Japan, whereby PI 459025B was collected from China, representing geographically distanced regions (Table 3). These data indicate that the *Rpp* gene of PI 423972 is allelic to *Rpp4*, and the Soybean Genetics Committee has approved the designation *Rpp4-b* for the resistance of PI 423972. *Rpp4-b* appears to provide resistance to a narrower range of pathotypes, but shows a greater level of resistance than that provided by *Rpp4*.

The *Rpp4-b* (PI 423972) haplotype at the *Rpp4* locus was used to examine PI 423972, a panel of diverse genotypes including the 32 North American soybean ancestors, known sources of *Rpp* resistance, Prichard, Williams 82, PI 605791A (that had BSA hits within 5 cM of the *Rpp4* locus), and two other unmapped sources of resistance (PI 566984 and PI 567188). PI 423972, PI 605791A, PI 566984, and PI 567188 all possess a unique 5-SNP haplotype that no other PI in the panel possessed, including PI 459025B (*Rpp4*) (Table 3). This suggests that they may all possess *Rpp4-b*.

Interestingly, PI 423972 did not cluster with the other genotypes that had the unique haplotype, including PI 605791A (which had BSA hits within 5 cM of the *Rpp4* locus) (Supplementary Fig. 1; Table 3). PI 605791A had a unique isolate pattern when compared to PI 423972 (*Rpp4-b*) and PI 459025B (*Rpp4*) and therefore may harbor another allele or tightly-linked gene at the *Rpp4* locus and needs to be investigated further. PI 566984 and PI 567188 have not been tested with a panel of isolates (Table 3).

The 187.5 Kb interval (51,397,064–51,584,617), to which *Rpp4-b* has been mapped, contains 10 Glyma.Wm82.a2.v1 annotated genes in the Williams 82 reference genome (SOYBASE.ORG) (Supplementary Table 2). Of these, Glyma.18g226300 and Glyma.18g226500 are possible candidate genes, as they belong to the NBS-LRR gene family that has been associated with *Rpp* genes (Meyer et al. 2009; Yu et al. 2015). Similar to *Rpp4C4*, that was identified in the PI 459025B source of *Rpp4* resistance, *Rpp4-b* is likely a sequence or copy number variant of one of these Williams 82 candidate genes in the PI 423972 genotype (Meyer et al. 2009). Further studies should be done to identify the sequences encoding resistance in *Rpp4-b*.

This study has mapped the resistance of PI 423972 (*Rpp4-b*) and demonstrated that *Rpp4-b* could be a valuable resistance allele for cultivar development in the southeastern USA. The KASP SNP assays developed here, including the GSM0543 and GSM0387 SNP markers, offer a tool to introgress *Rpp4-b* into elite germplasm.

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