# Molecular mapping of Asian soybean rust resistance in soybean landraces PI 594767A, PI 587905 and PI 416764

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Asian soybean rust (ASR), caused by *Phakopsora pachyrhizi*, is one of the most serious diseases of soybean. The soybean landraces PI 594767A, PI 587905 and PI 416764 previously showed high levels of resistance to a wide range of ASR fungus, while the genetic basis of the resistance has yet to be understood. In this study, the ASR resistance loci were mapped using three independent mapping populations, POP-1, POP-2 and POP-3 derived from crosses BRS184 × PI 594767A, BRS184 × PI 587905 and BRS184 × PI 416764, respectively. In each population, the resistance to ASR segregated as a single gene, but the resistance was dominant in PI 594767A and PI 587905 and incompletely dominant in PI 416764. The resistance genes from both PI 594767A and PI 587905 were mapped on chromosome 18 corresponding to the same location as known resistance locus *Rpp1*. Quantitative trait locus (QTL) analysis performed on POP-3 identified the putative ASR resistance locus in PI 416764 on the defined region of chromosome 6 where *Rpp3* was located. The QTLs detected by the mapping explained about 67–72% of the phenotypic variation in POP-3 Cluster analysis based on disease reactions to 64 ASR populations demonstrated the presence of at least two types of functional resistant *Rpp1* alleles: strong and weak allele(s), e.g. soybean accession PI 594767A and PI 587905 carry the strong resistant *Rpp1* allele(s). Introducing or pyramiding strong *Rpp1* allele(s) in elite soybean cultivars is expected to be useful against the South American rust population.

*Keywords:* cluster analysis, genetic mapping, pathogenic diversity, *Phakopsora pachyrhizi*, QTL analysis

## Introduction

Asian soybean rust (ASR) caused by the fungus *Phakopsora pachyrhizi* is rapidly invasive and one of the most damaging plant diseases. The disease was first reported in Japan and initially limited to tropical and subtropical areas of Asia and Australia, but it has spread to Africa, South America and the USA over the course of the last hundred years. Now the disease has become the greatest potential threat to major soybean-growing regions of Brazil, Paraguay, Bolivia and northern Argentina. The disease has also been predicted to be an emerging risk to soybean producers in the USA (Pivonia *et al.*, 2005). Since the introduction of soybean, the ASR fungus has been notorious for its great negative effect on plant growth and yield. The pathogen is capable of infecting soybean plants at any stage of development (Melching *et al.*, 1989). In severe infections, plants show considerable leaf yellowing and premature defoliation that results in yield reductions from both fewer and smaller seeds (Bromfield, 1984). Recent assessment suggests yield losses as high as 10–80% depending on the soybean growth stage and environmental conditions at which ASR infection occurs (Ogle *et al.*, 1979; Bromfield, 1984; Hartman *et al.*, 2005). To avoid large reduction of soybean yield from ASR infection, a significant shift in production cost and management practices would be required.

The obligate biotrophic basidiomycete *P. pachyrhizi* is known to have several distinct characteristics among rusts. While most rust fungi have complicated life cycles, occurring in five stages, only two spore-producing stages – telial and uredinial – are known for *P. pachyrhizi*. However, like other rusts, the uredinial stage is the main infective stage for ASR. Hundreds of urediniospores are produced asexually from a single uredinium as early as 7–10 days after infection that cause repeated infection of the same soybean plant during the same season (Marchetti *et al.*, 1975). The pathogen *P. pachyrhizi* is one of a few rusts that enters the host by direct penetration of the cuticle and epidermal cell wall, making it one of the

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most aggressive invaders (Miles *et al.*, 2003). The fungus has, exceptionally among rusts, a broad host range, infecting more than 150 species of plants from more than 53 genera including soybean, related *Glycine* species, and other hosts in the Fabaceae (Hartman *et al.*, 2011). Hence, the pathogen shows less dependence on stochastic events of time and host-specific dissemination, favouring building up inoculum on a common host and then dispersing onto a second host in high numbers. This combination of reproduction, dispersal and survival features makes it a difficult pathogen to control.

Several management tactics have been employed to control soybean rust and to reduce the impact of the disease. Chemical treatment with fungicides has been perceived as the most effective strategy to reduce the impact of soybean rust (Levy, 2005). Although there are significant benefits, the limited number of appropriate fungicides, 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 environmentally friendly, cost-effective and long-term management of the disease can be achieved through use of disease resistance (Hartman *et al.*, 2005; Ribeiro *et al.*, 2007). Development of rust resistant cultivars has been an important aspect of recent genetic improvement programmes in soybean and would be enhanced by the identification of the gene(s) controlling ASR resistance. Until now, six dominant resistance genes (*Rpp1*–*Rpp6*) to *P. pachyrhizi* have been reported. These genes may also act in a recessive manner, depending on the *P. pachyrhizi* isolates (Calvo *et al.*, 2008; Garcia *et al.*, 2011; Ray *et al.*, 2011). All the *Rpp* genes have been mapped to particular chromosomes or linkage groups (LGs): *Rpp1* on LG-G (chromosome 18; Ray *et al.*, 2011), *Rpp2* on LG-J (chromosome 16; Silva *et al.*, 2008), *Rpp3* on LG-C2 (chromosome 6; Hyten *et al.*, 2009), *Rpp4* on LG-G (Silva *et al.*, 2008), *Rpp5* on LG-N (chromosome 3; Garcia *et al.*, 2008) and *Rpp6* on LG-G (Li *et al.*, 2012). Resistance alleles of these loci confer either immunity or resistance, producing reddish-brown (RB) lesions with little or no sporulation, compared to susceptibility alleles that lead to tan-coloured lesions with abundant sporulation (TAN reaction). Despite the fact that these resistance genes confer high levels of ASR resistance, they function in a race-specific manner. In addition, while lack of sexual reproduction should limit variability of the rust fungus, there is substantial pathogenic variation in the rust populations (Akamatsu *et al.*, 2013). As such, soybean cultivars with resistance to all known races of *P. pachyrhizi* are not yet available. These circumstances have greatly increased the need for continuous efforts to find noble resistance gene(s) or allele(s) with a broader spectrum of activity to the rust fungus and to incorporate them into commercial soybean cultivars.

Previously, screening by Miles *et al.* (2006) had identified two Chinese soybean landraces PI 594767A (Zhao Ping Hei Dou) and PI 587905 (Xiao Huang Dou) and one Japanese landrace PI 416764 (Akasaya) with resistance to a mixture of four ASR isolates. Subsequent adult plant field screening in Paraguay demonstrated that PI 594767A was among the 10 accessions with the highest level of resistance, while PI 587905 was the most resistant accession in maturity group VII to naturally occurring ASR infection (Miles *et al.*, 2008). When tested against the Japanese rust population, all three of these accessions were found to be highly resistant (Yamanaka *et al.*, 2010). In a comparative screening against 59 South American and five Japanese rust populations, PI 594767A, PI 587905 and PI 416764 showed resistant reactions to 96, 84 and 34% of the tested populations, respectively (Akamatsu *et al.*, 2013). This demonstrates that the resistance from these accessions can be effective over a wide range of ASR isolates and could be useful in breeding programmes. However, the genetic basis of ASR resistance in these accessions has yet to be understood. Hence, in an effort to exploit this valuable resistance, in the present study the mode of inheritance was investigated and the resistance loci in PI 594767A, PI 587905 and PI 416764 were mapped.

## Materials and methods

### Plant materials

The parental soybean genotypes used in this study were a Brazilian soybean rust-susceptible cultivar BRS184 and three resistant lines, PI 594767A, PI 587905 and PI 416764. The parental genotypes used in the crosses were obtained from Embrapa Soybean Germplasm Collection, located at Londrina, PR, Brazil. The resistant lines were used as male and were crossed with susceptible cultivar BRS184 to develop $F_2$ mapping populations. Three mapping populations consisting of 82, 117 and 86 $F_2$ plants were derived from three crosses, BRS184 × PI 594767A (POP-1), BRS184 × PI 587905 (POP-2) and BRS184 × PI 416764 (POP-3), respectively. The three mapping populations and the parental genotypes were grown as described by Yamanaka *et al.* (2010).

### Pathogen multiplication and inoculation

The Japanese ASR isolate T1-2 (Yamanaka *et al.*, 2013) was used in this study for inoculation of soybean plants. When the plants reached V3 to V4 growth stage (*c*. 3 weeks after sowing), they were inoculated with T1-2 urediniospores. The optimal spore concentration used for inoculation of soybean plants was $5 \times 10^4$ urediniospores per mL. Preservation, multiplication and adjustment of spore concentration were carried out by following the instructions at www.jircas.affrc.go.jp/english/manual/soybean_rust/soybean_rust.html. The parental genotypes and POP-1, POP-2 and POP-3 plants were first sprayed with distilled water containing 0·04% v/v polyoxyethylenesorbitan monolaurate (Tween 20) using an atomizer. After air-drying for 15 min, plant leaves were inoculated by spraying them with freshly prepared spore suspension until run-off. Plants were allowed to air dry before being placed in a humid chamber maintaining high humidity and dark conditions overnight and then transferred to a growth chamber with the same conditions as described previously (Yamanaka *et al.*, 2010).

### Resistance evaluation

Two weeks after inoculation, plants were evaluated for ASR phenotype reactions by examining the number of uredinia per lesion (NoU) and sporulation level (SL). Three infected leaflets were detached from each inoculated plant, and the abaxial side was examined microscopically for determining NoU and SL in a maximum of 30 lesions in total, 10 lesions from each leaflet. 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. Qualitative phenotypes of ASR resistance in the $F_2$ population were estimated based on quantitative data of NoU and SL. Infection phenotypes with lesions with NoU and SL <2·0 were classified as resistant (R) and those with lesions with NoU and SL ≥2·0 were classified as susceptible (S) (Yamanaka *et al.*, 2011).

### SSR marker analysis

DNA was extracted from the parental genotypes as well as individual $F_2$ plants using a modified CTAB (cetyltrimethyl ammonium bromide) method (Lemos *et al.*, 2011). Simple sequence repeat (SSR) markers linked to six *Rpp* (1–6) loci were used to investigate the linkage between the known *Rpp* loci and unknown loci of ASR resistance in three accessions. Two SSR markers linked to each of the known ASR resistance genes (*Rpp1–Rpp6*) 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 to be polymorphic between the mapping parents of POP-1, POP-2 and POP-3 and used for initial screening of a small subset (24 $F_2$ plants) of each population. Once the potential locations of the resistance genes were identified, the full mapping population was tested for confirmation. Additional markers around the regions where the potential ASR resistance genes were mapped were chosen from SoyBase (http:// soybase.org). Primer sequences of the SSR markers were also available in SoyBase. All SSR markers used in this study were co-dominant for the parents. PCR and subsequent electrophoresis were performed following the procedures described at www. jircas.affrc.go.jp/english/manual/soybean_rust/soybean_rust.html.

### Genetic mapping and statistical analysis

Goodness-of-fit between observed and expected segregation ratios of ASR phenotypes and genotypes of SSR markers in $F_2$ populations was tested using chi-square analysis. Analysis of variance and regression analysis were used to test the significance of the association between ASR phenotype (NoU and SL) and flanking markers and to estimate how much phenotypic variation could be explained by flanking markers, respectively. Linkage analysis was performed to map SSR markers and the loci for ASR resistance in each mapping population using MAPMAKER/EXP v. 3.0b software (Lander *et al.*, 1987). A logarithm of the odds (LOD) score of >3·0 and a maximum genetic distance of 37·2 cM was used as a threshold to test linkage among markers. The Kosambi mapping function was used to convert recombination values into map distances. Degree of dominance of the putative resistant locus in POP-1 and POP-2 was calculated from additive and dominance effects of the resistance alleles relative to the susceptible alleles as determined by nearest linked markers of the loci. Genomic region(s) associated with resistance characters NoU and SL in POP-3 were mapped as quantitative trait loci (QTLs) using the interval mapping

functions in WINDOWS QTL CARTOGRAPHER v. 2.5 (Wang *et al.*, 2007). The estimated order of markers determined by MAP-MAKER was used for QTL analysis. The LOD score threshold for declaring a putative locus as significant was determined by permutation testing using 1000 permutations of the data. The QTL positions for NoU and SL were defined as the peaks of maximum LOD score.

### Cluster analysis

Cluster analysis was applied to the data set of disease reactions from 16 differential soybean accessions against 64 ASR populations obtained in previous studies (Akamatsu *et al.*, 2013). The resistant, intermediate and susceptible infection types were coded as 0, 1 and 2, respectively. Distance matrices were prepared by calculating the Euclidean distance between samples using R software v. 2.13.0 (R Development Core Team, 2011), and the resulting matrices were put into a hierarchical clustering function of the software. A dendrogram based on the unweighted pair group method with arithmetic mean (UPGMA) was also constructed with R software. An R package, PVCLUST, was run to assess uncertainty in the hierarchical cluster analysis, which calculates probability values (*P* values) for each cluster using bootstrap resampling techniques (Suzuki & Shimodaira, 2006).

## Results

### Evaluation of Asian soybean rust phenotypic trait

Significant differences were observed in NoU and SL between the susceptible and resistant parents of the three populations. ASR isolate T1-2 infection of susceptible parent BRS184 resulted in development of the expected tan-coloured lesions and the production of many uredinia with abundant sporulation. The NoU in BRS184 ranged from 3·30 to 3·60, while the SL was the highest level (3·00) of production (Fig. 1a,b). Inoculation of PI 594767A with the same ASR isolate gave rise to strong resistance or almost immune reaction, showing imperfect formation of a few RB lesions with no uredinium production. The mean value of both NoU and SL in this resistant parent was therefore scored as 0·0. On the cross involving BRS184 × PI 594767A, all the $F_1$ plants were shown to exhibit identical levels of resistance to PI 594767A (data not shown), suggesting that the gene controlling the resistant phenotype was completely dominant. Phenotypic analysis of POP-1 showed a segregation of 69 plants with resistant phenotype and 13 plants with the susceptible phenotype. In the 69 resistant plants, 67 plants showed a highly resistant reaction without any uredinium production, as shown by PI 594767A, while only two plants were found to form lesions with very few NoU (0·1) and little sporulation (0·1). A chi-square test revealed that the observed segregation fitted the expected segregation ratio of a single dominant resistance gene, 3:1 (resistant: susceptible) in the $F_2$ generation (Table 1). The degree of dominance for both NoU and SL is 1·00, indicating that the putative ASR resistant locus in POP-1 is completely dominant (Table 2).
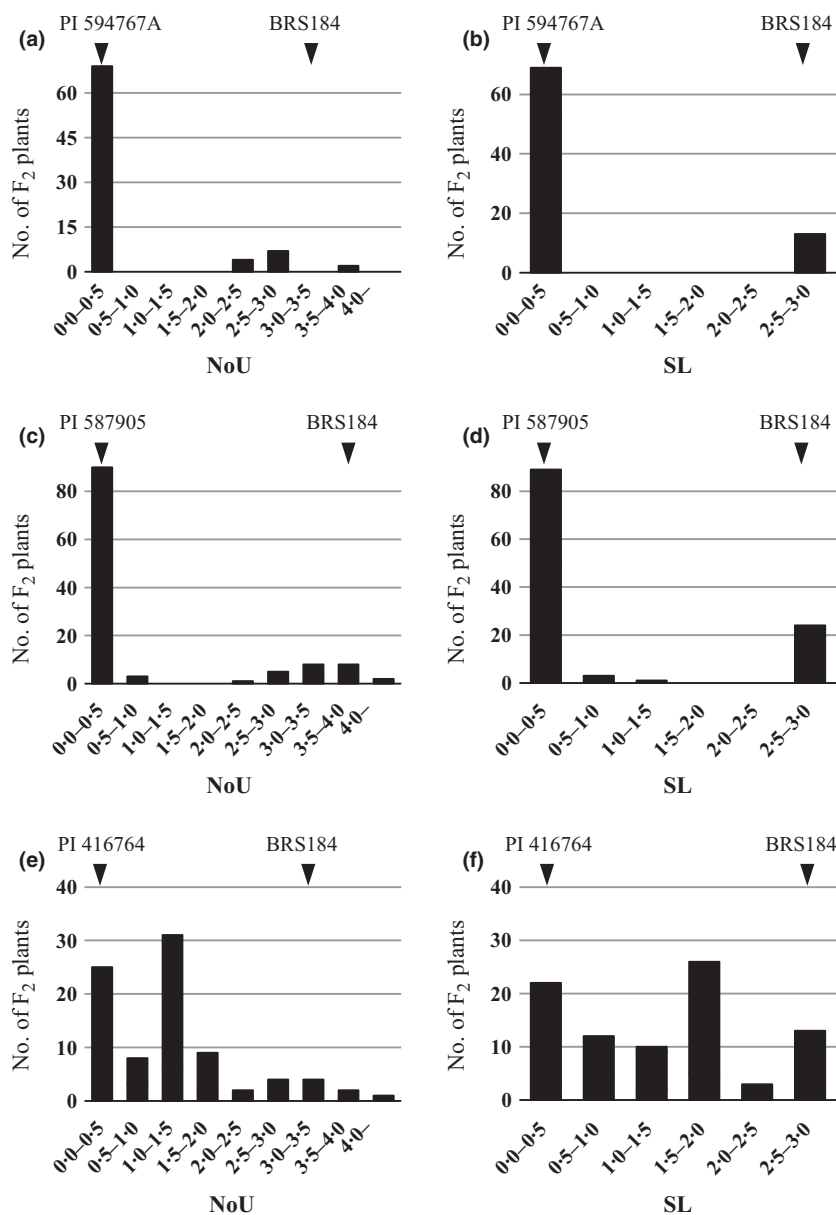
**Figure 1** Frequency distributions of number of uredinia per lesion (NoU) (a, c & e) and sporulation level (SL) (b, d & f) in POP-1 (BRS184 × PI 594767A) (a & b), POP-2 (BRS184 × PI 587905) (c & d) and POP-3 (BRS184 × PI 416764) (e & f) against Asian soybean rust (ASR) isolate T1-2. Triangles indicate the values of respective resistant and susceptible parents.

Parental line PI 587905 of POP-2 was found to exhibit a resistant phenotype to T1-2 infection and produce typical RB lesions with a mean NoU and SL value of 0·5 (Fig. 1c,d). All $F_1$ plants derived from the cross BRS184 × PI 587905 were also resistant like the resistant parent (data not shown). The POP-2 plants segregated into two clearly separated ranges, 93 with resistant and 24 with susceptible phenotype (Table 1). The observed segregation fitted the 3:1 (resistant:susceptible) ratio ($\chi^2 = 1.15$, $P = 0.28$) in the $F_2$ generation, suggesting that a single dominant gene controls the resistance in PI 587905. The degree of dominance for NoU and SL is 0·92 and 0·90, respectively, indicating that the putative resistant locus for both traits is completely dominant in POP-2 (Table 2).

The ASR reaction of the parental line PI 416764 of POP-3 was also highly resistant to T1-2 and differed significantly from BRS184 with regard to both NoU and SL, resulting in a formation of typical RB lesions with fewer uredinia and little sporulation. The $F_1$ plants from the cross BRS184 × PI 416764 had intermediate resistance which was closer to that of the resistant parent than that of the susceptible line (data not shown). However, $F_2$ progenies derived from this cross (POP-3) showed a wider variation in values of both NoU and SL. The NoU in the $F_2$ population ranged from 0·03 to 4·27, while that in parental line PI 416764 and BRS184 were 0·13 and 3·30, respectively (Fig. 1e). Similarly, the minimum mean value of SL in the $F_2$ population was 0·03, while the resistant parent had a mean SL of 0·13

**Table 1** Segregation of disease reaction to Asian soybean rust (ASR) isolate T1-2 in two $F_2$ populations: POP-1 (BRS184 × PI 594767A) and POP-2 (BRS184 × PI 587905)

| Population | Number of $F_2$ plants | | $\chi^2$ of the expected ratio of R:S (3:1) | $P^a$ |
|---|---|---|---|---|
| | R | S | | |
| POP-1 (BRS184 × PI 594767A) | 69 | 13 | 3·66 | 0·06 |
| POP-2 (BRS184 × PI 587905) | 93 | 24 | 1·15 | 0·28 |

R: resistant; S: susceptible.

[a]$P$ = probability, $P > 0.05$ means the observed segregation fits the 3:1 model.

(Fig. 1f). However, the $F_2$ progenies that fell beyond the high or low mean of the two parents were only few in number (<5). The occurrence of a few extreme $F_2$ individuals is expected due to environmental rather than genetic reasons because the $F_2$ population was much larger than the parental populations. Nevertheless, the phenotype data of the $F_2$ population showed no clearly differentiated classes, i.e. resistant segregating from the susceptible phenotype; rather it appeared to follow a continuous distribution for both traits examined. Thus, it was decided to perform QTL analysis for mapping of ASR resistance in POP-3.

## Mapping of resistance loci to ASR in three populations

Initial genotyping of a small subset of POP-1 (24 $F_2$ plants) with one marker linked to each of the *Rpp1* to

*Rpp6* genes and subsequent testing by ANOVA showed that the $F_2$-inferred NoU data were significantly associated with Sat_064 linked to the *Rpp1* gene (result not shown). Genotype data of Sat_064 in the full set of POP-1 also revealed significant ($P < 0.0001$) association with NoU and SL (Table 2). The coefficient of determination ($R^2$) of each phenotypic variation by this marker was 0·56 for NoU and 0·57 for SL, respectively (Table 2). Additional SSR markers Sat_372, Sat_117, Satt191 and Sct_199 around *Rpp1* were found to be polymorphic between the mapping parents, while Sct_187 and SSR66 (Kim *et al.*, 2012) were nonpolymorphic and showed dominant inheritance of a polymorphic band, respectively. Genotyping of the full set of POP-1 with SSR markers Sat_064, Sat_372, Sat_117, Satt191 and Sct_199 followed by a chi-square test revealed that all SSR markers mapped in POP-1 satisfactorily fitted the expected ratio for co-dominant inheritance (1:2:1). The resistance locus of PI 594767A was mapped at 11·4 cM from Sat_117 and at 0·0 cM from Sat_064 (Fig. 2).

Similar to POP-1, primary genotyping of a small subset of POP-2 (24 $F_2$ plants) with six known gene-linked SSR markers followed by an ANOVA test showed that SSR marker Sat_064 had significant association with NoU (data not shown). The linkage was further confirmed by testing the full mapping population with this marker and three additional SSR markers, Sat_372, SSR66 and Sat_117 near *Rpp1*. A chi-square test revealed that all SSR markers used to map the resistance in POP-2 fitted the expected ratio for co-dominant inheritance and following a 1:2:1 ratio. The ASR resistance locus of PI 587905 was mapped between markers Sat_064 and

**Table 2** Association between Asian soybean rust (ASR) resistance and simple sequence repeat (SSR) markers in POP-1 (BRS184 × PI 594767A) and POP-2 (BRS184 × PI 587905) determined by one-way ANOVA and regression analysis

| Population | Resistance characters | Marker and genotype[a] | Mean | SD | $F$-value | $P^b$ | $R^{2\ c}$ | Additive effect (a)[d] | Dominance effect (d)[d] | d/a[e] |
|---|---|---|---|---|---|---|---|---|---|---|
| POP-1 | NoU | Sat_064: A | 2·80 | 0·46 | 1341·02 | <0·0001 | 0·56 | −1·40 | −1·40 | 1·00 |
| | | Sat_064: H | 0·00 | 0·02 | | | | | | |
| | | Sat_064: B | 0·00 | 0·00 | | | | | | |
| | | All | 0·45 | 1·04 | | | | | | |
| | SL | Sat_064: A | 2·92 | 0·16 | 11069·54 | <0·0001 | 0·57 | −1·46 | −1·45 | 1·00 |
| | | Sat_064: H | 0·00 | 0·02 | | | | | | |
| | | Sat_064: B | 0·00 | 0·00 | | | | | | |
| | | All | 0·46 | 1·07 | | | | | | |
| POP-2 | NoU | SSR66: A | 3·37 | 0·55 | 1297·45 | <0·0001 | 0·64 | −1·68 | −1·55 | 0·92 |
| | | SSR66: H | 0·14 | 0·19 | | | | | | |
| | | SSR66: B | 0·02 | 0·07 | | | | | | |
| | | All | 0·76 | 1·36 | | | | | | |
| | SL | SSR66: A | 2·98 | 0·08 | 2439·83 | <0·0001 | 0·67 | −1·48 | −1·33 | 0·90 |
| | | SSR66: H | 0·17 | 0·25 | | | | | | |
| | | SSR66: B | 0·02 | 0·08 | | | | | | |
| | | All | 0·70 | 1·18 | | | | | | |

NoU: the number of uredinia per lesion; SL: sporulation level; SD: standard deviation.

[a]Genotype: A, homozygous susceptible parent; H, heterozygous; B, homozygous resistant parent.

[b]$P$, probability of significance, calculated by single-factor analysis of variance.

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

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

[e]Degree of dominance: 1 complete dominance for resistance; 0 lack of dominance; −1 complete dominance for susceptibility.
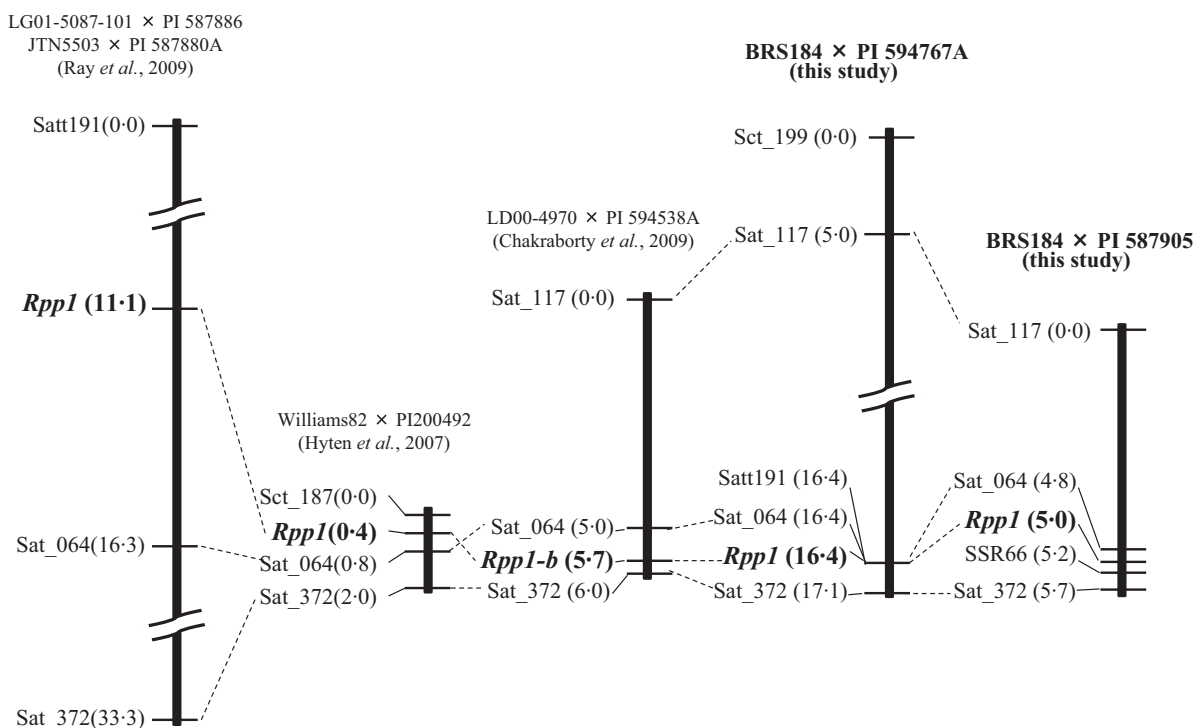
**Figure 2** Genetic linkage map around *Rpp1*, the resistance locus against *Phakopsora pachyrhizi* on linkage group G (chromosome 18) constructed from POP-1 (BRS184 × PI 594767A) and POP-2 (BRS184 × PI 587905). The values in parentheses next to marker names are cumulative distances (cM) generated using Kosambi's mapping function. The linkage maps of Ray *et al.* (2009), Hyten *et al.* (2007) and Chakraborty *et al.* (2009) are included for reference.

SSR66 (Fig. 2). Genotype data of SSR66, the nearest marker to *Rpp1* in the full set of POP-2 showed significant (*P* < 0·0001) association with NoU and SL (Table 2). The coefficient of determination ($R^2$) of each phenotypic variation by this marker was 0·64 for NoU and 0·67 for SL, respectively (Table 2).

In contrast, in POP-3, initial marker-trait analysis of 24 F$_2$ plants by ANOVA indicated a significant linkage between NoU and SSR marker Sat_263 (data not shown). The linkage was confirmed by subsequent screening and analysis of the full set of POP-3 with Sat_263 and additional markers from the same location. SSR markers Sat_251, Sat_238 and Satt307 around *Rpp3* were chosen based on their high polymorphism in amplicon size between the parents, whereas SSR marker Satt460 was found to be nonpolymorphic between them. A chi-square 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 four SSR markers that spanned a total length of 10·1 cM. An interval mapping using QTL CARTOGRAPHER was performed and the causative QTLs for two resistant characters, NoU and SL were identified. As shown in Figure 3, a significant QTL for NoU was detected in the interval between the SSR markers Sat_238 and Sat_263 with a peak at 4·4 cM on LG-C2. Similarly, the significant QTL associated with SL was indicated by a peak in

the interval between Sat_263 and Satt307 (Fig. 3; Table 3). The peak LOD scores for these QTLs were 20·81 and 23·68, respectively (Table 3). Composite interval mapping also generated a single QTL for each trait with similar peak position of interval mapping (data not shown), indicating that a single locus for each of NoU and SL is located in this region. The additive effects of these two QTLs were estimated to reduce NoU and SL by 1·17 and 1·02, respectively. The degree of dominance for the QTL associated with NoU and SL was 0·06 and 0·02, respectively, indicating that both QTLs were incompletely dominant. The QTLs detected by the mapping explained about 66·7 and 71·6% of the variations for NoU and SL in POP-3, respectively (Table 3).

## Cluster analysis

Cluster analysis yielded a dendrogram based on the disease reaction profile of the 16 soybean differentials to 64 ASR isolates from Argentina, Brazil, Paraguay and Japan (Fig. 4). The dendrogram showed clustering of the resistant parental varieties PI 594767A and PI 587905, of POP-1 and POP2 respectively, with known *Rpp1* variety PI 587880A, indicating that the ASR resistance gene in the accessions had much in common. Similarly, resistant parent PI 416764, of POP-3, grouped with known *Rpp3*-carrying variety PI 462312, as expected.
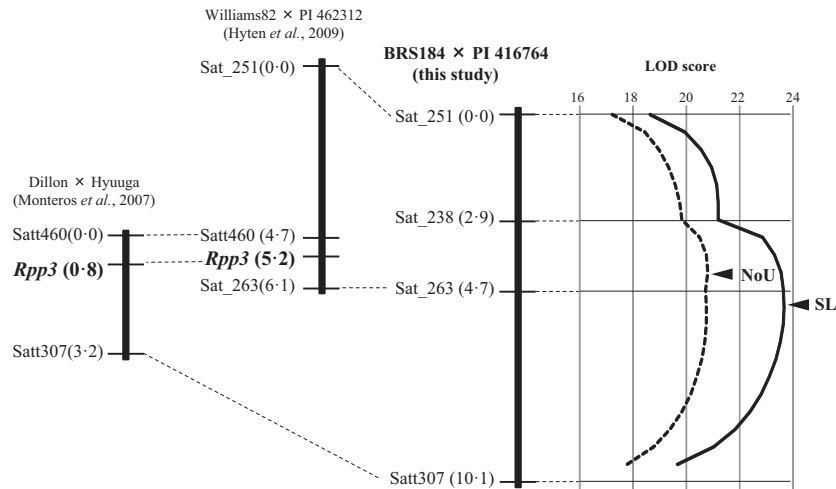
**Figure 3** Genetic linkage maps and logarithm of the odds (LOD) curves of quantitative trait loci (QTLs) for number of uredinia (NoU) and sporulation level (SL) in the *Rpp3* region of soybean linkage group C2 (chromosome 6). Genetic linkage map was generated using Kosambi's mapping function from the $F_2$-inferred Asian soybean rust (ASR) resistance phenotypic data of POP-3 (BRS184 × PI 416764). The cumulative cM distances from top of the linkage group were indicated in parenthesis next to each marker. The linkage maps of Hyten *et al.* (2009) and Monteros *et al.* (2007) are included for reference. LOD curves of QTLs for NoU and SL were generated using the same $F_2$ population by applying interval mapping analysis. The closed triangle indicates the QTL peak position.

The other differentials are clustered according to the similar mode of resistance (*Rpp1–Rpp5*). The UPGMA cluster analysis further revealed that 16 differentials were divided into two major groups. The first group was composed of accessions that showed resistant reactions to 78 to 96% of the tested ASR populations. This accession group was named 'Strong R', reflecting the strong ASR resistant reaction, and included the accessions that carry *Rpp1* (PI 594767A, PI 587905 and PI 587880A), *Rpp5* (PI 200526) and an unknown resistance gene (PI 587855). In contrast, the remaining 'Weak R' group contained accessions that showed resistant reactions to 0–42% of the tested ASR populations. The 'Weak R' included accessions carrying *Rpp4* (PI 459025), *Rpp3* (PI 416764 and PI 462312), *Rpp2* (PI 417125 and PI 230970), *Rpp-1b* (PI 587886), *Rpp1* (PI 368039 and PI 200492) as well as susceptible varieties that may carry

no major ASR resistance gene. This result showed that soybean accessions carrying *Rpp1* were distributed in both the 'Strong R' and 'Weak R' groups, suggesting that *Rpp1* may have at least two kinds of functional resistant allele, which largely differ from each other in the degree of resistance to South American ASR populations. Thus, the hierarchical clustering analysis proved useful to locate the superior resistance genes, alleles or resources against South American ASR populations with diverse pathogenicity.

## Discussion

Landrace genotypes are often found to have high resistance to various biotic and abiotic stresses. Natural variation found in these genotypes has played a vital role in the breeding of resistance to biotic and abiotic stresses.

**Table 3** Summary of quantitative trait loci (QTLs) for the number of uredinia per lesion (NoU) and sporulation level (SL) detected in POP-3 (BRS184 × PI 416764)

| QTL | Flanking marker 1 | Position (cM)[a] | Flanking marker 2 | Position (cM)[a] | QTL position (cM)[a] | LOD[b] | Additive effect (a)[c] | Dominance effect (d)[c] | d/a[d] | Variance explained (%)[e] |
|---|---|---|---|---|---|---|---|---|---|---|
| NoU | Sat_238 | 2·9 | Sat_263[f] | 4·7 | 4·4 | 20·80 | −1·17 | 0·07 | −0·06 | 66·7 |
| SL | Sat_263[f] | 4·7 | Satt307 | 10·1 | 5·2 | 23·68 | −1·02 | −0·20 | 0·20 | 71·6 |

[a]Positions are cumulative cM distances from the top of the linkage group constructed in the present study.
[b]LOD, logarithm of the odds score for each QTL.
[c]Additive and dominance effects of QTLs are those of the resistance alleles relative to the susceptible alleles.
[d]Degree of dominance: 1 complete dominance for resistance; 0 lack of dominance; −1 complete dominance for susceptibility calculated by additive and dominance effects from the interval mapping.
[e]Variance explained is the proportion of variance explained by each QTL.
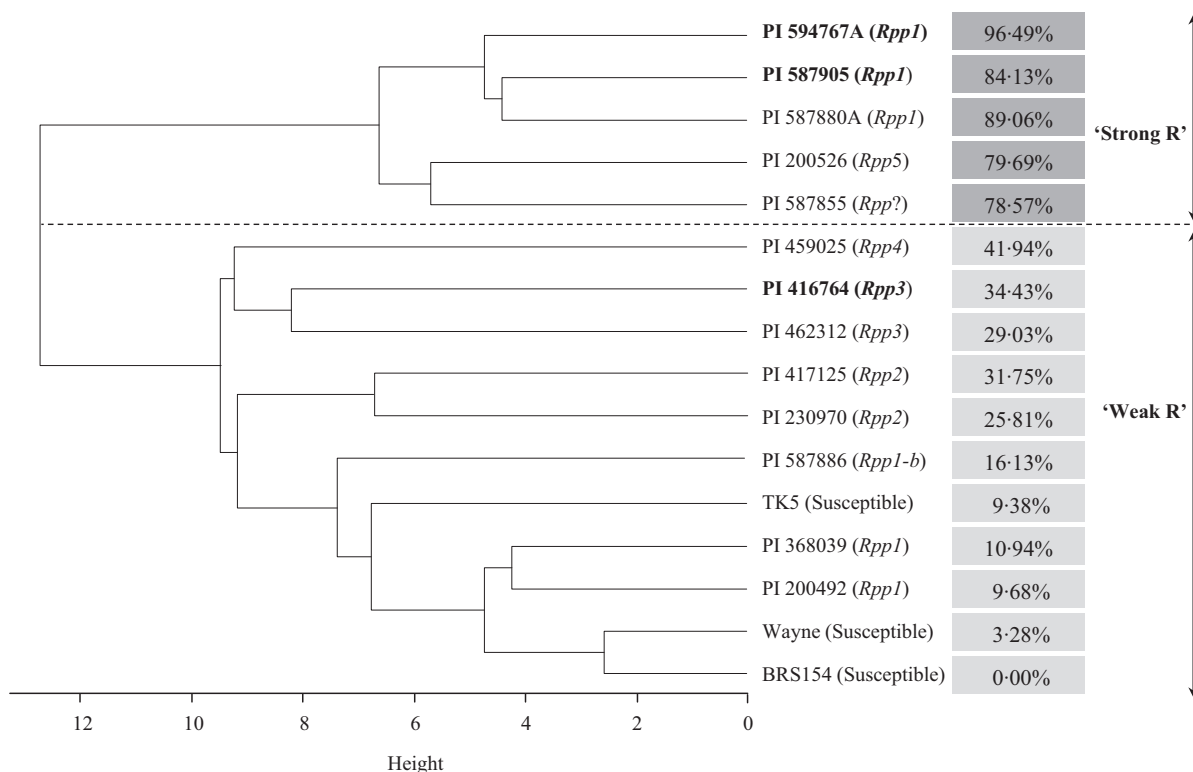[f]Nearest marker to the QTL.

**Figure 4** UPGMA dendrogram based on Asian soybean rust (ASR) disease reactions of 16 soybean differentials to 16 Argentinian, 24 Brazilian, 19 Paraguayan and five Japanese soybean rust populations (Akamatsu *et al.*, 2013). The gene conditioning the ASR resistance is indicated in parentheses next to each name of accession. The percentage values represent the percentage of resistant reaction to tested ASR populations. The arrows indicate the range of arbitrary groups of 'Strong R' and 'Weak R' accessions.

Soybean landrace genotype PI 594767A was observed as having a highly resistant, almost immune phenotype to one Japanese ASR isolate T1-2. Similarly, PI 587905 and PI 416764 displayed resistant phenotypes to this isolate. This is consistent with findings of previous studies using ASR isolates from Thailand, Zimbabwe, Brazil, Argentina, Paraguay and Japan (Miles *et al.*, 2006, 2008; Yamanaka *et al.*, 2011; Akamatsu *et al.*, 2013). The genetic basis of the ASR resistance in these genotypes was not determined in previous studies. The results of the present study suggested that single genes primarily control ASR resistance in these three accessions, where the resistance was dominant in PI 594767A and PI 587905 and incompletely dominant in PI 416764. The soybean rust resistance gene in PI 594767A and PI 587905 was identified as *Rpp1*. The *Rpp1* gene in PI 594767A was located between SSR markers Satt191 and Sat_064, while that in PI 587905 was found as mapping between SSR markers Sat_064 and SSR66 onto the soybean chromosome 18 (LG-G). Previously, *Rpp1* from other soybean accessions were also mapped to the same region on chromosome 18 (Fig. 2; Hyten *et al.*, 2007; Chakraborty *et al.*, 2009; Ray *et al.*, 2009; Kim *et al.*, 2012). The marker order and position in the linkage maps of the present study closely match those in these earlier investigations. The *Rpp1* linkage map was previously reported to differ from the soybean consensus map of Song *et al.* (2004) by showing an inversion at Sat_372 and Sat_064 (Hyten *et al.*, 2007; Ray *et al.*, 2009; Kim *et al.*, 2012). Similar inversion at these marker positions was also confirmed in the linkage maps of the present investigation, indicating the *Rpp1* linkage maps of the present and past studies were highly corroborated.

Although the resistance gene from both PI 594767A and PI 587905 was mapped to the *Rpp1* region, these two PIs were not identical in their resistance to the tested isolate T1-2 in the present study. Differential responses to some rust populations by these two accessions were also reported in a multi-isolate screening by Akamatsu *et al.* (2013). These two PIs also differed from other *Rpp1* carrying accessions, PI 587880A (Ray *et al.*, 2009), PI 587886 (Ray *et al.*, 2009) and PI 200492 (Hyten *et al.*, 2007) in their reaction to a number of South American and Japanese isolates during the same study (Akamatsu *et al.*, 2013). These results indicate that the soybean rust resistance observed in these accessions could be controlled by different alleles of *Rpp1*. Allelic variations in the *Rpp1* gene have been previously reported between PI 200492 (*Rpp1*) and PI 594538A (*Rpp1-b*) (Chakraborty *et al.*, 2009). Although the current data could not distinguish the mode of resistance of PI 594767A and PI 587905 from

PI 594538A or those reported by Garcia *et al.* (2011), it is probable that additional *Rpp1* alleles or tightly linked loci are present on the same genomic region. The cluster analysis performed in the present study using disease reaction profiles of 16 soybean differentials demonstrated that at least two types of resistant *Rpp1* alleles were functioning against ASR populations; the strong and the weak allele. The former allele was carried by PI 594767A, PI 587905 and PI 587880A, while the latter was found in PI 200492, PI 587886 and PI 368039. The strong resistant *Rpp1* allele conferred superior resistance to South American rust populations, while the weak *Rpp1* allele was less effective against them. The *Rpp1* allele from PI 594767A seemed to be the strongest candidate gene so far, as this was the only accession that did not show a susceptible phenotype to any of the tested South American and Japanese isolates. Until now, no soybean cultivar has been reported to be resistant to all known races of ASR. Thus, the discovery of this novel *Rpp1* allele from PI 594767A will have a far ranging impact on soybean breeding.

Analysis of the third accession, PI 416764, revealed that the degree of dominance and phenotypic expression of the ASR resistance gene in this accession was different from that of the other two accessions. The NoU and SL observed in the ASR resistance expressed in POP-3 varied across a continuous spectrum and application of interval mapping of the QTL suggested that nearly all of this variation was probably due to incomplete dominance of the single ASR resistance gene, *Rpp3*. It is possible that part of the variation in phenotype was due to the variation in genetic background of the progenies. The resistant locus *Rpp3* was previously reported to confer resistance in PI 462312 (Ankur) and Japanese cultivar PI 506764 (Hyuuga) (Hartwig & Bromfield, 1983; Monteros *et al.*, 2007; Kendrick *et al.*, 2011), and was mapped to chromosome 6 between SSR markers Sat_263 and Satt460 (Hyten *et al.*, 2009) and between SSR marker Satt460 and Satt307, respectively (Monteros *et al.*, 2007). A direct comparison between the map of the present study and those of Monteros *et al.* (2007) and Hyten *et al.* (2009) is limited, because the nonpolymorphic SSR marker Satt460 could not be used in the present investigation. However, the order of markers that were common among the maps was very similar. Moreover, the physical locations of markers in the present map were found to be in accordance with those on the *Glycine max* genome (assembly version 1.01; http://soybase.org). The cluster analysis also showed that PI 416764 formed a subcluster with *Rpp3*-carrying accession PI 462312, confirming the similar mode of resistance in both PIs. However, the reaction phenotypes in these two accessions were different from some ASR isolates (e.g. Argentinian isolates AP2-2 and AP3-3; Akamatsu *et al.*, 2013). Similarly, a *P. pachyrhizi* isolate collected from Brazilian fields was able to overcome the resistance found in PI 462312 although it was resisted in PI 506764 (Silva *et al.*, 2008). These findings suggest that the accessions share different resistant alleles of *Rpp3* or different resistance genes linked closely in this region. Any such allelic differentiation in the candidate region requires allelism tests against an appropriate ASR isolate. Moreover, it would be highly interesting to dissect this candidate region by high-resolution mapping and to clarify the relationship among them by gene cloning and functional characterization.

In conclusion, the present study successfully mapped the genes conditioning resistance against ASR in three soybean accessions. The cluster analysis identified useful resistant gene(s)/allele(s) against the recent rust populations in South America. Introducing or pyramiding useful gene(s)/allele(s) with other available ASR resistance gene(s) in a single soybean cultivar may provide more durable resistance against a complex pathogen population consisting of diverse *P. pachyrhizi* isolates.

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