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Characterization of three soybean landraces resistant to Asian soybean rust disease

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Abstract *Phakopsora pachyrhizi* is an obligatory biotrophic fungus that causes Asian soybean rust (ASR) disease. ASR control primarily involves chemical control and the use of resistant soybean cultivars carrying an *Rpp* (resistance to *P. pachyrhizi*) gene. This study aimed to characterize the ASR resistance of three soybean Asian landraces. By screening the world core collection (WC) of soybean, which consists of 80 varieties, three landraces were identified in Southeast Asia as resistant to ASR. Genetic mapping using the F_2 population derived from a cross with an ASR-susceptible variety, BRS 184, indicated that KS 1034 (WC2) has ASR resistance conferred by a single dominant resistance gene, mapped on chromosome 18, in the same region where *Rpp1* was mapped previously. The BRS 184 × WC61 (COL/THAI/1986/THAI-80) F_2 population, on the other hand, showed an ASR resistance locus mapped by quantitative trait locus analysis on chromosome 6, in the region where the resistance conferred by PI 416764 *Rpp3* resides, with a logarithm of the odds score peak at the same position as the marker, Satt079, while the BRS 184 × WC51 (HM 39) population showed the resistance to ASR allocated between Satt079 and Sat_263 markers, also in the region where *Rpp3* was mapped previously. Both WC51 and WC61 have the same infection profile as FT-2 and PI 462312 when tested against the same ASR isolate panel. These three WCs can be used in MAS programs for introgression of *Rpp1* and *Rpp3* and the development of ASR-resistant cultivars in the breeding program.

Keywords *Phakopsora pachyrhizi* · Genetic resources · Resistance locus · *Glycine max*

Introduction

Asian soybean rust (ASR), caused by the obligatory biotrophic Basidiomycota fungus *Phakopsora pachyrhizi* (Sydow & Sydow), is one of the most severe diseases affecting soybean (*Glycine max*), causing losses of up to 80% in ideal conditions in the various geographic regions where it has been reported, costing annually an estimated US $1.77 billion, on average (Godoy et al. 2016).

Currently, the strategies for ASR management and control include the application of chemical fungicides (Embrapa 2019) and the use of specific cultivation practices, such as the elimination of secondary hosts and the introduction of soybean-free growth periods

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(sanitary periods) (Langenbach et al. 2016). Additionally, genetic resistance has been explored by developing cultivars carrying resistant genes, such as cultivar Inox® from TMG (Tropical Breeding & Genetics) and BRSMS Bacuri, BRSGO, and BRS 511 from Embrapa (Empresa Brasileira de Pesquisa Agropecuaria) in Brazil.

To obtain resistant cultivars, the genetic resistance identification in different soybean genotypes as well as the elucidation of defense mechanisms that contribute to the resistance to attack by *P. pachyrhizi*, represents important strategies for ASR control. Eight different *P. pachyrhizi* resistance loci (resistance to *P. pachyrhizi*: *Rpp*) have been identified and mapped in the soybean genome (*Rpp1* to *Rpp7*): *Rpp1* from PI 200492 (Hyten et al. 2007), *Rpp1-b* from PI 594538A (Chakraborty et al. 2009), *Rpp2* from PI 230970 (Silva et al. 2008), *Rpp3* in PI 462312 (Hyten et al. 2009), *Rpp4* in PI 459025 (Silva et al. 2008), *Rpp5* in PI 200456 (Garcia et al. 2008), *Rpp6* in PI 567102B (Li et al. 2012), and *Rpp7* in PI 605823 (Childs et al. 2017).

Depending on the *Rpp* gene present in the soybean and the *Avr* gene of the *P. pachyrhizi* isolate involved in the interaction, different symptoms are observed: an incompatible-type interaction of the soybean plant in response to the pathogen; an expressed immune reaction governed by the resistance gene *Rpp1* (the plant shows no visible symptoms), or the formation of reddish-brown lesions (RB) governed by the other *Rpp* genes resulting from programmed cell death and promoting the limitation of sporulation and fungal growth; and the susceptible reaction, characterized by tan-colored lesions (TAN), resulting from the total sporulation of *P. pachyrhizi* pustules (Van de Mortel et al. 2007).

It has been observed that some ASR resistance genes in soybean have functional annotations as belonging to the leucine-rich repeat (LRR) receptor family (Meyer et al. 2009). Binding of the specific effectors from one isolate results in conformational changes in the receptor and, subsequently, activation of the signal cascade, culminating in the activation of plant defense genes. However, the limited set of receptors in soybean and a large number of ASR effectors, which change constantly, make the maintenance of resistance in the host challenging, making evident the importance of studies to identify new sources of resistance in soybeans and the development of new resistant cultivars.

For the selection of candidates for sources of new *Rpp* genes, world core collection (WC), assigned by the National Institute of Agrobiological Sciences (NIAS) (Kaga et al. 2012), has soybean genotypes with high genetic diversity and represents a source of potential candidates.

In this study, we aimed to determine the ASR resistance locus of WC germplasm landraces, identified as resistant to ASR after screening, which possibly have new *G. max Rpp* locus.

Materials and methods

Plant materials

The world soybean core collection (WC), which consists of 80 soybean varieties, was used to screen the ASR-resistant varieties in this study (Supplementary Sheet 1). The leaflets from a single plant of all varieties and the leaflets from three plants of the selected varieties were used in the primary and secondary screenings, respectively. The soybean genotypes used as parents in the present study included the Brazilian cultivar BRS 184 (used as a female), which has no ASR resistance *Rpp* gene (susceptible), and three Asian landraces WC2 (KS 1034 from Malaysia), WC51 (HM 39 from India), and WC61 (COL/THAI/1986/THAI-80 from Thailand) (used as males), identified in the screening steps as ASR-resistant materials.

The three selected resistant genotypes were crossed with an ASR-susceptible Brazilian cultivar BRS 184 (BRS 184 × WC2, BRS 184 × WC51, and BRS 184 × WC61), resulting in the F_2 mapping populations with 187, 152, and 137 plants from six, eight, and three F_1 plants, respectively, which were used in the present study. In each combination, a subset of 24 plants in the F_2 population was chosen randomly and tested from each crossing to check that there is an association between the segregations of the resistance phenotype and DNA markers tagging known *Rpp* loci.

All soybean plants used for the resistance evaluation in the present study were cultivated and maintained following the methodology described by Yamanaka et al. (2010).

Pathogen inoculation and resistance evaluation

The urediniospores used in this study were kept and multiplied in detached leaves of the susceptible soybean

genotype BRS 184. The collection, preservation, multiplication, and concentration adjustment of spores were performed following the methodology provided by the manual (Yamanaka et al. 2019). After collection, the spores were dehydrated in silica gel (overnight) and stored in an ultra-freezer at − 80 °C until use. Before use, spore dormancy was broken by heating to 39 °C for 60 s.

Three leaves of the first trifolium of each plant were inoculated with urediniospores when they reached the development stages V3–V4 (approximately 3 weeks old), according to the scale proposed by Fehr and Caviness (1977). The leaves were collected, cleaned in sterile deionized water, and kept in Petri dishes. Subsequently, the inoculum was performed by applying approximately 0.1 mL of a solution on the abaxial face of the leaf, consisting of urediniospores with a concentration of 5×10^4 spores mL^{-1}, resuspended in 0.04% Tween-20 (polyoxyethylene sorbitan monolaurate, Promega) using a sterile paintbrush. Concomitantly, drops of the inoculum solution were dripped under agar glass slides to evaluate viability by counting the germinated spores (300 spores or more). After inoculation, the germination test agar plate and leaves remained in the dark overnight, and after this period, they were kept in a growth chamber (Biotron, Nippon Medical & Chemical Instruments, Co.), according to the following parameters: temperature 21 °C, a photoperiod of 12 h of light, and a luminosity of approximately 3000 lx (Yamanaka et al. 2019).

Fourteen days after inoculation, the reactions to the ASR isolates were evaluated by analyzing the following parameters: number of uredinia per lesion (NoU) and sporulation level (SL) per the scales described in Yamanaka et al. (2010). For classification, 30 lesions (10 in each leaflet) were evaluated in the abaxial leaf surface, where plants with SL and NoU lesions with values ≤ 1.5 and 1.2, respectively, were considered resistant and values above them were considered susceptible.

For the selection of ASR-resistant soybean genotypes, 80 soybean accessions were selected from the WC with 96 genotypes (Kaga et al. 2012). The candidates went through two screening stages: first, by using the mixture of two Japanese isolates (E1-4-12 and T1-2) (Yamaoka et al. 2014). Second, a new round of inoculation, phenotyping, and selection was performed using a Brazilian isolate (BRP-2.6) (Yamanaka et al. 2013) and T1-2. For the initial screening, resistant genotype candidates were screened based on the visual scoring of SL: 0, 1, 2, or 3 through the naked eye using the scale described in Yamanaka et al. (2010). For the secondary screening of the selected genotypes, SL was determined microscopically based on 30 lesions.

Genotyping with simple sequence repeat markers

To determine the location of the putative resistance loci of each of the three screened landraces, a subset of 24 F_2 plants in each population was genotyped with the simple sequence repeat (SSR) markers representative of the *Rpp1* to *Rpp7* loci. For the WC2 population, the markers used were *Rpp1*: Sat_064; *Rpp2*: Satt620; *Rpp3*: Sat263; *Rpp4*: SSR18_1576; *Rpp5*: Sat_280; *Rpp6*: Satt324; and *Rpp7*: SSR19_1014. For the WC51 population, the markers used were *Rpp1*: Sat_064; *Rpp2*: SSR16_0908; *Rpp3*: Sat263; *Rpp4*: SSR18_1576; *Rpp5*: Sat_280; *Rpp6*: SSR18_0392; and *Rpp7*: SSR19_1014. For the WC61 population, the markers used were *Rpp1*: SSR66; *Rpp2*: SSR16_0908; *Rpp3*: Sat263; *Rpp4*: SSR18_1576; *Rpp5*: Sat_280; *Rpp6*: Satt324; and *Rpp7*: Satt076. These markers were selected from the known genetic maps of *Rpp*s (Yamanaka et al. 2019), and polymorphisms between parents were checked in advance.

If the genotyping resulted in one of the markers being significant for SL or NoU in the 24-plant subset, the other markers surrounding this marker were selected and used to genotype the entire F_2 population from each cross. For the BRS 184 × WC2 population, SSR markers were used for the *Rpp1* resistance locus: Sat_117, SSR18-1793, Sct_187, Sat_064, SSR24, and Sat_372. For the BRS 184 × WC51 and BRS 184 × WC61 populations with probable resistance at the *Rpp3* locus, SSR markers were used: Sat_251, Sat_238, Satt460, Satt079, Sat_263, SSR06_1554, and Satt_307. The DNA extraction, polymerase chain reaction (PCR), and electrophoresis were performed following the procedures described by Yamanaka et al. (2010).

Initially, DNA was extracted from the young unifoliate leaves of each plant, collected, and frozen in an ultra-freezer before the ASR inoculation step, using the modified CTAB (cetyl trimethylammonium bromide) method by Yamanaka et al. (2010). After extraction, the DNA concentration was determined by the reading on a NanoDrop spectrophotometer (Thermo Fisher Scientific; 260 nm) and diluted to the final concentration of 50 ng μL^{-1} for use in the PCR. After the reaction,

acrylamide gel electrophoresis was performed on all samples. Subsequently, images were obtained by scanning the gel and analyzed for the resulting band patterns.

Genetic mapping of ASR resistance

Frequency segregation of resistance data, phenotype data, and marker genotype data from each of the three F_2 populations were analyzed by performing the goodness-of-fit χ^2 (chi-square) test to compare with expected segregation rates. An analysis of variance (ANOVA) test and linear regression analysis were also performed to determine if there was significance in the association between ASR resistance and SSR markers used in genotyping and to determine genetic effects (additives and dominance).

The distances (cM), linkage, and SSR marker orders were calculated using Kosambi's function in the MAPMAKER/EXP v.3.0 software (Lander et al. 1987). A significance logarithm of the odds (LOD) score of 3.0 and a maximum genetic distance of 37.2 cM (centimorgan) for the linkage map were used as the threshold. The WC61 genomic region associated significantly with the NoU and SL was detected using interval mapping from the Windows QTL Cartographer software v.2.5.011 (Wang et al. 2012). Other parameters defined for the quantitative trait locus (QTL) analysis were 0.5 cM walk speed, 1000 permutations (permutation test), and a 0.01 significance level, following the methodology and parameters employed by Yamanaka et al. (2015). The resistance gene position in the QTL analysis was defined as the maximum LOD score.

WC2, WC51, and WC61 reaction profiles to the ASR isolates panel

A total of seven genotypes carrying the resistance genes *Rpp1* and *Rpp1-b* were compared to determine allelic variations of these loci in soybean chromosome 18, with the soybean landrace WC2 under study, against four ASR isolates from Brazil (BRP-2.1, BRP-2.5, BRP-2.6, BRP-2.49), two from Japan (E1-4-12 and T1-2), and one from Mexico (MRP-16). Candidate genotypes carrying the *Rpp1* gene included PI 200492, Himeshirazu, and PI 587886, while candidate genotypes carrying the *Rpp1-b* gene included PI 587905, PI 594767A, PI 587880A, and PI 587855 (Supplementary Sheet 1).

For candidates for the *Rpp3* gene sources, a comparison of infection reactions of a total of four *Rpp3* genotypes was performed to determine allelic variations of the locus between different sources, with soybean landraces WC51 and WC61 under study, against the same panel of isolates used in the evaluation of genotypes carrying *Rpp1* and *Rpp1-b*. Candidate genotypes carrying the *Rpp3* gene included Hyuuga, FT-2, PI 462312, and PI 416764 (Supplementary Sheet 2).

Results and discussion

Screening and selection of the world core collection

To select the resistant candidates, 80 soybean accessions from the WC were screened. After primary accession screening using two weak-virulent Japanese ASR isolates (E1-4-12 and T1-2), 13 accessions with resistance phenotypes were selected. Subsequently, a new round of inoculation, phenotyping, and selection was performed using a panel composed of a Brazilian isolate (BRP-2.6) and T1-2 (Supplementary Sheet 1).

Thirteen soybean accessions presented resistance to the mixture of two Japanese isolates in the first screening stage and were selected for the second screening stage (Supplementary Sheet 1). Three landraces, WC2 (KS 1034) from Malaysia, WC51 (HM 39) from India, and WC61 (COL/THAI/1986/THAI-80) from Thailand, presented an immune reaction to the Japanese isolates used in the first step of screening and resistance symptoms to the strong-virulent Brazilian isolate BRP-2.6. For this reason, these three Asian landraces were crossed with susceptible cultivar BRS 184 to generate the mapping populations.

ASR resistance of three resistant accessions and their progenies

A comparison of NoU and SL values by Japanese ASR isolates between susceptible BRS 184 and resistant parents WC2, WC51, and WC61 showed that there are significant differences between them (Supplementary Sheet 3).

The susceptible Brazilian cultivar BRS 184 presented a NoU above the threshold value of 1.2 and a maximum SL of 3.0, against both isolates tested. In contrast, WC2, WC51, and WC61 showed resistance against these isolates. WC51 and WC61 showed no formation of lesions

against the T1-2 isolate. Inoculation of the E1-4-12 isolate resulted in the formation of six RB-type lesions in WC2; however, this was without the formation of uredinia and spores (SL and NoU = 0.0). A higher susceptibility of BRS 184 against the T1-2 isolate (NoU = 2.40 to 2.80) than E1-4-12 (NoU = 2.30) was observed, which was the same result observed by Yamanaka et al. (2015) using the same isolates (Supplementary Sheet 3).

The results of the ASR resistance segregation for WC2 and WC51 F_2 populations are shown in Supplementary Sheet 2. In the F_2 population of BRS 184 × WC2, 125 plants had phenotypes that were classified as resistant and 38 plants exhibited susceptible lesions (Supplementary Sheet 3). The WC51 F_2 population showed a segregation of 95 plants with resistant phenotypes and 33 plants with susceptible phenotypes. These frequencies fit the expected segregation ratio for F_2 of 3:1, according to the χ^2 test, indicating that the ASR resistance observed in WC2 and WC51 was controlled by a single dominant gene (Supplementary Sheet 2). The degree of dominance (d/a) (Table 1) values of the complete dominance for resistance were evident for both populations.

Phenotypic analysis of the WC61 F_2 population revealed a wide distribution of NoU (parental 0.0–2.8 and F_2 plants 0.0–3.3) and SL (parental 0.0–2.4 and F_2 0.0–3.0) values as well as the presence of plants with intermediate phenotypes (plants with NoU values classified as resistant, SL values as susceptible, and vice versa), according to the scale provided by Yamanaka et al. (2010) (Supplementary Sheet 3). Due to a lack of clarity in the classification of the samples as resistant and susceptible, the segregation of the characteristics was unclear in the WC61 F_2 population. For this reason, a QTL analysis was performed to map the ASR resistance loci in WC61. As observed in Table 1, the WC61 F_2 population had incomplete dominance (d/a < 1) and a high value for the dominance effect (*d*), which may explain the undefined phenotypes in this population.

Genetic mapping of ASR resistance in WC2, WC51, and WC61

Genotyping of the 24 individual subsets from each F_2 population of BRS 184 × WC2, BRS 184 × WC51, and BRS 184 × WC61, with specific markers for *Rpp1* to *Rpp7* loci, indicated that resistance to WC2 is associated with the marker for *Rpp1*, whereas in WC51 and WC61, it was locus *Rpp3* (Supplementary Sheet 4).

Genotyping all 165 individuals of the WC2 F_2 population with five SSR markers from the *Rpp1* and *Rpp1-b* locus region (Sat_117, SSR18-1793, Sct_187, Sat_064, SSR24, and Sat_372) showed that there was a significant and highest association between Sct_187 and the variation of resistance characteristics NoU ($P = 4.29 \times 10^{-68}$) and SL ($P = 9.47 \times 10^{-70}$). The variance explained by Sct_187 was accounted for by $R^2 = 0.60$ for both NoU and SL, respectively (Table 1 and Supplementary Sheet 5). Genetic mapping of the resistance loci in WC2 with six markers allocated resistance loci in a 4.4-cM region on soybean chromosome 18, which includes the *Rpp1* resistance locus, mapped previously by Hyten et al. (2007), Ray et al. (2009), Kim et al. (2012), Yamanaka et al. (2015, 2016), and Hossain et al. (2015), and it was found in a region different from where *Rpp1-b* was mapped (Chakraborty et al. 2009). It was flanked on one side by the Sct_187 marker and on the other side by the Sat_064 and SSR24 markers and was present in the same region where Himeshirazu *Rpp1* was mapped previously by Yamanaka et al. (2015) (Fig. 1). The physical distance between these markers, based on the *G. max* genome (Gmax 2.0), was 149.9 kb (Soybase 2018). The additive effects of the single WC2 allele in contrast to BRS 184 on NoU and SL were − 0.81 and − 1.25, respectively (Table 1). The degree of dominance (d/a) was 1.15 and 1.16 for NoU and SL, respectively, demonstrating complete dominance of resistance at this locus (Table 1).

Genotypic data from all 128 individuals of the WC51 population tested with markers for *Rpp3* (Sat_251, Sat_238, Satt460, Satt079, Sat_263, and SSR06_1554) indicate a significant association of the Satt079 marker and the NoU and SL characteristics ($P = 5.41 \times 10^{-78}$ and 5.06×10^{-87}, respectively), as well as the phenotypic variations of each ($R^2 = 0.72$ and 0.73, respectively) (Table 2 and Supplementary Sheet 6). The WC51 ASR resistance locus was mapped between the Satt460 and Satt079 markers in the same region where *Rpp3* was mapped in the previous study with sources of this locus (PI 416764 by Hossain et al. (2015) and PI 462312 by Hyten et al. (2009); Fig. 2). The region has 0.8 cM of soybean chromosome 6 and represented a physical distance of 453.9 kb in the soybean variety Williams 82 (Soybase 2018). The additive effect of the WC51 allele in this locus reducing the NoU and SL is in the order of − 0.92 and − 1.26, respectively, when compared with that of the BRS 184 allele. The degree of dominance was 1.09 and 1.07 for the NoU and SL, respectively,

Table 1 Association between Asian soybean rust (ASR) resistance and simple sequence repeat (SSR) markers in BRS 184 × WC2, BRS 184 × WC51, and BRS 184 × WC61 F_2 populations, calculated by one-way ANOVA and regression analysis

Population	Resistance characters*	Markers[a]	Mean	SD	One-way ANOVA		R^{2c}	Genetic effect (B against A, single allele)		
					F value	*P*[b]		Additive effect (a)	Dominance effect (d)	d/a[d]
BRS 184 × WC2	NoU	Sct_187: A	1.86	0.22	476.134	4.3E−68	0.60	− 0.81	− 0.94	1.15
		Sct_187: H	0.14	0.35						
		Sct_187: B	0.01	0.63						
		All	0.46	0.79						
	SL	Sct_187: A	2.86	0.24	503.291	9.5E−70	0.60	− 1.25	− 1.45	1.16
		Sct_187: H	0.20	0.54						
		Sct_187: B	0.02	0.95						
		All	0.69	1.20						
BRS 184 × WC51	NoU	Satt079: A	2.13	0.43	476.134	5.4E−78	0.72	− 0.92	− 1.02	1.09
		Satt079: H	0.23	0.28						
		Satt079: B	0.01	0.74						
		All	0.67	0.94						
	SL	Satt079: A	2.86	0.52	503.291	5.1E−87	0.73	− 1.26	− 1.35	1.07
		Satt079: H	0.31	0.35						
		Satt079: B	0.01	0.89						
		All	0.90	1.25						
BRS 184 × WC61	NoU	Satt079: A	1.74	1.27	476.134	3.08E−30	0.67	− 1.17	− 0.34	0.28
		Satt079: H	1.12	0.63						
		Satt079: B	0.61	0.54						
		All	1.17	0.96						
	SL	Satt079: A	1.94	1.45	503.291	7.74E−27	0.65	− 1.30	− 0.17	0.13
		Satt079: H	1.51	0.78						
		Satt079: B	0.84	0.76						
		All	1.47	1.10						

SD, standard deviation; **NoU*, number of uredinia per lesion; *SL*, sporulation level

[a] Marker genotype: A: homozygous susceptible (BRS 184), H: heterozygous, B: homozygous resistant (WC varieties)

[b] *P*: probability of significance calculated by ANOVA

[c] R^2 : coefficient of determination calculated by regression analysis (for the selected marker)

[d] Degree of dominance: 1 = under complete dominance for resistance; 0 = lack of dominance; − 1 = under complete dominance for susceptibility

demonstrating complete dominance of resistance at this locus (Table 1).

Genotyping of all 113 plants in the WC61 F_2 population with the *Rpp3* markers allowed mapping of the locus to control NoU and SL resistance characteristics using the interval mapping of the QTL analysis. A LOD score peak was detected for the characteristics NoU (LOD value = 30.30) and SL (LOD value = 26.81) in the same position as Satt079 (Fig. 2). Thus, WC61 showed locus-controlling resistance for the NoU and SL characteristics to ASR in the same region as locus *Rpp3*, similar to WC51, whose resistance co-segregates with the Satt079 marker. The additive effect of the resistance of this locus reducing NoU and SL is in the order of − 1.17 and − 1.30 (Table 1), respectively. The degree of dominance was low, and it was 0.28 and 0.13 for NoU and SL, respectively (Table 1), showing incomplete dominance of resistance at this locus. This difference in the degree of dominance between WC51 (complete dominance) and WC61 (incomplete dominance) was evidence that their *Rpp3* allele was different, even though they shared a similar pattern of symptoms

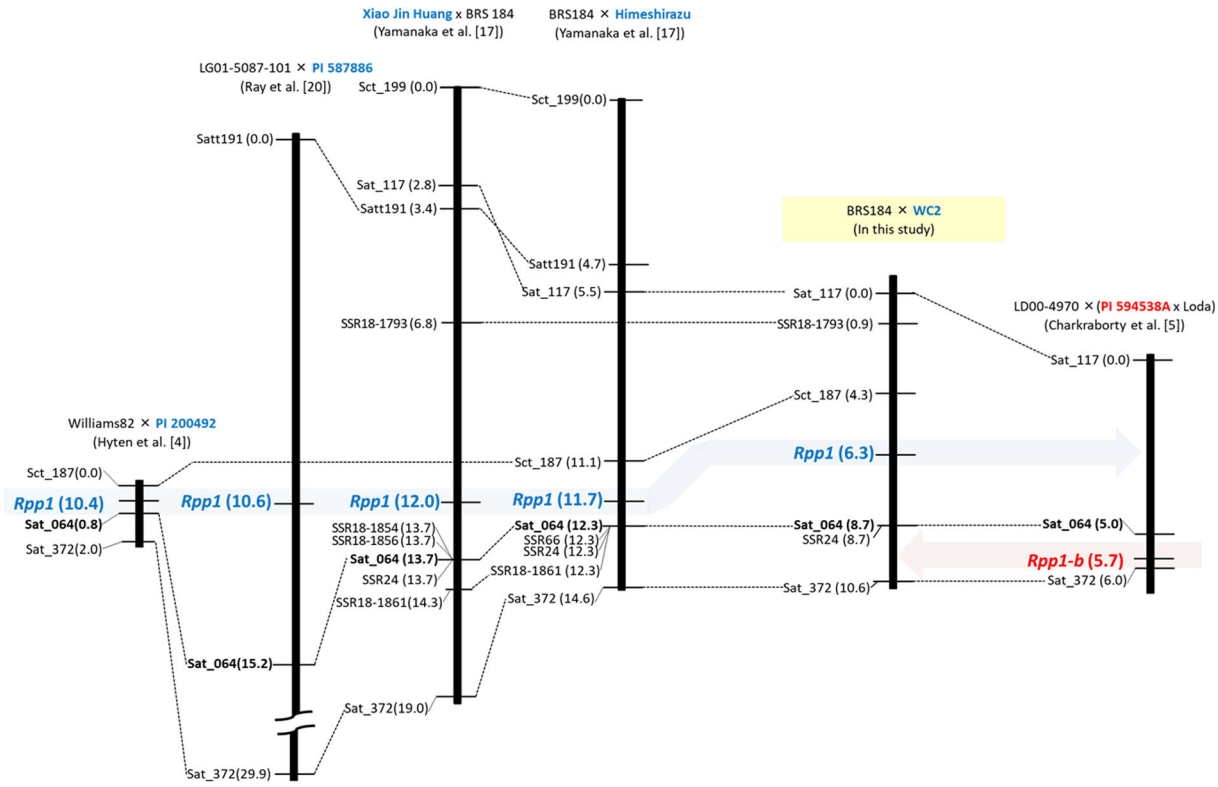

Fig. 1 Genetic linkage map location of *Rpp1* conferring resistance to Asian soybean rust (ASR) on chromosome 18 (linkage group G) in one mapping population in this study, compared with the location of *Rpp1-b* (map of the locus in PI 594538A as reported by Chakraborty et al. 2009) and *Rpp1* (map of the locus in PI 200492 reported by Hyten et al. 2007), in PI 587886 by Ray et al. (2009) and in Xiao Jin Huang and Himeshirazu by Yamanaka et al. (2015, 2019). Map location of *Rpp1* of WC2 based on the segregation of 163 F_2 plants from the BRS 184 × WC2 population. On the left is the name of the SSR markers used in the mapping process, along with the distances (cM) generated using Kosambi's function in the software MAPMAKER/EXP v.3.0

to the ASR panel. The variance explained by Satt079 was accounted for as $R^2 = 0.67$ for NoU and $R^2 = 0.65$ for SL (Table 1 and Supplementary Sheet 7). Incomplete dominance provided an advantage in breeding programs as it allowed the separation of resistant homozygous plants from heterozygous plants based on phenotypic data, with an intermediate phenotype in the latter case. Since complete dominance of resistance does not allow a distinction between homozygous and heterozygous plants, based only on the phenotype (the same as in this case), it is necessary to make a selection assisted by molecular markers or a progeny test in this case.

For the genetic mapping of ASR resistance in WC51 and WC61, phenotyping data by E1-4-12 inoculation were not used as it was observed that the inoculum and/or the inoculation process presented problems. This was observed in many samples in the F_2 population tested, where approximately 27% (WC61) to 67% (WC51) showed no symptoms after inoculation. This was true even with samples with the S genotype. Another possible explanation would be the effect of the genetic background, besides the presence of *Rpp3*, which would provide resistance against the weak-virulent isolate. For these reasons, the isolate E1-4-12 was not used to evaluate the WC51 and WC61 segregating populations.

Determination of putative resistance alleles in WC2, WC51, and WC61

A comparison of infection reactions of WC2 with genotypes carrying the *Rpp1* gene (PI 200492, Himeshirazu, and PI 587886) and the *Rpp1-b* gene (PI 587905, PI 594767A, PI 587880A, and PI 587855) showed that the candidate WC2 has the same profile of reactions as the isolate panel (BRP-2.1, BRP-2.5, BRP-2.6, BRP-2.49, E1-4-12, T1-2, and MRP-13.18 isolates) as the Himeshirazu genotype (Table 2). WC2 and

Table 2 Comparison of infection reactions of seven genotypes carrying the resistance loci *Rpp1* and *Rpp1-b* with soybean accession WC2 under study, against 4 ASR isolates from Brazil (BRP-2.1, BRP-2.5, BRP-2.6, BRP-2.49), 2 from Japan (E1-4-12 and T1-2), and 1 from Mexico (MRP-13.18)

Genotype	*Rpp* gene	Infection type to ASR isolates[1]						
		E1-4-12	T1-2	BRP-2.1	BRP-2.5	BRP-2.6	BRP-2.49	MRP-13.18
WC2	***Rpp1***	**I**	**S**	**S**	**S**	**S**	**S**	**HR**
BRS 184*	–	S	S	S	S	S	S	S
PI 587886	(*Rpp1*)	S	S	S	S	S	S	S
Himeshirazu	**(*Rpp1*)**	**I**	**S**	**S**	**S**	**S**	**S**	**HR**
PI 200492	(*Rpp1*)	I	HR	S	S	S	S	I
PI 587905	(*Rpp1-b*)	I	HR	R	S	HR	R	S
PI 594767A	(*Rpp1-b*)	I	I	HR	S	HR	I	S
PI 587880A	(*Rpp1-b*)	I	HR	HR	S	I	HR	S
PI 587855	(*Rpp1-b*)	I	HR	HR	S	I	I	S

[1] S: susceptible; SR: slightly resistant; R: resistant; HR: highly resistant; I: immune;
*BRS 184: susceptible control

Bold letters represent genotypes that share the same / similar phenotype pattern

Himeshirazu presented an E1-4-12 immune phenotype, susceptible to T1-2, BRP-2.1, BRP-2.5, BRP-2.6, and BRP-2.49 and highly resistant to MRP-13.18, a pattern observed only in these two accessions (Table 2). Yamanaka (2015) mapped ASR resistance in Himeshirazu in the same region, between Sct_187 and

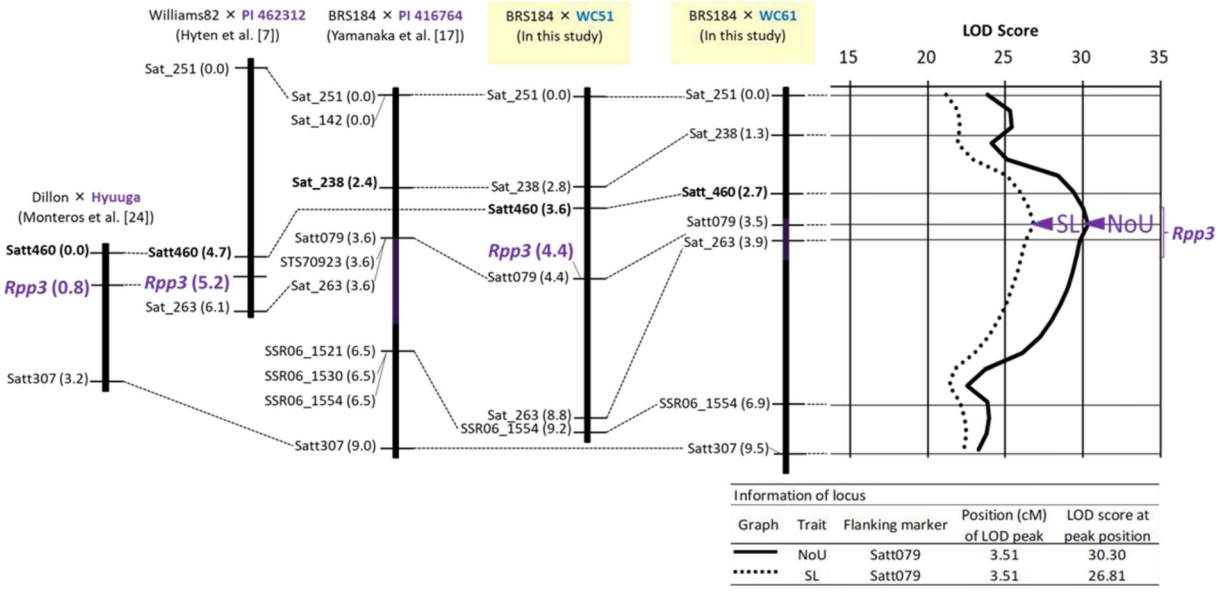

Fig. 2 Genetic linkage map location of *Rpp3* conferring resistance to Asian soybean rust (ASR) on chromosome 6 (linkage group C2) in two mapping populations in this study, compared with the location of *Rpp3* (map of the locus in Hyuuga reported by Monteros et al. 2007), in PI 462312 by Hyten et al. (2009) and in PI 416764 by Hossain et al. (2015) and Yamanaka et al. (2019). Map location of *Rpp3* was based on the segregation of 128 F_2 plants from the BRS 184 × WC51 population and 113 F_2 plants from the BRS 184 × WC61 population. On the left is the name of the SSR markers used in the mapping process, along with the distances (cM) generated using Kosambi's function in the software MAPMAKER/EXP v.3.0 (BRS 184 × WC51 population) and Windows QTL Cartographer v.2.5.011 (BRS 184 × WC61). The resistance locus of WC61 was estimated by peak positions of the logarithm of the odds (LOD) score curves obtained by the quantitative trait locus (QTL) analysis for NoU (number of uredinia) and SL (sporulation level)

Sat_064 markers, and although WC2 had a larger additive effect (a) than that observed in Himeshirazu (NoU = −0.76 and SL = −0.96), likely due to the higher susceptibility of BRS 184 against E1-4-12 in this study, the value of R^2 (NoU = 0.65 and SL = 0.66) and degree of dominance (d/a) (NoU = 1.0 and SL = 1.0) were very similar. This indicates that WC2 and Himeshirazu may share the same ASR resistance allele.

PI 587886 was the only genotype that showed susceptibility to all the isolates tested, as well as the susceptible cultivar BRS 184. Akamatsu et al. (2013) had already observed that PI 587886 has a susceptibility phenotype against Brazilian isolates and some Japanese isolates obtained in the same region in the state of Ibaraki as E1-4-12 and T1-2 (Yamaoka et al. 2014). The other genotypes tested presented their own unique patterns, but they most commonly showed susceptibility to the Brazilian isolate BRP-2.5. PI 200492 showed susceptibility to all Brazilian isolates. All genotypes carrying the resistance *Rpp1-b* locus showed common immunity to the Japanese isolate E1-4-12 and susceptibility to the isolate BRP-2.5 and to the Mexican isolate MRP-13.18 (although at different levels) compared with the other isolates tested (Table 2). The test results of the seven isolates indicated clear allelic differences in the region of *Rpp1* and *Rpp1-b* based on the different symptom patterns observed between the sources. There are even differences between the sources of *Rpp1* (Himeshirazu and PI 200492), for example, with two distinct symptom patterns to the tested ASR panel (Table 2). WC2 differed from the original source of *Rpp1* PI 200492 (Hyten et al. 2007), which was resistant to both the Japanese ASR isolates and has a similar symptom profile to Himeshirazu (resistant to E1-4-12 and susceptible to T1-2). *Rpp1* has already been observed to confer resistance to ASR in the USA (Paul et al. 2015; Miles et al. 2006), and against Japanese isolates by Akamatsu et al. (2013, 2017) and Hossain and Yamanaka (2019). However, *Rpp1* is susceptible to most South American isolates from Brazil, Argentina, and Paraguay, thus differing from soybean accessions that have the resistance locus *Rpp1-b* that showed high resistance to the ASR population of this region, between the 2007 and 2015 crops (Akamatsu et al. 2013, 2017). A previous study by Chakraborty et al. (2009) had already demonstrated differences between the *Rpp1* and *Rpp1-b* loci, in which *Rpp1-b* was allocated between Sat_064 and Sat_372 markers, almost 1 cM from which *Rpp1* was allocated previously (in a genetic map region above the marker Sat064) (Hyten et al. 2007). He also observed differences in symptoms between *Rpp1-b* (PI 594538A: resistant) and *Rpp1* (PI 200492: susceptible), after inoculation with the Zimbabwe isolate ZM01-1. Furthermore, differences between them were observed by Yamanaka (2015) in the reactions to different Brazilian ASR isolates, and Himeshirazu and Xiao Jing Huang (*Rpp1*) genotypes were mapped in a region different from the *Rpp1-b* locus from where Hossain et al. (2015) (PI 594767A and PI 587905) had mapped them. The results of the seven isolates' symptom test and the location where the resistance was mapped corroborate the observations of Yamanaka (2015), which found clear differences in reactions to different isolates between accessions with the *Rpp1* locus and genotypes with the *Rpp1-b* locus, resulting from possible genetic differences between them (different alleles, differences in the location where resistance is allocated and the genes present in the region).

A comparison of infection reactions of WC51 and WC61 with genotypes carrying the *Rpp3* gene (PI 462312, PI 416764, Hyuuga, and FT-2) allowed us to observe that candidates WC51 and WC61 have a putative resistance locus that demonstrates the same reaction profile to the panel that isolates as the PI 462312 and FT-2 genotype (Table 3). Initially, only WC51 and PI 462312/FT-2 presented an identical phenotype set: immune to E1-4-12, resistant to T1-2, susceptible to BRP-2.1, BRP-2.5, BRP-2.6, and BRP-2.49, and resistant to MRP-13.18, with WC61 differing in symptoms from T-1-2 (no lesions). However, further phenotyping of WC61 along with the F_2 population derived from the BRS 184 × WC61 crossing revealed that this accession could present a few lesions without uredinia and spores (resistance phenotype) to the Japanese isolate T1-2. Thus, WC51 and WC61 have the same profile of symptoms as FT-2 and PI 462312. It is possible that the resistance present in WC61, despite being located in the same region as WC51 and presenting very similar symptom patterns, may be derived from different alleles. As observed in Table 3, unlike WC51, WC61 presented an allele with incomplete dominance (d/a < 1) (Table 1). In addition, the symptom pattern to the ASR panel of isolates varied slightly between them and was not identical in its entirety, which was expected since the varieties have different geographic origins (India and Thailand) and, consequently, have differences in their genetic basis and genealogy, which explain the differences in

the ability to recognize and respond to an isolate of ASR.

In this study, the *Rpp3* resistance loci (PI462312, PI 416764, Hyuuga, and FT-2 genotypes) showed high resistance to Japanese ASR isolates, which was observed by Yamanaka (2015) against a Japanese ASR population and by Akamatsu et al. (2017) and Hossain and Yamanaka (2019) against seven Japanese isolates, and susceptibility to Brazilian isolates. In this study, PI 416764 and Hyuuga showed resistance to all isolates tested. Hyuuga was a result of the crossing between PI 416764 (*Rpp3*) and the Japanese variety Asomusume (Yamanaka et al. 2019), which accounts for its similarity with PI 416764 in the symptom profile to the panel of isolates tested. PI 416764 had already shown greater resistance to ASR isolates in a study by Hossain and Yamanaka (2019), where 13 Bangladesh isolates were tested with fewer uredinia and urediniospores than PI 462312. Miles et al. (2019) tested two mixtures of USDA ASR isolates and observed that PI 462312 showed a susceptible phenotype for both mixtures, while PI 416764 showed a resistance phenotype in the same assays. The study by Akamatsu et al. (2013) showed that PI 416764 is resistant to 54% of the 24 South American isolates tested in its study, while PI 462312 was resistant to 29%. In a study by Hossain et al. (2015), the genotype PI 462312 presented a resistant phenotype mapped at the *Rpp3* locus with incomplete dominance, WC61, values of the additive effect (NoU = − 1.17 and SL = − 1.02), dominance effect (NoU = 0.07 and SL = − 0.20), and degree of dominance (d/a) value (NoU = − 0.06 and SL = 0.20), similar to those observed in the present study against the same Japanese T1-2 isolate (Table 1). These facts, along with the pattern of symptoms to the ASR panel shared between them, indicate that WC61 and PI 462312 may share the same ASR-resistant allele.

All results validate the genotyping data with specific markers for *Rpp1* and *Rpp3* from the results of genetic mapping and confirm that WC2 has resistance to ASR at locus *Rpp1*, while WC51 and WC61 have resistance to ASR located at locus *Rpp3*. Although resistance was allocated in the same region, WC51 and WC61 show different symptom patterns against the isolate T1-2 (unclear segregation in WC61) (Supplementary Sheet 3), as well as differences in the degree of dominance (d/a) (lack of dominance in WC61) (Table 1), indicating that they may differ as to the allele of resistance. A possible explanation for the difference in the ability to recognize and respond to the T1-2 isolate between these two genotypes is that they have different geographic origins, and consequently, they have different genetic differences that explain these variations in their ASR resistance. WC51 originates in India, whereas WC61 originates in Thailand, and both may have undergone different co-evolution processes with different ASR populations. They have undergone different selective pressures from different isolates present in the region in which they were located, and therefore, different alleles or genes present in the region of *Rpp3* were selected and maintained in each of them.

Table 3 Comparison of infection reactions of 4 genotypes carrying the resistance locus *Rpp3* with soybean accessions WC51 and WC61 under study, against 4 ASR isolates from Brazil (BRP-2.1, BRP-2.5, BRP-2.6, BRP-2.49), 2 from Japan (E1-4-12 and T1-2), and 1 from Mexico (MRP-13.18)

Genotype	*Rpp* gene	Infection type to ASR isolates[1]						
		E1-4-12	T1-2	BRP-2.1	BRP-2.5	BRP-2.6	BRP-2.49	MRP-13.18
WC51	***Rpp3***	**I**	**R**	**S**	**S**	**S**	**S**	**R**
WC61	***Rpp3***	**I**	**I**	**S**	**S**	**S**	**S**	**R**
BRS 184[*]	–	S	S	S	S	S	S	S
PI 462312	**(*Rpp3*)**	**I**	**R**	**S**	**S**	**S**	**S**	**R**
PI 416764	*(Rpp3)*	I	HR	SR	SR	SR	HR	R
Hyuuga	*(Rpp3)*	I	R	HR	SR	SR	HR	R
FT-2	***(Rpp3)***	**I**	**R**	**S**	**S**	**S**	**S**	**R**

[1] S: susceptible; SR: slightly resistant; R: resistant; HR: highly resistant; I: immune;
[*]BRS 184: susceptible control

Bold letters represent genotypes that share the same / similar phenotype pattern

Conclusion

The present study allowed the mapping of resistance to ASR present in accessions WC2, WC51, and WC61, which now represent new varieties that are sources of loci *Rpp1* and *Rpp3* in marker-assisted soybean breeding. Although none of the three accessions was resistant to isolates from Brazil, they were resistant to isolates from Japan (E1-4-12 and T1-2) and Mexico (MRP-13.18) and may be used as sources of resistance to these isolates by introgression in breeding programs in combination with other cultivars and *Rpp* genes, to improve the resistance against a wider range of ASR isolates. Combining two or more *Rpp* by gene pyramiding may be an interesting way to ensure long-term resistance to ASR and against a broad group of different pathogen isolate populations, while avoiding chemical control and its environmental and economic impacts. In addition, ASR has high diversity in its pathogenicity (Zhang et al. 2012), proven by the identification of several different isolates (Yamaoka et al. 2014; García-Rodríguez et al. 2017; Hossain and Yamanaka 2019), and the limited resistance of each *Rpp* against the different ASR populations (Akamatsu et al. 2017) makes it difficult to maintain long-term resistance and highlights the importance of finding new sources of *Rpp* for use in breeding programs.

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Author contributions All authors LNA, YM, and NY contributed to designing the research, performing the experiments, analyzing the data, and writing the manuscript, even though LNA contributed the most to performing the experiments.

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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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