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ORIGINAL ARTICLE

A novel *Phakopsora pachyrhizi* resistance allele (*Rpp*) contributed by PI 567068A

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Abstract

Key message **The *Rpp6* locus of PI 567102B was mapped from 5,953,237 to 5,998,461 bp (chromosome 18); and a novel allele at the *Rpp6* locus or tightly linked gene *Rpp[PI567068A]* of PI 567068A was mapped from 5,998,461 to 6,160,481 bp.**

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 5,953,237 to 5,998,461 bp (LOD score of 58.3) and the resistance from PI 567068A from 5,998,461 to 6,160,481 bp (LOD score of 4.4) (Wm82. a1 genome sequence). QTL peaks were 139,033 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, which 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.

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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, b; Ono et al. 1992; Slaminko et al. 2008a, b). 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, b; 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, 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 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; 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. 2015). 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 Southeastern USA, *Rpp1*, *Rpp2*, *rpp2*, *Rpp3*, *Rpp?*(Hyuuga), *Rpp4*, and *Rpp6* have been shown to condition varying levels of resistance to SBR (Walker et al. 2014a, b). 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, b). 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 an 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 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 was originally believed to have a novel allele at the *Rpp3* locus (Monteros et al. 2007). A combination of recombinant inbred line (RIL) 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 mapping *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* 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 50 K 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. (2015) 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 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. 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. 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 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 $F_{5:6}$ 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 $F_{2:3}$

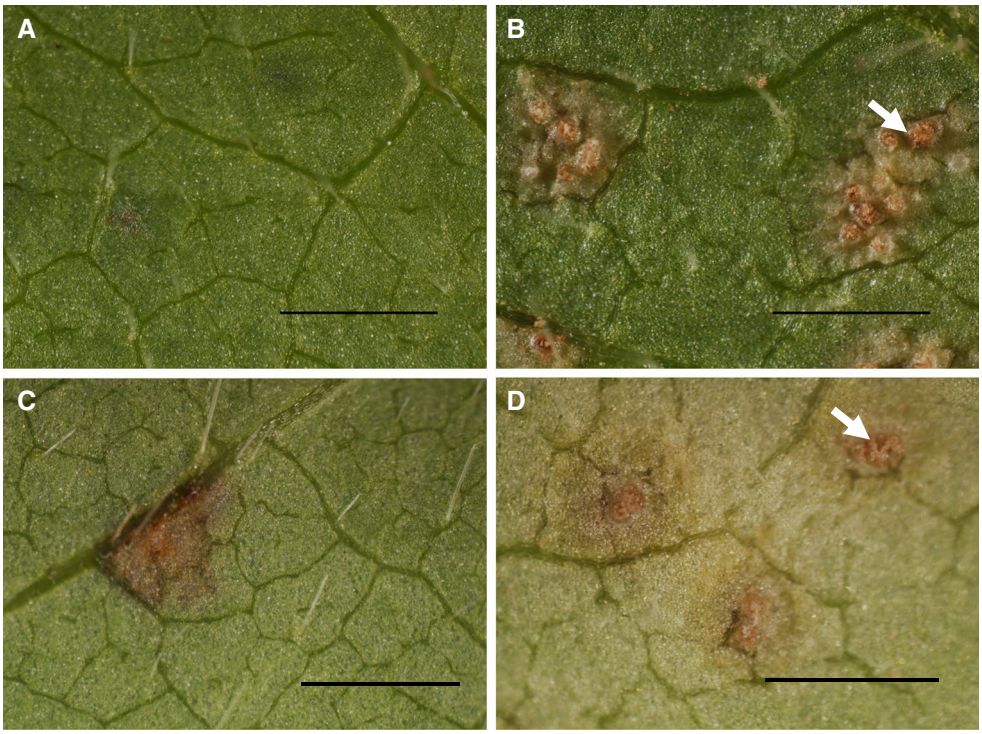

Fig. 1 The reactions of mapping population parents to the Georgia 2012 (GA12) *P. pachyrhizi* bulk isolate: **a** PI 567102B (*Rpp6*), **b** G00-3213, **c** PI 567068A, and **d** Prichard. G00-3213 and Prichard had TAN, highly sporulating lesions (**b**, **d**). PI 567102B (*Rpp6*) and PI 567068A had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

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 SBR 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 SBR 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 × 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 and a representative lesion reaction for each mapping population parent is shown in Fig. 1.

Due to variable seed germination, 10–12 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

Table 1 Reactions of mapping population parents to *Phakopsora pachyrhizi* isolates and sources of resistance

Germplasm	*Phakopsora pachyrhizi* isolate and lesion reactions[a]			
	SA01-1	ZM01-1	AU79-1	GA12[b]
PI 518671 (Williams 82)	TAN	TAN	TAN	TAN
G00-3213	–	–	–	TAN
PI 612157 (Prichard)	–	–	–	TAN
PI 567102B (*Rpp6*)	RB	RB	RB[cd]	RB
PI 567068A	TAN[e]	TAN	RB/INT[f]	RB[g]

– not tested

[a] 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

[b] The Georgia 2012 (GA12) bulk isolate was collected from field-grown kudzu and soybean in 2012

[c] Harris et al. (2015) demonstrated an all-TAN reaction of PI 567102B to AU79-1

[d] One TAN plant in replication two

[e] One replication showed an intermediate phenotype

[f] Two replications showed intermediate phenotype, two replications showed a reddish-brown phenotype

[g] A few lesions showed sporulation

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 3 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 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 each 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 h 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, 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 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 (http://www.lgcgroup.com). 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 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 an LOD score of 3.0 (Figs. 2, 3, 4). JoinMap was used to calculate Chi-square values for both populations (Table 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, 2000 permutations, and a 0.001 level of significance.

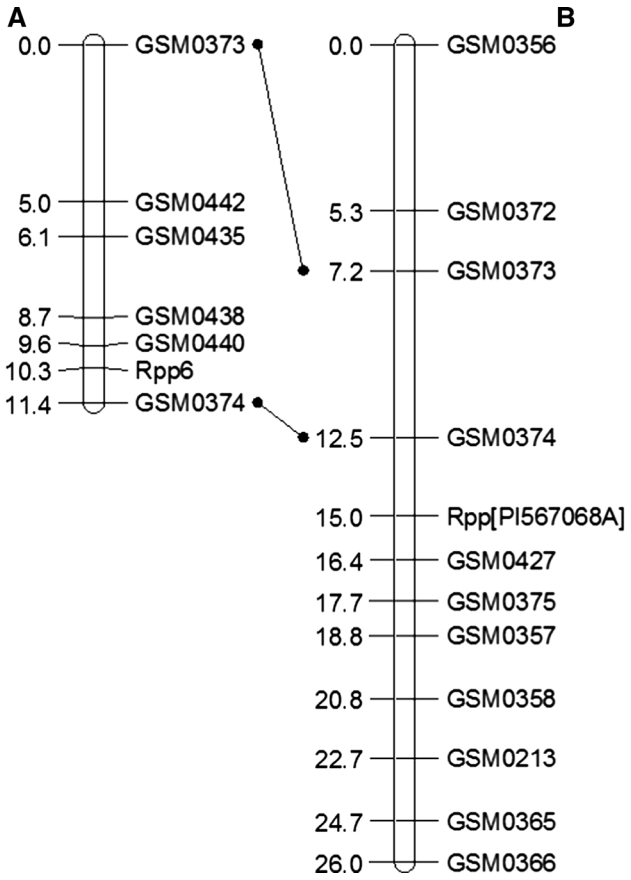

Fig. 2 Linkage map constructed with SNP markers: **a** recombinant inbred line of G00-3213 × PI 567102B (*Rpp6*) and **b** $F_{2:3}$ population of Prichard × PI 567068A. The *left side* of the linkage map displays distance in centiMorgans and the *right side* shows the KASP SNP assay ID (Table 4). Note *Rpp6* and *Rpp[PI567068A]* map to different intervals. *Solid lines* highlight shared SNP markers used to assay both mapping populations

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 Chr 18. The estimated positions of *Rpp6* and *Rpp[PI567068A]* were determined using linkage mapping. 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

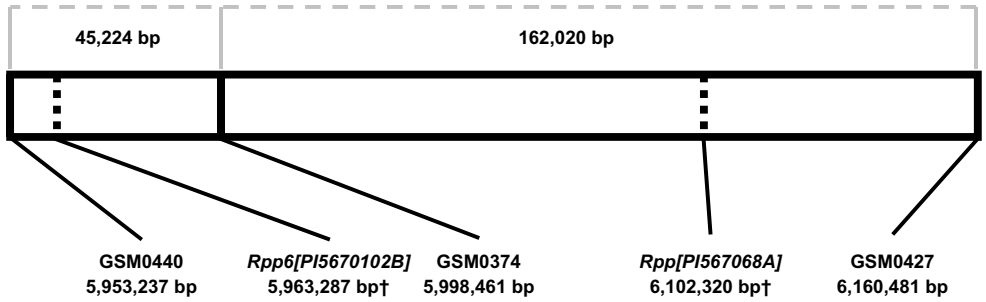

Fig. 3 Physical interval where *Rpp6* and *Rpp[PI567068A]* mapped to Chr 18. The physical locations of GSM markers correspond to SNP positions in the Wm82.a1 genome sequence. The positions of *Rpp6* and *Rpp[PI567068A]* were assigned based on linkage maps generated in this study, and therefore represent estimated positions (*dagger*). The *solid gray lines* represent the physical intervals that harbor *Rpp6* (45,224 bp) and *Rpp[PI567068A]* (162,020 bp) determined by SNP markers. The interval that harbors both *Rpp6* and *Rpp[PI567068A]*, shown with the *dotted gray line* is 139,033 bp

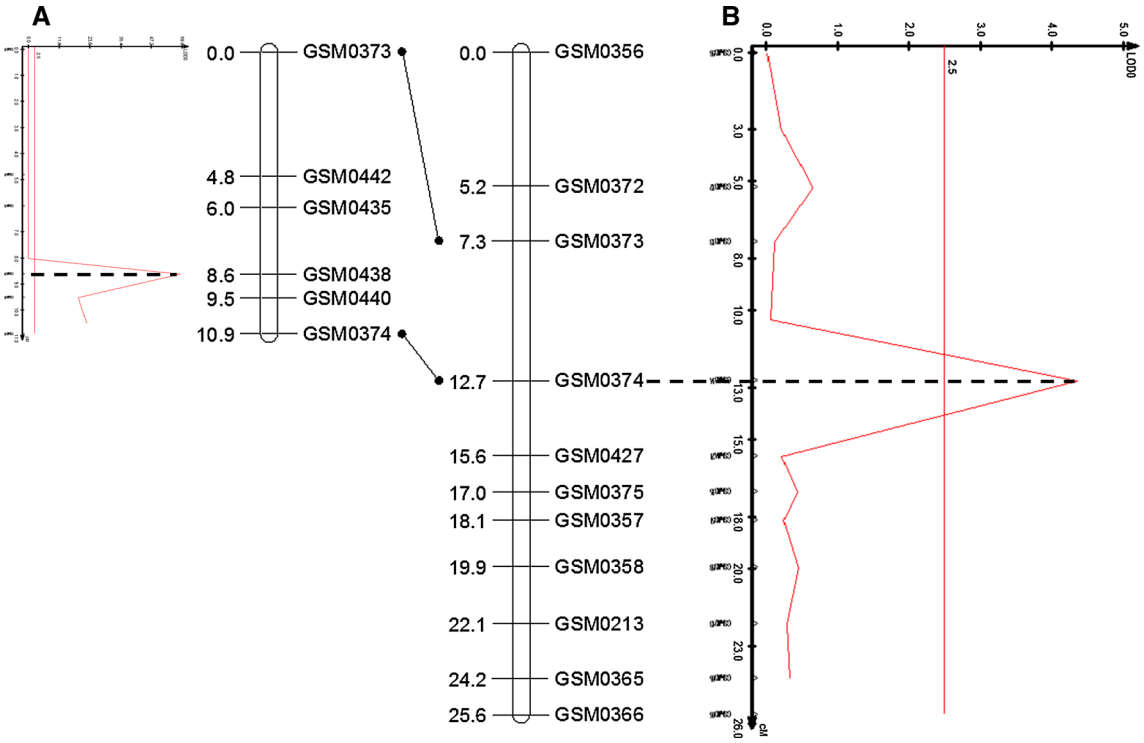

Fig. 4 The linkage map and QTL likelihood plot for **a** RILs of G00-3213 × PI 567102B (*Rpp6*) and **b** $F_{2:3}$ population of Prichard × PI 567068A. Displayed on the *left side* of the linkage map is genetic distance in centiMorgans. The *right side* displays the KASP SNP assay ID (Table 4). Linkage 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

et al. 2015), several elite U.S. cultivars (Williams 82, Prichard, 5601T, and Boggs), and PIs that possessed SBR 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 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 4, which uses a function to create a hierarchical cluster analysis of dissimilarities across all the SoySNP50K SNPs being analyzed. Missing data was not counted as a dissimilarity, and heterozygous locus data are treated as a 50 % match to homozygous allele calls.

Table 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[a]					
	R	H	S	Total	Expected segregation	χ^2
G00-3213 × PI 567102B	91	NA[b]	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

NS: non-significant result when compared to the expected Chi-square value ($p > 0.05$)

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

[b] Heterozygous genotypes and individuals with missing phenotypic data were considered not applicable (NA) from the Chi-square analysis and were excluded

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. 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). 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; http://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 3). Therefore, SBSA using the Prichard × PI 567068A $F_{2:3}$ families was able to detect an 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 an $F_{2:3}$ 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 3; Fig. 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 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 (Figs. 2, 3; Table 3). *Rpp6* contributed by PI 567102B mapped from 5,953,237 to 5,998,461 bp (Figs. 2, 3; Table 3). *Rpp6* had a peak LOD score of 58.3 over SNP GSM0438 (5,930,715 bp; Fig. 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

Table 3 KASP assay ID and primer sequences that were used for mapping resistance from PI 567102B (*Rpp6*) and PI 567068A

Assay ID[c]	DBSNP ID[A]	SNP ID[b]	Resistance source	PI SNP allele	Forward primer 1 5′–3′ (FAM)	Forward primer 2 5′–3′ (HEX)	Reverse primer 5′–3′
GSM0213	ss715632657	BARC_1.01_ Gm18_7515726_T_C	PI 567068A	C	GAAGGTGACCAAG TTCATGCTCTG CTCGCTGATGCTGc	GAAGGTCGGAGTC AACGGATTCCTG CTCGCTGATGCTGt	CGCCCCGTATTC TTCAAAAC
GSM0356[c]	ss715630605	BARC_1.01_ Gm18_4451304_C_T	PI 567068A	T	GAAGGTGACCAAG TTCATGCTTCTCG AAGGCCAAGTTGTGc	GAAGGTCGGAGTCA ACGGATTGATCTCGA AGGCCAAGTTGTGt	AGCATTGGTCGTCGT TGTTC
GSM0357	ss715632549	BARC_1.01_ Gm18_6697094_C_T	PI 567068A	T	GAAGGTGACCA AGTTCATGCTCTCGT CCGGTGCACTGTCc	GAAGGTCGGAGTCA ACGGATTCTATCT CGTCCGGT GCACTGTCt	TGAAAATAGGCTGA ATAGTGACGAG
GSM0358	ss715632566	BARC_1.01_ Gm18_6840290_T_C	PI 567068A	C	GAAGGTGACCAAGT TCATGCTATTGCCA CCACCAGTAAGCAc	GAAGGTCGGAGTCA ACGGATTATTGCCA CCACCAGTAAGCAt	GGCCTCCCATGCAAT TGTTA
GSM0365	ss715632812	BARC_1.01_ Gm18_8777288_A_G	PI 567068A	G	GAAGGTGACCAAG TTCATGCTCGATGG GGAACTGGCAGAa	GAAGGTCGGAGTC AACGGATTCGATGG GGAACTGGCAGAg	CTCGCTTGACGG AAAACTTTGA
GSM0366	ss715632832	BARC_1.01_ Gm18_8928479_A_G	PI 567068A	A	GAAGGTGACCAAG TTCATGCTACACCC TGAAGGCCAGACAa	GAAGGTCGGAGTC AACGGATTACACCC TGAAGGCCAGACAg	GGTTGATTTGCCCAG AATAGGA
GSM0372	ss715630789	BARC_1.01_ Gm18_4784486_C_A_RC	PI 567068A	A	GAAGGTGACCAAG TTCATGCTAGGAG AGTGAATTAATTAGA AGTGACAa	GAAGGTCGGAGTC AACGGATTAGGAG AGTGAATTAATTAGA AGTGACAc	TCCCAACCCAAGA GAAATC
GSM0373	ss715630802	BARC_1.01_ Gm18_4792076_G_A_RC	PI 567068A/PI 567102B	A	GAAGGTGACCAAGT TCATGCTTCAATAA TAGAATTCTTTACCC AAATAGa	GAAGGTCGGAGTC AACGGATTCAATAA TAGAATTCTTTAC CCAAATAGg	GTTGAATTTGTGGTC AATCCAG
GSM0374	ss715632179	BARC_1.01_ Gm18_5961229_C_T_RC	PI 567068A/PI 567102B	C	GAAGGTGACCA AGTTCATGCTGG GGCATTGCTACG TACACc	GAAGGTCGGAG TCAACGGATT TGGGGCATTGCT ACGTACACt	ACCTGTGCCCGTTTA CCCA
GSM0375[c]	ss715632525	BARC_1.01_ Gm18_6384548_A_C_RC	PI 567068A	C	GAAGGTGACCAAG TTCATGCTTCCTT CTTCTTTACCCTA CTTGCCAAa	GAAGGTCGGAGTCAA CGGATTTCCTTCTT CTTTACCCTACTT GCCAAc	GAGGGACCAAGCCAT ATCAACG
GSM0427	NA	BARC_1.01_ Gm18_6122387_G_A_RC	PI 567068A	T	GAAGGTGACCAAG TTCATGCTTGGCA CTATGACATTTGTTA AGGAAc	GAAGGTCGGAGTCA ACGGATTGTGGCA CTATGACATTTGTTA AGGAAt	GGATAAGTGTCAAAA AGACCATGGAC

Table 3 continued

Assay ID[c]	DBSNP ID[A]	SNP ID[b]	Resistance source	PI SNP allele	Forward primer 1 5′–3′ (FAM)	Forward primer 2 5′–3′ (HEX)	Reverse primer 5′–3′
GSM0435	NA	BARC_1.01_ Gm18_5627744_C_A_RC	PI 567102B	A	GAAGGTGACCAAGT TCATGCTCCCTAGA GGTGTTATGTGGTA TCACAa	GAAGGTCGGAG TCAACGGATTACCT AGAGGTGTTTATGTG GTATCACAc	AAAACTTTGTGTCACA TGATTCCA
GSM0438	NA	BARC_1.01_ Gm18_5893484_G_A	PI 567102B	G	GAAGGTGACCAAGT TCATGCTGATGAT TGTCCCAGCAATAA TGTTa	GAAGGTCGGAGTCA ACGGATTTGATTGT CCCAGCAATAA TGTTg	TTGCTGGTGACTAA AGGGGG
GSM0440	NA	BARC_1.01_ Gm18_5916005_T_A	PI 567102B	T	GAAGGTGACCAAGT TCATGCTCTTATTTCC ATCAACACTTGCACa	GAAGGTCGGAGTCA ACGGATTTCTTATT TCCATCAACACT TGCACt	TCGCTGTTTTGGA CTTTTCTCC
GSM0442	ss715631635	BARC_1.01_ Gm18_5517202_G_A_RC	PI 567102B	A	GAAGGTGACCAAGT TCATGCTGGGCAC AGAGATTTGGATCAa	GAAGGTCGGAGTC AACGGATTGGGCAC AGAGATTTGGATCAg	CCTTCAACACTCCA ACCACCAA

[a] dbSNP ID found at http://www.soybase.org/dlpages/index.php#snp50k (Song et al. 2013). Those SNPs listed as not applicable (NA) were not assigned dbSNP IDs

[b] 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

[c] Indicates marker was dominant as opposed to co-dominant with the PI 567068A population

marker interval shown below, or a recombination on either side of the *Rpp6* locus. Of the 184 RILs, two had recombinations flanking each side of the *Rpp6* locus.

Rpp[PI567068A] was fine mapped from 5,998,461 to 6,160,481 bp, spanning a 161,158 bp interval (3.9-cM) flanked by KASP markers GSM0427 and GSM0374 (Figs. 2, 3; Table 3). *Rpp[PI567068A]* had an LOD score of 4.4 over SNP GSM0374 (5,998,461 bp). *Rpp6* and *Rpp[PI567068A]* are both flanked by the SNP marker GSM0374 (Fig. 3; Table 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. 3). Based on the SoySNP50K Infinium Chip (Song et al. 2013), PI 567102B and PI 567068A have identical haplotypes from 5,961,788 (ss715632113) to 6,406,710 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 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 an RB reaction to GA12. The mapping population parents G00-3213 and Prichard were susceptible to the GA12 isolate (Fig. 1; Table 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 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 4; Harris et al. 2015).

PI 567102B and PI 567068A have an identical haplotype allele in the interval of ss715630691 (4,734,471 bp)

Table 4 Haplotypes of the PIs having *Rpp* genes indicated to be at the *Rpp6* locus, soybean ancestors and PIs with known *Rpp* genes

PI[a]	Cultivar	Collection location (year)	MG[b]	*Phakopsora pachyrhizi* reaction[c]	Maps to known locus	Phenotyped with isolate panel if *Rpp6* locus	Phenotyping results	Known gene	Ss715632362 6,126,354[d]	Ss715632523 6,393,112	Ss715632525 6,406,710
PI 567102B	NA	Indonesia, East Java (1993)	IX	R	*Rpp6*	Yes[f]	*Rpp6* like[f]	*Rpp6*	C	A	G
PI 567129	NA	Indonesia, East Java (1993)	IX	R[e]	*Rpp6*[f]	No	NA	NA	C	A	G
PI 567104B	NA	Indonesia, East Java (1993)	IX	R[e]	*Rpp4*/*Rpp6*[f]	Yes[f]	*Rpp4* and *Rpp6* like[fg]	NA	C	A	G
PI 567090	NA	Indonesia, East Java (1993)	IX	R[e]	hits on several chr [f]	Yes[f]	Unique pattern[f]	NA	C	A	G
PI 567068A	NA	Indonesia, East Java (1993)	VII	R	*Rpp6*	Yes[f]	*Rpp[PI 567068A]* like[f]	NA	C	A	G
PI 476905A	Nguu mao hong	China, unknown (1983)	V	R[e]	*Rpp6*[f]	Yes[f]	Unique pattern[f]	NA	C	A	G
PI 567076	NA	Indonesia, East Java (1993)	VII	R[e]	*Rpp6*[f]	Yes[f]	*Rpp[PI567068A]* like[f]	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, Northeast China (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

Table 4 continued

PI[a]	Cultivar	Collection location (year)	MG[b]	*Phakopsora pachyrhizi* reaction[c]	Maps to known locus	Phenotyped with isolate panel if *Rpp6* locus	Phenotyping results	Known gene	Ss715632362 6,126,354[d]	Ss715632523 6,393,112	Ss715632525 6,406,710
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

Table 4 continued

PI[a]	Cultivar	Collection location (year)	MG[b]	*Phakopsora pachyrhizi* reaction[c]	Maps to known locus	Phenotyped with isolate panel if *Rpp6* locus	Phenotyping results	Known gene	Ss715632362 6,126,354[d]	Ss715632523 6,393,112	Ss715632525 6,406,710
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[e]	*Rpp1*	NA	NA	*Rpp1*	C	A	T
PI 594538A	Minhoubaishawandou	China, Fujian (1996)	IX	NA	*Rpp1*	NA	NA	*Rpp1-b*	C	A	T
PI 230970	NA	Japan, unknown (1956)	VII	R[e]	*Rpp2*	NA	NA	*Rpp2*	C	A	T
PI 224270	Howgyoku	Japan, Hyogo (1955)	VII	R[e]	*Rpp2*	NA	NA	*rpp2?*	C	G	T
PI 462312	Ankur	India, Uttar Pradesh (1981)	VIII	R[e]	*Rpp3*	NA	NA	*Rpp3*	C	A	T
PI 506764	Hyuuga	Japan, Kyushu (1986)	VII	R[e]	*Rpp3*	NA	NA	*Rpp?[Hyuuga]*	C	H	T
PI 459025B	Bing nan	China, Fujian (1981)	VIII	R[e]	*Rpp4*	NA	NA	*Rpp4*	C	G	G

Table 4 continued

PI[a]	Cultivar	Collection location (year)	MG[b]	*Phakopsora pachyrhizi* reaction[c]	Maps to known locus	Phenotyped with isolate panel if *Rpp6* locus	Phenotyping results	Known gene	Ss715632362 6,126,354[d]	Ss715632523 6,393,112	Ss715632525 6,406,710
PI 471904	Orba	Indonesia, Java (1982)	IX	R[e]	*Rpp5*	NA	NA	*Rpp5* allele	C	G	T
PI 200526	Shira Nuhi	Japan, Shikoku (1952)	VIII	S[e]	*Rpp5*	NA	NA	*Rpp5* allele	C	G	T
PI 200487	Kinoshita	Japan, Shikoku (1952)	VIII	R[e]	*Rpp5*	NA	NA	*Rpp5* allele	C	G	T
PI 200456	Awashima Zairai	Japan, Shikoku (1952)	VIII	NA	*Rpp5*	NA	NA	*rpp5?*	C	G	T

All isolate reaction data are 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, is associated with the *Rpp6* locus using this panel of genotypes

Not applicable (NA), was not tested

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

[b] MG, maturity group

[c] 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

[d] The genomic locations are from chromosome 18 of the Wm82.a2.v1 sequence and indicate the dbSNP location (e.g., ss715632525) in bp. These data are available online at http://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

[e] Data extrapolated from Walker et al. 2014b

[f] Data extrapolated from Harris et al. 2015

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

to ss715632534 (6,591,476 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 (5,961,788–6,406,710 bp), spanning 444,922 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 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 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 3, 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 4). None of the 32 North American soybean ancestors, 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 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 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 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 (Fig. 5; Table 4).

PI 567102B and PI 567068A clustered together and were 77.4 % similar (Fig. 5; 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 4; Fig. 5).

Discussion

The resistance gene *Rpp6* contributed by PI 567102B and *Rpp[PI567068A]* from PI 567068A were both mapped using a relatively high density pannel of SNP markers, and each *Rpp* gene is flanked by the GSM0374 SNP identified in this study (Fig. 3; Table 3). Through linkage mapping, *Rpp6* was mapped from 5,953,237 to 5,998,461 bp and *Rpp[PI567068A]* was mapped from 5,998,461 to 6,160,481 bp (Fig. 3; Table 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 (data not shown). QTL peaks for *Rpp6* (LOD score of 58.3), and *Rpp[PI567068A]* (LOD score of 4.4) were 139,033 bp apart (Fig. 2; Table 3). This suggests that *Rpp6* and *Rpp[PI567068A]* are either tightly linked or possibly allelic. An allelism test or further fine mapping may help resolve between these two possibilities.

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 an 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 1). The mapping population parents G00-3213 and Prichard were also susceptible (TAN) to the GA12 bulk isolate, as expected (Fig. 1; Table 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. 1; Table 1). When PI 567102B and PI 567068A were challenged with SA01-1, and ZM01-1, and AU79-1 again in this

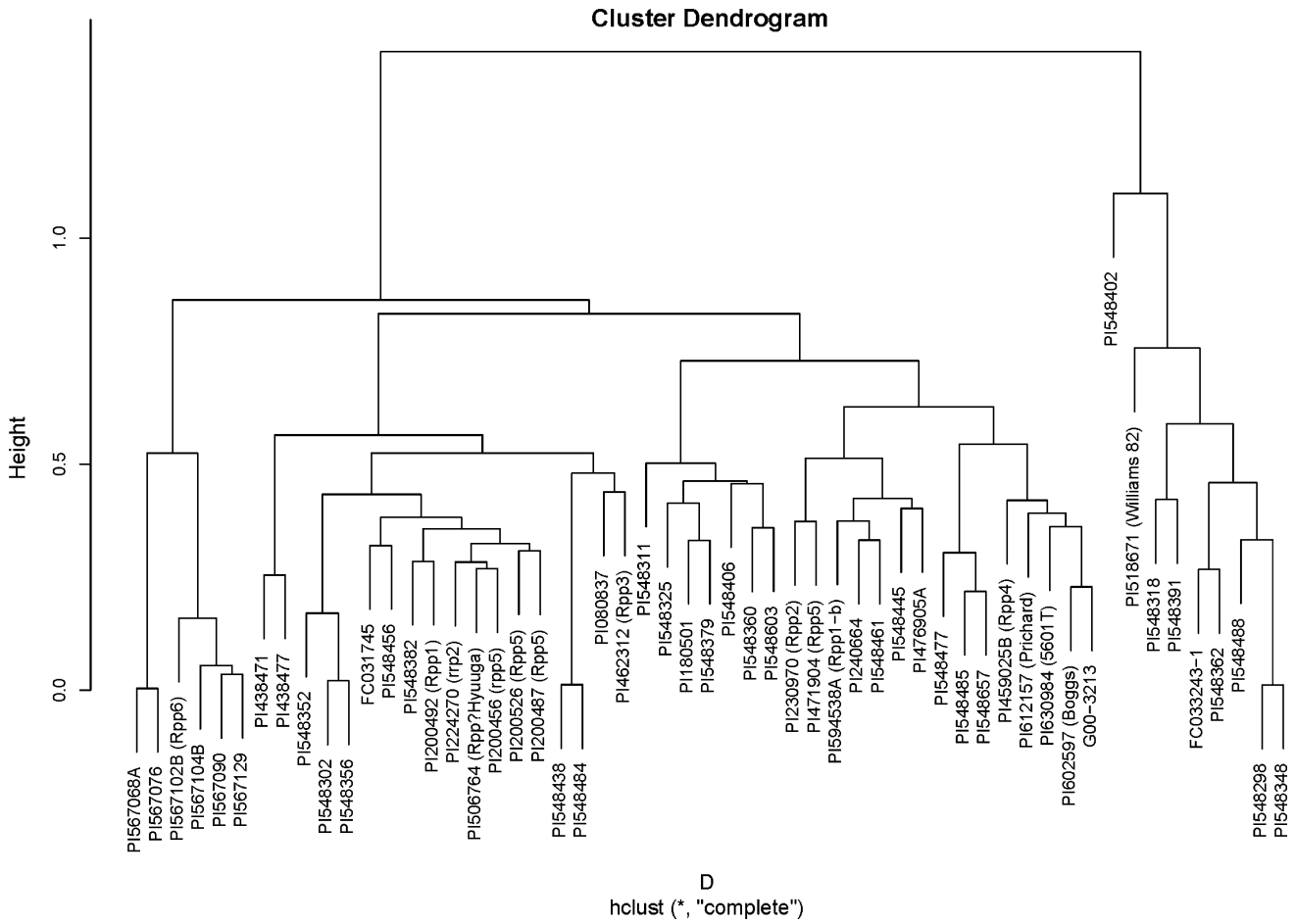

Fig. 5 A dendrogram describing the relationship of all lines listed in Table 4 based on the SoySNP50K SNPs

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 lesions on the 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 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 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 4; Fig. 5).

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 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.

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. Zenglu Li and Roger Boerma interpreted the results, provided oversight for experiments, and edited the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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