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Molecular mapping of Asian soybean rust resistance in Chinese and Japanese soybean lines, Xiao Jing Huang, Himeshirazu, and Iyodaizu B

Naoki Yamanaka · Md. Motaher Hossain · Yuichi Yamaoka

Abstract Asian soybean rust (ASR), caused by the fungus *Phakopsora pachyrhizi*, is one of the most serious soybean diseases in South America and other tropical and sub-tropical areas. The soybean lines, 'Xiao Jing Huang,' 'Himeshirazu,' and 'Iyodaizu B' were previously identified for their resistance to ASR fungus, while the genetic basis of the resistance has yet to be known. In this study, we mapped the ASR resistance loci in these three lines using three independent mapping populations derived from crosses with an ASR susceptible variety, BRS184. In each population, resistance to ASR appeared to be primarily controlled by a single major gene. The resistance genes with largest effect from Xiao Jing Huang and Himeshirazu were genetically mapped on chromosome 18 corresponding to the same location of known resistant locus, *Rpp1*. On the other hand, quantitative trait locus analysis mapped the major ASR resistance locus of Iyodaizu B to the region of chromosome 16 where *Rpp2* was previously mapped. Genetic mapping with DNA markers and disease reactions of seven candidates carrying *Rpp1* to four Brazilian ASR isolates revealed a significant variation in their ASR resistance reaction, indicating that they share different resistance genes tightly linked to each other or different resistant alleles of a single *Rpp1* gene. Therefore, these seven soybean lines could be clearly separated into at least two functional groups.

Keywords Allelic variation · Genetic mapping · *Glycine max* · *Phakopsora pachyrhizi* · QTL · Resistance gene

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Introduction

Asian soybean rust (ASR) caused by biotrophic basidiomycete *Phakopsora pachyrhizi* (H. Sydow and Sydow) occurs in all major soybean-growing regions of the world. The disease is considered as one of the most serious economic threats for soybean growers (Goellner et al. 2010) and severe yield losses are common in South America when environmental conditions are conducive for disease development (Yorinori 2008). Several management tactics have been employed to control ASR and to minimize the impact of this disease. Chemical treatment with fungicide has been perceived as the first line of

defense against the disease (Levy 2005). However, limited number of appropriate fungicide, specific application requirements, increased production costs, environmental pollution and development of fungicide resistant races are the main concerns of using fungicides (Schneider et al. 2008). Hence, an environment friendly, cost-effective and long-term management of the disease can be achieved through utilization of host genetic resistance to the disease (Ribeiro et al. 2007). A major impediment to breeding for ASR resistance is the existence of the high pathogenic variability in the pathogen population and the length of time that takes to incorporate effective and novel *Rpp* genes into new cultivars. Therefore, rational management of ASR in sustainable cropping systems should be based on integrated management strategy that includes host resistance and fungicide.

Genetic resistance to ASR has been identified. Six dominant genes (*Rpp1* to *Rpp6*) controlling pathotype-specific resistance to ASR have so far been mapped at different loci (Hyten et al. 2007; Garcia et al. 2008; Silva et al. 2008; Hyten et al. 2009; Li et al. 2012). Incompatible interactions mediated by some of these genes have an immune phenotype, whereas resistances conferred by other R genes are characterized by limited fungal growth and sporulation and the formation of reddish-brown lesions. Compatible interactions typically are characterized by tan-colored lesions with fully sporulating uredinia. Partial or rate-reducing resistance to ASR has also been documented in soybean, but it has not been widely employed because of complexities in assessment. Although these major *Rpp* genes are now available for breeding, they rarely offer durable resistance to highly variable ASR pathogen (Oliveira et al. 2005). These genes are effective to specific *P. pachyrhizi* isolates, while they were ineffective when challenged with other isolates. This has limited the use of single genes for resistance in soybean especially in South America, where ASR populations are highly virulent and divergent (Yamanaka et al. 2011; Akamatsu et al. 2013). These commonly encountered problems associated with the ineffectiveness of the specific resistance genes and identification of the durable resistance against highly variable ASR population have led to the continuous search for new resistant gene as a breeding remedy for ASR. Development of ASR resistant cultivar has been an important aspect of genetic improvement programs

in soybean in the current days and would be augmented by the identification of gene conditioning the ASR resistance in wide range of soybean varieties. However, very limited sources of soybean germplasm are known to be resistant to ASR. In order to identify new sources of resistance in soybean, Miles et al. (2006) evaluated the entire germplasm collection (16,000 accessions) of the United States Department of Agriculture (USDA) against a mixture of four *P. pachyrhizi* isolates. After two rounds of evaluation, only 850 accessions were identified with partial tolerance or resistance reactions to *P. pachyrhizi*, which correlates to less than 5 % of the USDA Soybean Germplasm Collection. Although specific *P. pachyrhizi* races are virulent on these single gene resistant sources, it may be beneficial to pyramid these known resistant genes into modern cultivars to create broad spectrum resistance to ASR.

Previously intensive screening of Chinese and Japanese soybean genetic resources had identified a Chinese land race, Xiao Jing Huang and Japanese cultivar, Himeshirazu with resistance to Brazilian ASR population (Yamanaka et al. 2008). Resistance to Japanese and Brazilian ASR population in Xiao Jing Huang was confirmed in subsequent experiments (Yamanaka et al. 2010, 2011). ASR resistance in Iyodaizu B was identified against the infection by Japanese ASR population (Benitez et al. 2008). Although these three lines do not possess a very high level of resistance against the tested ASR population, they could be beneficial for pyramiding broad spectrum ASR resistance. Therefore, the aims of the present study were to study the mode of inheritance and identify the resistance loci in Xiao Jing Huang, Himeshirazu, and Iyodaizu B.

Materials and methods

Plant materials

The parental soybean lines used in this study include an ASR susceptible Brazilian variety BRS184 and three ASR resistant Asian lines, Xiao Jing Huang (China, Chinese ID number, ZDD: 00880), Himeshirazu (Japan, Japanese ID number, JP: 67990), and Iyodaizu B (Japan, no national ID number). The resistant lines were crossed with susceptible Brazilian variety BRS184 to develop three F_2 mapping

populations; POP-A, POP-B, and POP-C. The mapping population POP-A was consisted of 90 F_2 plants derived from the cross of Xiao Jing Huang × BRS184. The mapping population POP-B with a pooled collection of 120 F_2 plants was developed from the cross of BRS184 × Himeshirazu while in POP-C, a population of 143 F_2 plants was produced from the cross of Iyodaizu B × BRS184 (for POP-C).

A total of ten soybean lines were tested against four Brazilian ASR isolates to determine allelic variation within *Rpp1* and *Rpp2* loci. The candidate lines carrying *Rpp1* included Komata (PI 200492), Huang Dou (PI 587880A), Bai Dou (PI 587886), Xiao Huang Dou (PI 587905), Zhao Ping Hei Dou (PI 594767A), Xiao Jing Huang (PI number not assigned) and Himeshirazu (PI 594177). The evaluated soybean lines which may carry *Rpp2* were No. 3 (PI 230970), Hougyoku (PI 224270), and Iyodaizu B (PI number not assigned) (Table 1) The three mapping populations, the parentals and the other ten candidates carrying *Rpp1* or *Rpp2* were grown as described by Yamanaka et al. (2010).

Pathogen inoculation and resistance evaluation

The Japanese ASR isolate T1-2 (Yamanaka et al. 2013b; Hossain et al. 2014) was used for inoculation of POP-A, while isolate E1-4-12 was used for inoculating POP-B and POP-C. ASR isolate T1-2 was obtained from ASR population T1 via single-lesion isolation by Yamaoka et al. (2014). ASR population T1 was originally collected from soybean cultivar 'Tachinagaha' in Tsukuba, Japan on September 20, 2007 (Yamaoka et al. 2014). ASR isolate E1-4-12 was obtained from Japanese ASR isolate E1-4 (Yamaoka et al. 2014) by single-lesion isolation following the manual (Yamanaka et al. 2013a). E1-4 has previously been known to be either avirulent or have only weak pathogenicity on differential varieties carrying different ASR resistance genes (*Rpp1* to *Rpp6*) (Yamaoka et al. 2014). Hence E1-4-12 was expected to be suitable for mapping weak ASR resistance genes in Himeshirazu, and Iyodaizu B.

When the plants of three mapping populations reached V3 to V4 growth stage (approximately 3 weeks after sowing), they were inoculated with the urediniospores of T1-2 and E1-4-12. The optimal spore concentration used for inoculation of soybean plants was 5×10^4 urediniospores/mL. Preservation,

multiplication and adjustment of spore concentration were carried out according to the manual (Yamanaka et al. 2013a). The inoculation of parental lines and POP-A, POP-B and POP-C plants was done following the same technique as described by Hossain et al. (2014). Inoculated plants were kept overnight in the humid chamber and then transferred to the growth chamber under the condition described in the previous study (Yamanaka et al. 2013b). Two weeks after inoculation, three populations and their parental lines were evaluated for ASR reactions based on number of uredinia per lesion (NoU) and sporulation level (SL). Three infected leaflets were detached from each inoculated plant and examined the abaxial side of each leaflet microscopically following the same methods of previous study (Hossain et al. 2014). For determining NoU and SL, a maximum of 30 lesions in total, 10 lesions from each leaflet were observed. The SL was rated on a scale of 0–3 as described by Yamanaka et al. (2010) where 0 = none, 1 = little, 2 = moderate and 3 = abundant sporulation. In T1-2 inoculated POP-A, infection phenotypes with lesions with NoU and SL < 2.0 were classified as resistant (R) and those with lesions with NoU and SL $\geq$ 2.0 were classified as susceptible (S) (Hossain et al. 2014). The boundary value of 1.0 was applied for E1-4-12 infection to POP-B, since the infection with less virulent ASR isolate, E1-4-12 produced lower NoU and SL than T1-2 infection and made the gaps $(0.0 < \text{NoU} < 1.1$, $0.0 < \text{SL} < 1.53)$ between R and S phenotypes in the frequencies distributions (Fig. 1). In contrast, the gaps between R and S were not observed in POP-C even though frequency distributions of NoU and SL seemed to be bimodal (Fig. 1). Therefore, boundary values of NoU and SL for R and S phenotypes were not determined and quantitative trait locus (QTL) analysis was applied for genetic mapping in POP-C.

In order to compare the ASR resistance among the ten selected *Rpp1* or *Rpp2* lines, we have used four Brazilian ASR isolates, BRP-2.1, BRP-2.5, BRP-2.6, and BRP-2.49 for inoculation. A detached-leaf method was used for this experiment using experimental condition as described in the previous study (Yamanaka et al. 2013b). Two weeks after inoculation, a total of 30 lesions from four infected leaflets of each line were evaluated by examining frequency of lesions with uredinia (%LU), NoU, and SL. Classification of infection types was done following the same criteria as used in our previous study (Yamanaka et al. 2013b).

Table 1 Resistance loci to Asian soybean rust (ASR) mapped with molecular markers

Name of line	PI number	Rpp[a]	Origin	Dif. no.[b]	Reference
Komata	PI 200492[c]	Rpp1	Japan	1	Hyten et al. (2007)
Bai Dou	PI 587886	Rpp1	China	11	Ray et al. (2009)
Xiao Jing Huang	(Not assigned)	Rpp1	China	–	(In this study, POP-A)
Himeshirazu	PI 594177	Rpp1	Japan	–	(In this study, POP-B)
Huang Dou	PI 587880A	Rpp1-b [?]	China	10	Ray et al. (2009)
Xiao Huang Dou	PI 587905	Rpp1-b [?]	China	12	Hossain et al. (2014)
Zhao Ping Hei Dou	PI 594767A	Rpp1-b [?]	China	13	Hossain et al. (2014)
Min Hou Bai Sha Wan Dou	PI 594538A	Rpp1-b	China	–	Chakraborty et al. (2009)
Gou Jiao Huang Dou	PI 594760B	Rpp1, rpp1	China	–	Garcia et al. (2011)
Jin Yun Dou	PI 561356	Rpp1	China	–	Kim et al. (2012)
No. 3	PI 230970[d]	Rpp2	Japan	3	Silva et al. (2008)
Hougyoku	PI 224270	rpp2	Japan	–	Garcia et al. (2008)
Iyodaizu B	(Not assigned)[e]	Rpp2	Japan	–	(In this study, POP-C)
Ankur	PI 462312	Rpp3	India	5	Hyten et al. (2009)
Akasaya	PI 416764	Rpp3	Japan	8	Hossain et al. (2014)
Hyuuga[f]	PI 506764	Rpp3[g]	Japan	–	Monteros et al. (2007)
FT-2[h]	PI 628932	Rpp3	Brazil	–	Brogin et al. (Brogin 2005)
MARIF 2740	PI 567099A	Rpp3[i]	Indonesia	–	Ray et al. (2011)
Bing Nan	PI 459025	Rpp4	China	6	Silva et al. (2008)
Shiranui	PI 200526	Rpp5	Japan	7	Garcia et al. (2008)
Awashima Zairai No.106	PI 200456	Rpp5	Japan	–	Garcia et al. (2008)
Kinoshita	PI200487	Rpp5	Japan	–	Garcia et al. (2008)
Orba[j]	PI 471904	rpp5	Indonesia	–	Garcia et al. (2008)
MARIF 2767	PI 567102B	Rpp6	Indonesia	17	Li et al. (2012)

Information for some lines was obtained from the websites of United States Department of Agriculture (USDA) (http://www.ars-grin.gov/npgs/acc/acc_queries.html) and National Institute of Agrobiological Sciences (NIAS) (http://www.gene.affrc.go.jp/index_en.php)

[a] Resistant genes are represented as Rpp (resistant allele is dominant) or rpp (resistant allele is recessive), respectively

[b] Identification number of differential varieties (Yamanaka et al. 2013a)

[c] PI 368039 (Tainung 4: Dif. No. 2) is derived from PI 200492 and may share same Rpp1 allele (Boerma et al. 2011)

[d] PI 197182, PI 230971, and PI 417125 (Dif. No. 4) are reported to carry same Rpp2 as PI 230970 has (Laperuta et al. 2008)

[e] Several PIs are present for 'Iyodaizu'

[f] PI 506764 (Hyuuga) is reported to carry Rpp5, too (Kendrick et al. 2011)

[g] 'Hyuuga' is derived from 'Asomusume' × 'Akasaya' and may share same Rpp3 allele as 'Akasaya'

[h] ASR-resistant variety, 'BRSMS-Bacuri' is derived from 'FT-2' and may share same resistant allele, Rpp3

[i] PI 567099A is reported to carry Rpp3 or another gene close to Rpp3 (Ray et al. 2011)

[j] ASR-resistant Taiwanese variety, 'TK 5' (Dif. No. 15) was developed from 'Orba'

SSR marker analysis

The same mapping strategy was adopted and followed in the present study as we did in our previous study (Hossain et al. 2014) for identifying the major ASR resistance loci in the three mapping population.

Briefly, DNA was extracted from the parental lines as well as individual F$_2$ plant. Simple sequence repeat (SSR) markers linked to six Rpp (1–6) loci were employed to investigate the linkage to unknown loci of ASR resistance in three lines. Although several SSR markers linked to each of the known ASR resistance
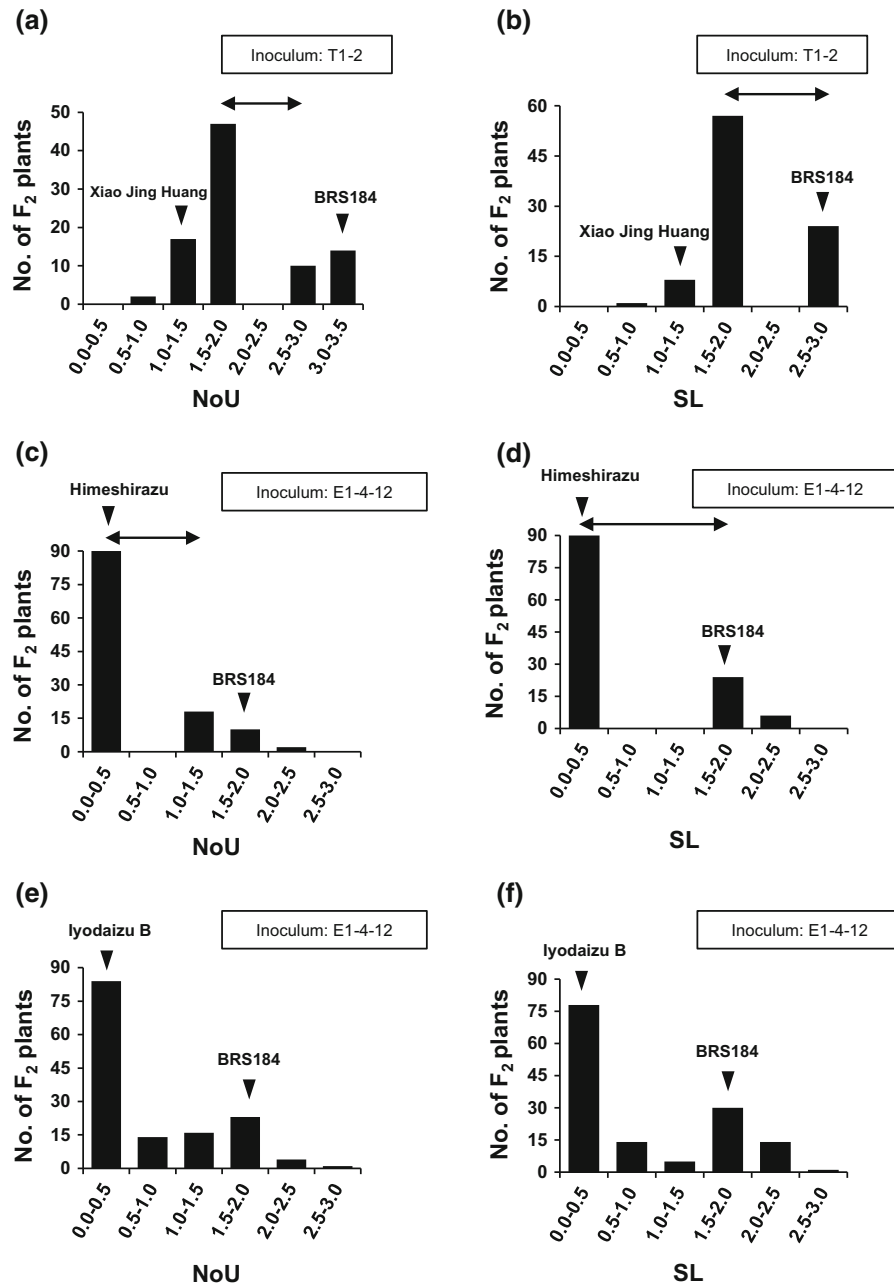
Fig. 1 Frequency distributions of number of uredinia per lesion (NoU) (**a**, **c** and **e**) and sporulation level (SL) (**b**, **d** and **f**) in POP-A (Xiao Jing Huang × BRS184) (**a** and **b**), POP-B (BRS184 × Himeshirazu) (**c** and d) and POP-C (Iyodaizu B × BRS184) (**e** and **f**) against Japanese Asian soybean rust (ASR) isolates. ASR isolates, T1-2 and E1-4-12 were used for POP-A, and other two populations: POP-B and POP-C, respectively. *Triangles* indicate the values of respective resistant and susceptible parents. Estimated boundary values between resistant and susceptible phenotypes for NoU and SL in POP-A and POP-B are shown by *arrows*

genes (*Rpp1–6*) were tested; one was selected as a polymorphic marker for each gene. SSR marker Sat_064, Satt380, Sat_263, Satt288, Sat_280 and

Satt324 linked to *Rpp1, 2, 3, 4, 5,* and *6,* respectively were found polymorphic between mapping parents of POP-A and used for initial screening of a small subset

(24 F_2 plants) of this population. In POP-B, SSR marker Sat_064, Satt380, Sat_263, AF162283, Sat_275 and Satt324 were polymorphic between mapping parents and selected for initial screening of a small subset of this population. In POP-C, the selected polymorphic SSR markers for initial screening of its small subset population were Sat_064, Satt380, Satt460, AF162283, Sat_280 and Satt324. Once significant association between NoU and genotypes of marker linked to *Rpp* loci was found, full mapping population was tested for confirmation. Additional markers around the regions where the potential ASR resistance gene was mapped were chosen from SoyBase (Grant et al. 2010). All SSR markers used in this study were co-dominant for parents. PCR and subsequent electrophoresis were performed following the procedures described in the manual (Yamanaka et al. 2013a).

Genetic mapping and statistical analysis

Goodness-of-fit between observed and expected segregation ratios of ASR resistance phenotypes and SSR marker genotypes in F_2 populations was tested using Chi square (χ^2) analysis. ANOVA and regression analysis were applied to identify the significance of the association between ASR phenotype (NoU and SL) and nearest flanking markers and to estimate the genetic effects and the portion of the phenotypic variation explained by the resistance genes based on genotypes of flanking markers. MAPMAKER/EXP 3.0b (Lander et al. 1987) was used to determine the order of loci for SSR markers and ASR resistance in each mapping population. The Kosambi mapping function was used to convert recombination values into map distances. The minimum logarithm of the odds (LOD) score and the maximum genetic distance for linkage map construction were adjusted to 3.0 and 37.2 cM, respectively. In POP-C, genomic region associated with NoU and SL was identified by quantitative trait loci (QTLs) analysis using Windows QTL Cartographer v2.5 (Wang et al. 2007). The interval mapping with the walk speed of 0.5 cM was applied for the QTL analysis. The estimated order of markers and distances between markers determined by MAPMAKER were used for QTL analysis. The LOD score threshold for declaring a putative locus as significant was determined by permutation testing using 1,000 permutations and significant at the 0.01 level. The threshold level of the LOD score was set at 1.78 for NoU and 1.75 for SL. The loci for NoU and SL were defined as the peaks of maximum LOD score. The nearest flanking marker was chosen according to the distance between peak of LOD score and markers.

In order to compare the linkage map around *Rpp1* obtained from this study with those of previous studies more precisely, the linkage maps from two independent F_2 populations derived from BRS184 × PI 594767A and BRS184 × PI 587905, respectively (Hossain et al. 2014) were re-constructed by the progeny test using F_3 populations. In both populations, fifteen or fewer (0–15) $F_{2:3}$ progeny from each resistant F_2 plant was cultivated and evaluated for their resistance to T1–2, as it was done in F_2 (Hossain et al. 2014). Firstly, we started this evaluation with a few F_3 plants, and then the numbers of evaluated plants were enlarged according to their phenotype. If a susceptible phenotype was observed among $F_{2:3}$ progeny, the *Rpp1* genotype of the F_2 plant was considered to have been heterozygous. If all the $F_{2:3}$ progeny were resistant, the F_2 plant was considered to have been either homozygous for the resistance allele or heterozygous. These genotype data for resistance loci were used for reconstruction of previous genetic maps.

Results

Evaluation of Asian soybean rust resistance

Significant differences were observed in NoU and SL between the susceptible parent BRS184 and three resistant parents, Xiao Jing Huang, Himeshirazu, and Iyodaizu B (Fig. 1). The susceptible parent BRS184 showed the highest susceptibility among the four varieties to both T1-2 and E1-4-12. However, the susceptibility was relatively higher to T1-2 (NoU = 3.13, SL = 3.00) than E1-4-12 infections (NoU = 1.62, SL = 1.97) (Fig. 1). Inoculations of Himeshirazu and Iyodaizu B with E1-4-12 isolate gave rise to strong resistance or almost immune reaction, showing imperfect formation of RB lesions with no uredinia production. The mean values of both NoU and SL in these varieties were therefore, scored as zero. On the other hand, Xiao Jing Huang produced RB lesions with limited uredinia and spore production (NoU = 1.13, SL = 1.43) after T1–2 infection.

Phenotypic analysis of the POP-A showed a segregation of 66 plants with resistant phenotype and 24 plants with the susceptible phenotype. In the 66 resistant plants, only 7 plants showed higher resistance (both NoU and SL) than the resistant parent Xiao Jing Huang but all resistant plants produced uredinia and spores. A Chi square (χ^2) test revealed that the observed segregation fitted the expected segregation ratio of single dominant resistance gene: 3:1 (resistant: susceptible) in the F_2 generation (Table 2). The degree of dominance for NoU and SL were 1.09 and 1.12, indicating that the putative ASR resistant allele primary controlling resistance in Xiao Jing Huang is completely dominant with a little heterosis (Table 3).

The POP-B plants segregated into two clearly separated ranges, 90 with resistant and 30 with susceptible phenotype (Table 2). The observed segregation perfectly fitted the 3:1 (resistant: susceptible) ratio ($\chi^2 = 0.00$, $P = 1.00$) in the F_2 generation, suggesting that a single dominant gene controls the resistance in Himeshirazu mainly. However, 7 of 30 susceptible F_2 plants showed higher NoU and SL than susceptible parent BRS184 (Fig. 1c, d). The degree of dominance for both NoU and SL was 1.00, indicating that the resistant loci is completely dominant in Himeshirazu (Table 3).

The F_2 progeny of POP-C showed a wider variation in values of both NoU and SL than that of POP-A and POP-B. The NoU in the F_2 population ranged from 0.00 to 2.53, while that in parental Iyodaizu B and BRS184 were 0.00 and 1.62, respectively (Fig. 1e). Similarly, the value of SL in F_2 population ranged from 0.00 to 2.90, while the resistant and susceptible parents had mean SL of 0.00 and 1.97, respectively (Fig. 1f). As a result, the frequency distributions of NoU and SL in the F_2 population were bimodal, however, some F_2 plants couldn't be classified into two differentiated classes, i.e. resistant and susceptible phenotype because no clear boundary between resistant and susceptible phenotype was not observed in these plants. Thus, we decided to perform a QTL analysis for mapping of ASR resistance in POP-C.

Mapping of resistance loci to ASR in three populations

Initial genotyping of a small sub set of POP-A (24 F_2 plants) with one marker linked to each of the Rpp1 to Rpp6 and subsequent test by ANOVA showed that the F_2-inferred NoU data were significantly associated with Sat_064 linked to the Rpp1 gene ($P < 0.0001$, data not shown). Genotype data of full set of POP-A with Sat_064 also revealed significant ($P < 0.0001$) association with variation in NoU and SL (Table 3). The coefficient of determination (R^2) of each phenotypic variation by this marker was 0.48 for NoU and 0.45 for SL, respectively (Table 3). Additional SSR markers Sat_372, Satt191, Sat_117, and Satt199 around Rpp1 were found polymorphic between the parents of POP-A. Genotyping of the full set of POP-A with Sat_064, Sat_372, Satt191, Sat_117, and Satt199, followed by a χ^2 test revealed that all SSR markers mapped in the POP-A satisfactorily fitted the expected ratio for co-dominant inheritance (1:2:1). The major resistance locus of Xiao Jing Huang controlling the phenotypic variation of NoU and SL was mapped between Satt191 and Sat_064 where Rpp1 was previously mapped by Ray et al. (2009) (Fig. 2).

Similar to POP-A, primary genotyping of a small subset of POP-B (24 F_2 plants) with SSR markers linked to six Rpp loci followed by ANOVA test showed that the genotype at SSR marker Sat_064 was significantly associated with variation in NoU ($P < 0.0001$, data not shown).

Table 2 Segregation of disease reaction to Asian soybean rust (ASR) isolate T1-2 and E1-4-12 in the F_2 population, POP-A (Xiao Jing Huang × BRS184) and POP-B (BRS184 × Himeshirazu), respectively

Population	Number of F_2 plants[a]		χ^2 of the expected ratio of R:S (3:1)	P[b]
	R	S		
POP-A (Xiao Jing Huang × BRS184)	66	24	0.13	0.72
POP-B (BRS184 × Himeshirazu)	90	30	0.00	1.00

[a] R resistant; S susceptible

[b] P = probability, P > 0.05 means the observed segregation fit the 3:1 model

Table 3 Association between Asian soybean rust (ASR) resistance and Simple Sequence Repeat (SSR) markers in POP-A (Xiao Jing Huang × BRS184), POP-B (BRS184 × Himeshirazu), and POP-C (Iyodaizu B × BRS184) determined by one-way ANOVA and regression analysis

Population	Resistance characters[a]	Marker and genotype[b]	Mean	SD	F value	P[c]	R^{2d}	Additive effect (a)[e]	Dominance effect (d)[e]	d/a[f]
POP-A	NoU	Sat_064: A	3.04	0.09	152.06	<0.0001	0.48	−0.68	−0.75	1.09
		Sat_064: H	1.61	0.44						
		Sat_064: B	1.67	0.20						
		All	1.98	0.69						
	SL	Sat_064: A	3.00	0.00	128.18	<0.0001	0.45	−0.55	−0.62	1.12
		Sat_064: H	1.82	0.40						
		Sat_064: B	1.89	0.17						
		All	2.13	0.58						
POP-B	NoU	Sct_187: A	1.51	0.25	1698.60	<0.0001	0.65	−0.76	−0.76	1.00
		Sct_187: H	0.00	0.00						
		Sct_187: B	0.02	0.07						
		All	0.38	0.67						
	SL	Sct_187: A	1.89	0.15	6978.33	<0.0001	0.66	−0.94	−0.94	1.00
		Sct_187: H	0.00	0.00						
		Sct_187: B	0.00	0.00						
		All	0.47	0.82						
POP-C	NoU	Satt620: A	1.63	0.40	321.14	<0.0001	0.70	−0.81	−0.51	0.63
		Satt620: H	0.31	0.31						
		Satt620: B	0.01	0.02						
		All	0.65	0.73						
	SL	Satt620: A	1.93	0.35	307.12	<0.0001	0.72	−0.96	−0.53	0.55
		Satt620: H	0.44	0.44						
		Satt620: B	0.01	0.03						
		All	0.80	0.84						

SD standard deviation

[a] Resistance characters, *NoU* the number of uredinia per lesion, SL: sporulation level

[b] Genotype, A: Homozygous susceptible parent, H Heterozygous, B: Homozygous resistant parent

[c] *P* probability of significance, calculated by single-factor analysis of variance

[d] R^2, coefficient of determination calculated based on the nearest marker by regression analysis

[e] Additive and dominance effects are those of the resistance alleles relative to the susceptible alleles

[f] Degree of dominance: 1 complete dominance for resistance; 0 lack of dominance; −1 complete dominance for susceptibility

The linkage was further confirmed by testing the full mapping population with this marker and six additional SSR markers, Sct_199, Satt191, Sat_117, Sct_187, SSR66, and Sat_372 near *Rpp1*. A χ^2 test revealed that all SSR markers used to map the resistance in the POP-B fitted the expected ratio for co-dominant inheritance and followed a 1 resistant: 2 segregating: 1 susceptible ratio. The major ASR resistance locus of Himeshirazu was mapped between Sct_187 and Sat_064 where *Rpp1* was previously mapped in PI200492 by Hyten et al. (2007) and in PI 587886 by Ray et al. (2009) (Fig. 2). Genotype data of POP-B with Sct_187, the nearest linked marker to *Rpp1* showed significant ($P < 0.0001$) association with variations in NoU and SL (Table 3). The R^2 of each phenotypic variation by this marker was 0.65 for NoU and 0.66 for SL, respectively (Table 3).

Rpp1 group   **Rpp1-b group**
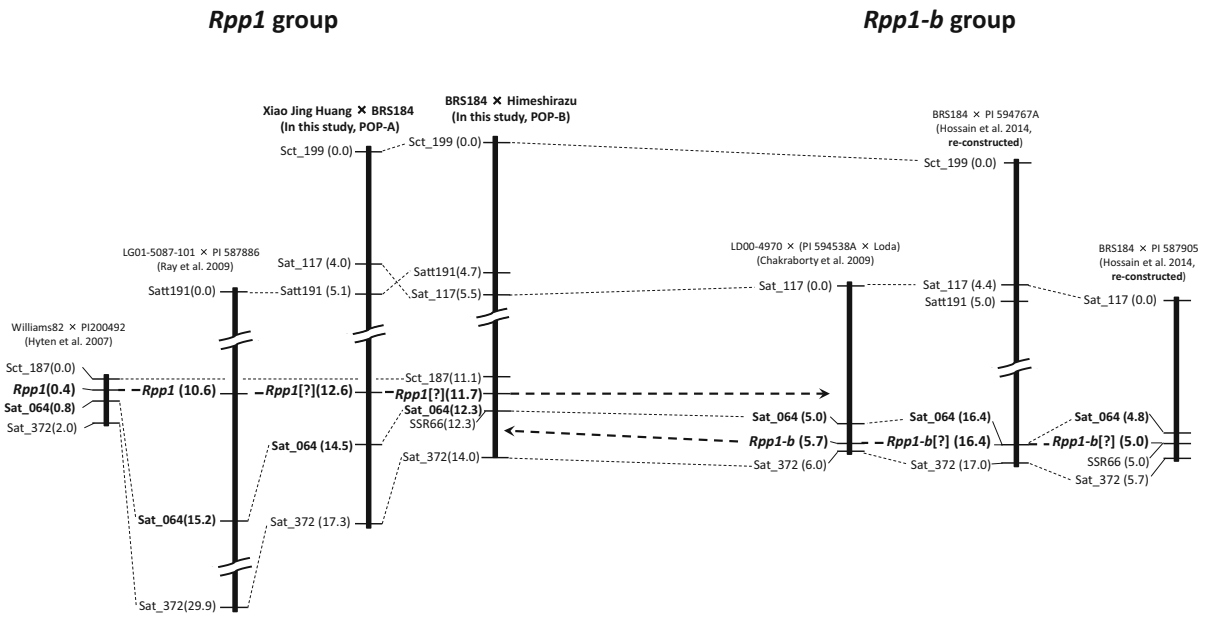

Fig. 2 Genetic linkage map around *Rpp1,* the resistance locus against *Phakopsora pachyrhizi* on linkage group G (chromosome 18) constructed from POP-A (Xiao Jing Huang × BRS184) and POP-B (BRS184 × Himeshirazu). The values in *parenthesis* next to marker name are cumulative distances (cM) from the *top* of linkage group in each mapping population. The linkage maps of Hyten et al. (2007), Chakraborty et al. (2009), Ray et al. (2009), and Hossain et al. (2014) are included for comparison reference. The *maps* of PI 594767A and PI 587905 by Hossain et al. (2014) were re-constructed in this study

On the contrary in POP-C, initial marker-trait analysis of 24 F_2 plants by ANOVA indicated a significant linkage between NoU and SSR marker Satt380 for *Rpp2* ($P = 0.0114$, data not shown). The linkage was confirmed by subsequent screening and analysis of full set of POP-C with Satt380 and additional markers from the same region. SSR markers Satt380, Sct_001, Sat_255, Satt620, and Sat_366 around *Rpp2* were polymorphic and used for mapping. A χ^2 test revealed that all the tested SSR markers satisfactorily fitted the expected ratio for co-dominant inheritance (1:2:1). A linkage map was constructed with the five SSR markers that spanned a total length of 15.9 cM (Fig. 3). An interval mapping was performed and the loci for the resistant characters, NoU and SL were identified. As shown in Fig. 3, a peak of LOD score for NoU was detected in the interval between the SSR markers Satt620 and Sat_366 at 9.6 cM from the top of this linkage group. Similarly, a peak of LOD score for SL was detected in the similar position of NoU between Satt620 and Sat_366 at 10.1 cM from the top of this linkage group (Fig. 3). The peak LOD scores for the loci for NoU and SL were

57.05 and 58.23, respectively (Fig. 3). Composite interval mapping also generated a single locus for each trait with same peak position of interval mapping (data not shown). These results indicated that a single locus located close to Satt620 where *Rpp2* was previously mapped (Garcia et al. 2008; Silva et al. 2008) contributes to reduce each of NoU and SL mainly in Iyodaizu B. The additive effects in this locus were estimated to reduce NoU and SL by 0.81 and 0.96, respectively (Table 3). The degree of dominance for NoU was 0.63 and that for SL was 0.55; indicating that resistance allele of this locus was dominant but incomplete. The R^2 of each phenotypic variation was 0.70 for NoU and 0.72 for SL, respectively (Table 3).

Infection types of ten candidates carrying *Rpp1* or *Rpp2*

ASR reaction profile of the ten candidates carrying *Rpp1* or *Rpp2* to four Brazilian ASR isolates was obtained by detached leaf method as shown in Table 4. Soybean lines PI 587905, PI 594767A, and PI 587880A which were previously mapped as *Rpp1* in
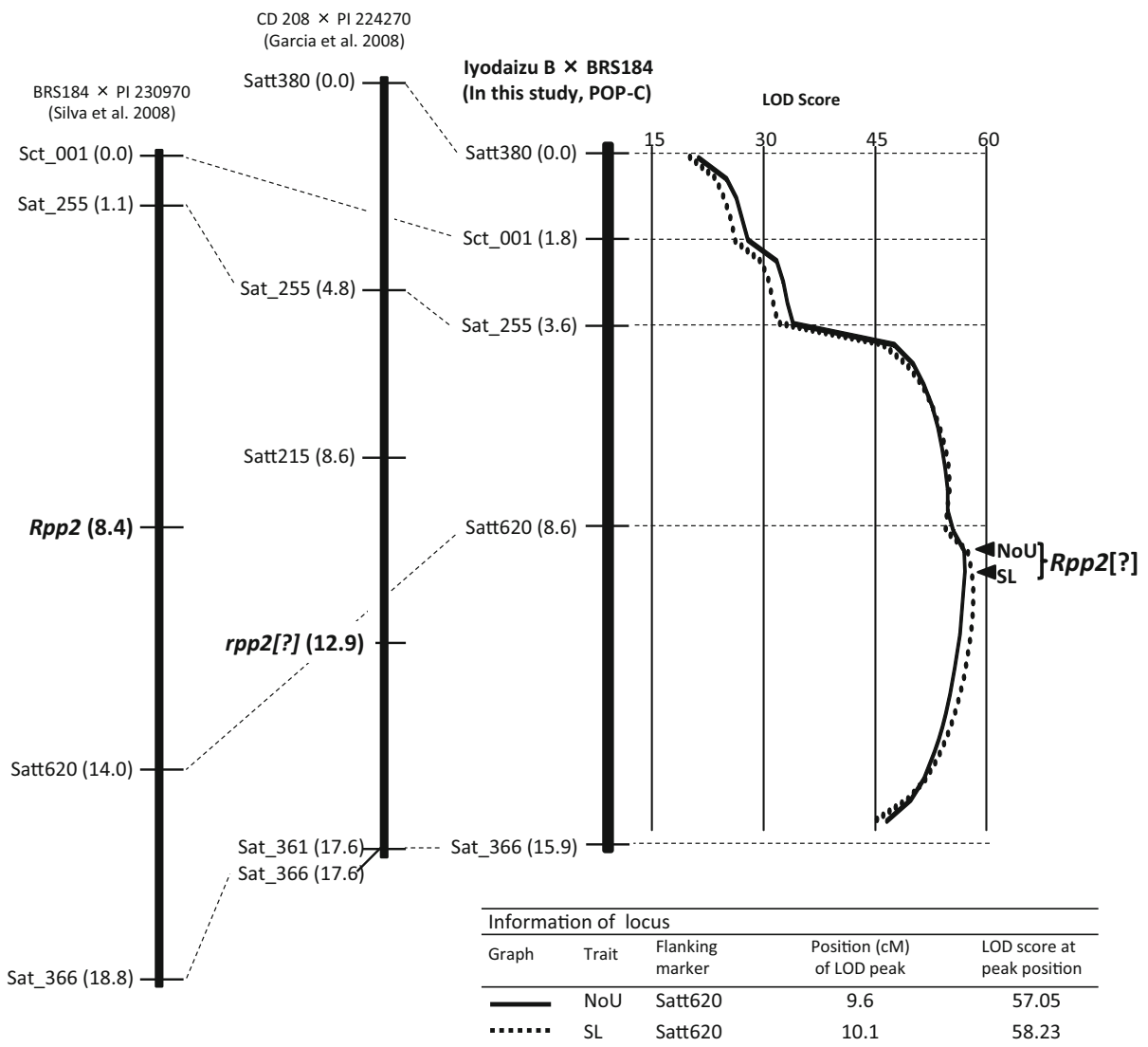
Fig. 3 Genetic linkage map around *Rpp2,* the resistance locus against *Phakopsora pachyrhizi* on linkage group J (chromosome 16) constructed from POP-C (Iyodaizu B × BRS184). Resistance locus of Iyodaizu B is estimated by peak positions (*closed triangles*) of logarithm of the odds (LOD) curves obtained by quantitative trait loci (QTLs) analysis for number of uredinia (NoU) and sporulation level (SL). The values in parenthesis next to marker name are cumulative distances (cM) from the *top* of linkage group in each mapping population. The linkage maps of Silva et al. (2008) and Garcia et al. (2008) are included for comparison reference

separate studies by Hossain et al. (2014) and Ray et al. (2009) showed the same "Highly resistant" phenotypes against two of four tested ASR isolates: BRP-2.49 and BRP-2.1 (Table 4). They also showed resistant phenotype to BRP-2.6 but degree of resistance was different for each. The infection by BRP-2.5 produced the only susceptible reaction in these three lines. On the other hand, the resistant parental lines,

Xiao Jing Huang and Himeshirazu of POP-A and POP-B, and the two other known *Rpp1* lines, PI 200492 and PI 587886 showed susceptible reaction to all four Brazilian ASR isolates. Therefore, these seven lines clearly revealed two different reaction patterns against infection by four tested ASR isolates.

Iyodaizu B, the resistant parent of POP-C, and the known *Rpp2* carrying lines, PI 230970 and Hougyoku

Table 4 Infection types of 10 resistant lines estimated to carry *Rpp1*, *Rpp1-b*[?], and *Rpp2* against four Brazilian Asian soybean rust (ASR) isolates, BRP-2.1, BRP-2.5, BRP-2.6, and BRP-2.49

Rpp gene	Resistant line	Infection type to ASR isolate [a]			
		BRP-2.49	BRP-2.1	BRP-2.6	BRP-2.5
Rpp1	PI587886	S	S	S	S
	Xiao Jing Huang	S	S	S	S
	Himeshirazu	S	S	S	S
	PI200492	S	S	S	S
Rpp1-b [?]	PI587905	HR	HR	R	S
	PI594767A	HR	HR	HR	S
	PI587880A	HR	HR	I	S
Rpp2	PI230970	S	S	S	S
	Iyodaizu B	R	SR	S	S
	Hougyoku	R	R	SR	R

[a] S : susceptible;   SR : slightly resistant;   R : resistant;   HR : highly resistant;   I : immune

showed different ASR reaction profile to the tested ASR isolates (Table 4). Hougyoku was resistant to all ASR isolates, except for BRP-2.6 against which "Slightly resistant" phenotype was observed. PI 230970 was clearly susceptible to all tested ASR isolates. Iyodaizu B showed "Resistant" against BRP-2.49 and "Slightly resistant" against BRP-2.1, while susceptible phenotype was observed against two other ASR isolates, BRP-2.6 and BRP-2.5.

Discussion

Since only six kinds of resistant genes against ASR have been identified so far and South American ASR pathogens are highly virulent and diverse (Yamanaka et al. 2010, 2011; Akamatsu et al. 2013), new ASR resistance resources are needed for soybean breeding in South America. On the other hand, pyramiding available ASR resistance genes in a single soybean cultivar is known to provide highly resistance against the ASR by candidate interactions among *Rpp* genes (Lemos et al. 2011; Yamanaka et al. 2013b). Therefore, new resistance genes may be useful for gene-pyramiding even though they don't show strong resistance individually. Soybean lines, Xiao Jing Huang, Himeshirazu, and Iyodaizu B were identified

as having ASR resistance in the previous studies (Benitez et al. 2008; Yamanaka et al. 2008, 2010, 2011). However, the genetic basis of the ASR resistance in these lines was not determined in previous studies. Our results suggested that single dominant gene, candidate *Rpp1* primarily control the ASR resistance in Xiao Jing Huang and Himeshirazu However, 'Iyodaizu B' had a single gene at or near the *Rpp2* locus which was incompletely dominant. The level of observed dominance might depend on the *P. pachyrhizi* isolate used to obtain rust reaction data. It seems quite possible that the degree of dominance against a more aggressive isolate may be less than towards a less aggressive isolate.

Chakraborty et al. (2009) suggested that PI 594538A carries ASR resistance gene *Rpp1-b* that is likely a different resistant allele or closely linked different gene from *Rpp1* of PI 200492 based on their slightly different map positions and different reactions to Zimbabwe ASR isolate. Hossain et al. (2014) also reported clearly different reactions by two kinds of *Rpp1* carrying lines against 64 South American ASR populations. *Rpp1*-carrying lines with weak resistance included PI 200492, PI 368039, and PI 587886, while those with strong resistance were PI 594767A, PI 587905, and PI 587880A. These lines were predicted to share different resistant alleles of *Rpp1* or tightly

linked loci on the same genomic region. In this study, resistance gene of Xiao Jing Huang was mapped between Satt191 and Sat_064, and resistance gene of Himeshirazu was mapped between Sct_187 and Sat_064 onto the soybean chromosome 18 (LG-G), where *Rpp1* of PI 200492 and PI 587886 were previously mapped (Hyten et al. 2007; Ray et al. 2009) (Fig. 2). These *Rpp1* loci are slightly different from *Rpp1* (*Rpp1-b*[?]) loci of PI 594538A, PI 594767A, and PI 587905 which were mapped between two SSR markers Sat_064 and Sat_372 (Chakraborty et al. 2009; Hossain et al. 2014). According to those previously reported studies and the present study, soybean lines PI 200492, PI 368039, PI 587886, Xiao Jing Huang, and Himeshirazu may carry the original *Rpp1* as identified by Hartwing & Bromfield (1983) that shows weak resistance to South American ASR pathogens. In contrast, PI 594538A, PI 594767A, PI 587905, and PI 587880A may carry the *Rpp1-b* as identified by Chakraborty et al. (2009) that shows strong resistance to South American ASR populations. ASR reaction profile of seven *Rpp1* carrying candidates in this study also supported this assumption by showing clear differences between *Rpp1* and *Rpp1-b* carrying lines in their reaction to three Brazilian ASR isolates (Table 4). However, Kim et al. (2012) suggested that ASR resistance genes from PI 587880A and PI 587886 were located in the same region between Satt191 and Sat_064. In addition, the order of molecular markers between genetic maps sometimes differs among the maps constructed by different parental combinations in soybean. As the *Rpp1* linkage maps of Hyten et al. (2007) and Ray et al. (2009) were previously reported to be different from the soybean consensus map of Song et al. (2004) by showing an inversion at Sat_372 and Sat_064. Furthermore, disease-resistance genes are known to cluster in some chromosomal regions in soybean (Jeong et al. 2001). *Rpp1/Rpp1-b* carrying lines may share different genes in such gene cluster. Thus, allelism test, fine mapping of resistant loci with DNA markers, or map-based cloning of genes are needed to be carried out to conclude if these two groups share different resistance alleles of same *Rpp* gene or tightly linked different ASR resistance genes.

The NoU and SL data in POP-C produced imperfect bimodal distributions, but interval mapping indicated that most of the variation is likely to be due to the incomplete dominance of a single gene that mapped to the vicinity of the *Rpp2* locus on chromosome 16. This incomplete dominance may be brought by the virulence of E1-4-12, since the kinds of ASR isolates are known to influence the level of dominance (Garcia et al. 2011; Lemos et al. 2011). It is also possible that some portion of the phenotypic variation was due to the different genetic backgrounds of the progeny and/or environmental factors. The resistant locus *Rpp2* was previously reported to confer resistance in PI 230970 and PI 224270 (Hougyoku), which was placed on chromosome 16 between SSR marker Sat_255 and Satt620 (Silva et al. 2008) and between SSR marker Satt215 and Sat_361 respectively Garcia et al. 2008). A direct comparison between our map and those of Silva et al. (2008) and Garcia et al. (2008) revealed that the order of markers that were common among the maps and resistance locus of these three maps were very similar except that *Rpp2* locus of Silva et al. (2008) seems to be far from Sat_366 compared with other two maps (Fig. 3). However, significant difference in reactions to ASR isolates was observed among three *Rpp2* carrying varieties. Resistance of PI 230970 was weakest to show susceptible against all four Brazilian ASR isolates, while Hougyoku was resistant against all tested isolate even though degree of resistance was different among ASR isolates used for inoculation (Table 4). Reaction profile of Iyodaizu B was different from those of PI 230970 and Hougyoku. Since resistance genes of them were mapped in the same region (Fig. 3), these three *Rpp2* lines may carry different *Rpp2* alleles, different tightly linked resistance genes or some other unknown genes except for *Rpp2*.

In conclusion, our study successfully mapped genes conditioning the resistance against ASR in three soybean lines, Xiao Jing Huang, Himeshirazu, and Iyodaizu B mainly. Since resistant alleles of these lines do not possess very high level of resistance against highly virulent Brazilian ASR isolates, these resistance genes may not be solely useful for ASR resistance in South America. However, pyramiding these genes with other available ASR resistance gene(s) in a commercial soybean cultivar may be proved useful in providing durable and enhanced resistance to strong and diverse *P. pachyrhizi* population in South America.

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