

# Novel Sources of Partial Resistance against *Phytophthora sojae* in Soybean PI 399036

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## ABSTRACT

With the rapid emergence of novel pathogen pathotypes, durable sources of resistance against *Phytophthora sojae* in soybean [*Glycine max* (L.) Merrill] are needed to manage *Phytophthora* stem and root rot (PRR). This study used an inoculum source composed of multiple isolates of the pathogen to characterize partial resistance (PR) to *P. sojae* in plant introduction (PI) 399036. Two recombinant inbred line (RIL) F<sub>5:7</sub> populations developed by crossing PI 399036 (high PR), with two germplasm lines (low PR), AR2 (AX20925) and AR3 (AX20931), were screened. Corrected dry weights (CDW—root and shoot) and percentage root rot data were collected. Inclusive composite interval mapping identified one putative quantitative trait locus (QTL) on chromosome 2 by all disease assessment parameters in the AX20925 population that explained 6 to 14% of the total phenotypic variation and five putative minor QTL on 5 chromosomes (3, 6, 12, 15, and 19) that individually explained 5 to 9% of the phenotypic variation. In the AX20931 population, two QTL, mapped to chromosome 9 and 17, were commonly identified by CDW while the QTL identified on chromosome 9 accounted for 21 to 30% of the total phenotypic variation. Additional QTL identified by CDW on chromosomes 9, 13, 14, and 15 individually accounted for 5 to 8% of the phenotypic variation. PI 399036 contributed the favorable alleles for the majority of the identified QTL. This study demonstrates the usefulness of PI 399036 as a novel source for breeding for resistance to PRR and that an inoculum mixture of *P. sojae* isolates with varying pathotypes can be used to identify putative QTL for PR to *P. sojae* using the rice (*Oryza sativa*) method.

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**Abbreviations:** CDRW, corrected dry root weight; CDSW, corrected dry shoot weight; CDW, corrected dry weight; CIM, composite interval mapping; %DRL, percentage of diseased root length; %DSA, percentage of diseased root surface area; ICIM, inclusive composite interval mapping; LOD, logarithm of odds; PI, plant introduction; PR, partial resistance; PRR, *Phytophthora* root rot; QTL, quantitative trait loci; RIL, recombinant inbred line; %RR, percentage of root rot; SNP, single nucleotide polymorphism.

SOYBEAN [*Glycine max* (L.) Merrill] is an important oilseed crop grown across the world that accounted for 59% of the total oilseed production worldwide in 2014 (Soystats, 2015). Like other important crop species, soybean is prone to yield-reducing diseases caused by plant pathogens. *Phytophthora* stem and root rot (PRR) is the second-most yield-reducing disease in soybean and sometimes can cause up to 100% yield losses in individual fields (Draper and Chase, 2001; Wrather and Koenning, 2009). The disease is caused by *Phytophthora sojae* Kauf. & Gerd. (syn. *P. megasperma* f. sp. *glycinea*), a very diverse species with over 200 pathotypes (formally known as races) (Dorrance et al., 2003). The pathotype of an isolate is determined by screening its pathogenicity on a standard set of soybean differentials, each containing a different *Rps* gene (Schmitthenner and Bhat, 1994; Dorrance et al., 2004). Owing to high mutation rates and sexual outcrossing in the pathogen populations, the number of pathotypes continues to increase (Ryley et al., 1998; Tyler 2007; Dou et al., 2010).

The *P. sojae*–soybean pathosystem exhibits two main types of resistance: monogenic resistance conferred by dominant *Rps*

Published in Crop Sci. 56:1–14 (2016).  
doi: 10.2135/cropsci2015.09.0578

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genes and partial resistance (PR), which is quantitatively inherited. Twenty-five *Rps* genes (*Rps1a*, *1b*, *1c*, *1d*, *1k*, *2*, *3a*, *3b*, *3c*, *4*, *5*, *6*, *7*, *8*, *9*, *10*, *YU25*, *YD29*, *YD25*, *YB30*, *UN1*, *UN2*, *ZS18*, *SN10*, *JS*) have been identified and mapped (Diers et al., 1992; Lohnes and Schmitthenner 1997; Demirbas et al., 2001; Weng et al., 2001; Gordon et al., 2004; Zhu et al., 2007; Ai-Ying et al., 2009; Yao et al., 2010; Yu et al., 2010; Sun et al., 2011; Wu et al., 2011, Lin et al., 2013, Zhang et al., 2013a,b; Sun et al., 2014). Although eight of these *Rps* genes (*Rps1a*, *Rps1b*, *Rps1c*, *Rps1k*, *Rps2*, *Rps3a*, *Rps6*, *Rps7*) have been incorporated into commercial cultivars, only six (*Rps1a*, *Rps1b*, *Rps1c*, *Rps1k*, *Rps3a*, *Rps6*) have been successfully deployed to commercial cultivation (Dorrance and Schmitthenner, 2000; Dorrance et al., 2007). *Rps8*, which provides broad-spectrum resistance to *P. sojae* isolates, is currently being introgressed into commercial cultivars or used in developing new cultivars (Cianzio et al., unpublished results, ISURF Docket # 03712, 22 Apr. 2009; Dorrance et al., 2007; Biofuels Journal, 2010). Among the *Rps* genes, only *Rps1k* has been isolated, and a candidate *Rps4* gene has been cloned (Gao et al., 2005; Sandhu et al., 2004). Both *Rps1k* and *Rps4* genes encode coiled coil-nucleotide binding-leucine rich repeat containing proteins. *Rps1k* has several paralogous sequences in the genome (Bhattacharyya et al., 2005).

Most of the commonly grown soybean varieties in Iowa contain *Rps1k*, although varieties carrying *Rps1c* and *Rps6* are also deployed to a lesser extent (Robertson et al., 2009). Management of PRR through the use of varieties with these *Rps* genes is not completely effective, since the pathogen population within a field can be very diverse. Pathotypes differ between fields, within regions, and even within the same field (Ryley et al., 1998; Dorrance et al., 2003; Jackson et al., 2004; Robertson et al., 2009). Pathogenic diversity within two commercial fields with a history of PRR in Iowa was evaluated and showed that 51.6% of isolates recovered from fields could infect soybean plants carrying *Rps1k*, 25.8% could infect plants with *Rps1c*, and 35.5% were able to cause disease in plants carrying *Rps6* (Robertson et al., 2009). This data suggest that the variability of pathogen diversity within and between fields can make selection of soybean cultivars challenging for producers. *Rps*-gene-mediated resistance is only effective against certain races of the pathogen and is durable for about 8 to 20 yr (Dorrance et al., 2003; Grau et al., 2004). On the other hand, PR provides resistance to all physiological races of the pathogen and has no negative effect on yield (Wang et al., 2012).

The evaluation of a recombinant inbred line (RIL) population developed from a cross of 'Conrad' × 'Hefeng 25' for PR to PRR across multiple environments showed a positive correlation between the accumulation of partial resistance loci and the decrease in percentage of disease severity (Li et al., 2010). Quantitative trait loci (QTL) for

PR to *P. sojae* have been reported in a number of studies, with the majority mapped to plant populations derived from crosses with Conrad (Burnham et al., 2003; Weng et al., 2007; Han et al., 2008; Lee et al. 2013a,b, 2014; Tucker et al., 2010; Wu et al., 2010; Wang et al., 2012). Attempts to dissect two soybean QTL, mapped to chromosome 19, through sequence and expression analysis, revealed that PR QTL can underlie a complex defense network with multiple mechanisms (Wang et al., 2012). It has been suggested that the best protection to PRR may be provided by either stacking *Rps* genes or using a combination of *Rps* gene(s) and PR QTL.

Quantitative trait loci for PR to *P. sojae* have been identified in research studies using different protocols for phenotyping (Han et al., 2008; Li et al., 2010; Wang et al., 2010). Stewart and Robertson (2012) developed a modified method to screen for PR to *P. sojae* in which isolates of the pathogen were grown on sterile rice (*Oryza sativa*) and soybean seedlings were forced to grow through a layer of colonized rice rather than a layer of *P. sojae* colonized agar. The same study reported corrected dry root weight (mean treatment dry root weight/mean dry root weight of the noninoculated control) to be negatively and significantly ( $p < 0.0001$ ) correlated with root rot severity ratings. Matthiesen et al. (2016) have shown that combining isolates able to cause disease on many *Rps* genes into one source of inoculum could be beneficial for identifying novel *Rps* genes. Using the hypocotyl inoculation method with a mixed inoculum source composed of multiple isolates, Matthiesen et al., (2016) identified lines of *Glycine max* and *G. sojae* with potential novel *Rps* genes.

The plant introduction (PI) line 399036 (USDA Soybean Germplasm Collection, <http://www.ars-grin.gov/npgs/>; accessed 27 May 2016) was identified as a putative source of novel *Rps* genes to *P. sojae* (Dorrance and Schmitthenner, 2000). In a different study, also with this PI line, it was suggested that the accession contains either multiple *Rps* genes, that is, *Rps1c*, *2*, *3* (a or b), and *4*, or a novel gene in addition to known *Rps* genes (Gordon et al., 2007a,b). Plant introduction 399036 was also identified as a high PR genotype to *P. sojae* in a preliminary study conducted by soybean pathologists in the North Central region of the United States (Cerra, 2007). In the present study, we evaluated two soybean populations derived from crosses between PI 399036 and each of two soybean germplasm lines with low PR to *P. sojae* but carrying resistance to iron deficiency chlorosis. The primary objective of the study was to characterize the PR in two RIL soybean populations, developed with PI 399036 as the common parent, using corrected dry root weight as the primary disease assessment parameter. An additional objective was to test the feasibility of using the infested rice protocol (Stewart and Robertson, 2012), containing multiple isolates of *P. sojae* representing different pathotypes, as a means to phenotype germplasm. Three

additional disease assessment parameters, namely, corrected dry shoot weight and root rot calculated as a percentage of diseased root length or diseased root surface area, were also tested as possible phenotypic measurements to quantify PR.

## METHODOLOGY

### Plant and Pathogen Material

Plant introduction 399036 (USDA Germplasm Collection) was crossed with two germplasm lines released by Iowa State University—AR2 (Cianzio et al., ISURF Docket # 03381, 8 Jan. 2006) and AR3 (Cianzio et al., ISURF Docket # 03380, 13 Jan. 2006), to generate two soybean populations. The cross was made in Puerto Rico at the Iowa State University research site located at the University of Puerto Rico's Isabela Substation (ISU-PR). Six  $F_1$  seeds were obtained for each cross. The identification of individual  $F_1$  plants was maintained throughout development of the RIL even though the hybrid nature of the  $F_1$  plants was confirmed using flower and pubescence colors.  $F_2$  seeds of the populations were planted in Puerto Rico, and each individual  $F_2$  plant was identified prior to harvest.  $F_{5,6}$  RILs were developed at the ISU-PR research site by advancing the original  $F_{2,3}$  lines through single-plant selections. After harvest of the RILs at the  $F_{5,6}$  generation, one more planting was performed to obtain ample seed supply for the research. The RILs used in this study were therefore at the  $F_{5,7}$  generation.

In this study, 232 RILs of PI 399036 × AR2 population, referred to as AX20925, and 277 RILs of the PI 399036 × AR3 population, referred to as AX20931, were evaluated. Leaf tissues were collected at the V2 growth stage (Pedersen, 2007) for DNA extractions. Tissues were ground with liquid nitrogen and DNA was extracted using the C-TAB (cetyltrimethylammonium bromide) protocol (CIMMYT, 2005).

Three *P. sojae* isolates, PT2004 C2.S1 (vir 1a, 1b, 1c, 1d, 1k, 2, 3c, 4, 6,7), 1005-2.9 (vir 1a, 1b, 1c, 1k, 3b, 7), and R7-2a (vir 1d, 2, 3a, 5, 6, 7), were used in this study. *Phytophthora sojae* isolate R7-2a was received from Dr. Anne Dorrance (Ohio State University), and PT2004 C2.S1, originally collected in Ohio by the Dorrance lab, was received from Dr. Brett Tyler (Virginia Tech). The isolate 1005-2.9 was collected from a soybean rotation study conducted in Iowa (Robertson et al., 2009).

### Inoculum Preparation

Cultures of the three *P. sojae* isolates, PT2004 C1.S2, R7-2a, and 1005-2.9, were each grown on half strength-V8 (DV8) plates amended with antibiotics (neomycin sulfate [50 µg/mL] and chloramphenicol [10 µg/mL]), for 5 to 7 d in the dark at 23°C and used either to make the rice-based inoculum or inoculum for hypocotyl testing. Rice-based inoculum was prepared by using a modified method of the protocol described in Stewart and Robertson (2012). Briefly, twice autoclaved par-boiled long grain rice (450 g) in clear autoclave bags (12x24, VWR International) were used to make the *P. sojae*-infested rice inoculum. Prior to the first round of autoclaving, 324 mL of distilled water was added to each bag. Half a plate of 5- to 7-d-old *P. sojae* culture was cut into small squares and mixed with the rice. Bags were sealed to keep them airtight and shaken every other day to avoid clumping of the rice. After

12 to 14 d, colonized rice of each isolate was mixed to a 1:1:1 ratio to make the final inoculum. Inoculum for the hypocotyl assay was prepared by macerating 5- to 7-d-old cultures grown on DV8 plates in a food chopper (Black and Decker Handy Chopper Plus—355-mL capacity) with 10 mL of sterile distilled water per 6 plates. Final inoculum for hypocotyl inoculation was made by mixing macerated mycelia of the three isolates in a 1:1:1 ratio, as done by Matthiesen et al. (2016).

### Phenotypic Assay

#### *Rps*-Gene Screen

Ten seeds of each of the RILs and the parental lines were planted in 237-mL Styrofoam cups filled with coarse vermiculite and kept at 25°C in the greenhouse with a 12-h photoperiod for a week. Cups were watered twice a day. Seven-day-old seedlings were then screened for resistance governed by *Rps* genes with the three *P. sojae* isolates, mixed in a 1:1:1 ratio, using the hypocotyl inoculation method as described by Dorrance et al. (2004). Each parental line was also screened using each of the three isolates individually. The experiment was replicated two additional times. A standard soybean differential line set was included with each replicate of the experiment (Matthiesen et al., 2016). 'Sloan', considered a universally susceptible soybean cultivar, was used to test the virulence of each individual isolate as well as the mixed inoculum. Plants were kept in a dew chamber at 25°C for 24 h in the dark following inoculations and then kept in a growth chamber with a 12-h photoperiod at 25°C. Plants were scored 7 d postinoculation as either resistant (<30% seedlings killed) or susceptible (≥70% seedlings killed).

#### Partial Resistance

Initially, the parental lines and the soybean cultivars Conrad (positive check) and Sloan (negative check) for PR were evaluated using the rice method. Conrad is known to have high PR to *P. sojae*, while Sloan shows low levels of PR (Lee et al., 2013a). Since both parental lines and the checks lacked complete root resistance, the RILs of the two populations were also evaluated for PR using the rice-based method.

Styrofoam cups (473 mL) were filled with A4 coarse vermiculite up to one-third and 10 mL of the mixed rice inoculum was placed on top of the vermiculite. Ten mL of twice autoclaved noninoculated rice was used in place of inoculum in the control. Inoculum was covered with approximately 160 mL of vermiculite. Cups were thoroughly watered until saturation, 10 seeds of each RIL were planted in the surface center of each cup and covered to the top of the cup with vermiculite. Cups were watered again after planting and they were arranged in a completely randomized design on a greenhouse bench. Soybean cultivars Conrad and Sloan, along with the parental lines of each of the populations, were included in each replication. Each experiment consisted of 20 seedlings per RIL, parental line, and check, of which 10 seedlings were inoculated and 10 seedlings were noninoculated. The entire experiment was replicated two more times.

Plants were watered once a day and greenhouse temperature was kept at 25°C with a 12-h light cycle. Stand counts were taken 21 d after planting, after which plants were dug and roots were thoroughly washed. Both roots and shoots of each plant were separated to obtain the corresponding dry weights. Roots were also



scanned using a WinRhizo root scanner (Regents Instruments Inc.) prior to drying. Corrected dry weights for both roots and shoots were calculated as follows (Stewart and Robertson, 2012):

$$\text{Corrected dry weight (CDW)} = \frac{\text{Mean dry weight of inoculated plants}}{\text{Mean dry weight of noninoculated plants}}$$

The roots were analyzed using the root image analyzing software WinRhizo (V 5.0A, Regents Instruments Inc.) to obtain root measurements of root length and root surface area. Percent root rot (%RR) for each RIL was calculated as a percentage of diseased root length (%DRL) as well as a percentage of diseased root surface area (%DSA).

## Marker Genotyping and Linkage Analysis

An array of 1536 single nucleotide polymorphisms (SNPs) of a universal soybean linkage panel 1.0 was used to screen the populations using the Illumina Golden Gate assay kit (Hyten et al., 2010). Single nucleotide polymorphism analysis was conducted at the Soybean Genomics and Improvement Laboratory in Beltsville, MD. The computer software Mapmaker for Macintosh V2.0 (Lander et al., 1987) with the Kosambi mapping function (Kosambi, 1944) was used for linkage analysis. Linkage groups were initially identified with the “two-point/group command” with a maximum  $\theta$  value of 0.40 and a minimum logarithm of the odds (LOD) threshold of 3.0. The “Ripple” command with an LOD of 3 was used to verify the marker order.

## QTL Mapping

Corrected dry weights as well as %RR data were used to map QTL using the software QTL IciMapping V 4 (Li et al., 2007; Wang et al., 2014). IciMapping uses inclusive composite interval mapping (ICIM), which is a modified algorithm from that used for composite interval mapping (CIM) (Li et al., 2007). Quantitative trait loci were detected with ICIM with significant genomewide LOD threshold determined by a 1000 permutation with an  $\alpha = 0.05$ , which was 3.03 for the AX20925 population and 3.22 for the AX20931 population. Quantitative trait loci with minor effects were identified with chromosomewide LOD thresholds determined by a 1000 permutation also with  $\alpha = 0.05$ . ICIM-ADD mode was used to find the gene action with a stepwise regression probability set at 0.001. ICIM-EPI mode was also tested to check for the presence of epistatic interactions between QTL.

## Statistical Analysis

The software packages SAS enterprise guide V 5.1 (SAS Institute), Graphpad (<http://www.graphpad.com/quickcalcs>; accessed 27 May 2016), and SigmaPlot V 12.3 (Systat Software) were used for statistical analysis. Chi squared test was conducted using Graphpad. SigmaPlot was used to check the normality and correlation of datasets, with the Shapiro-Wilk test and the Pearson product moment correlation, respectively. Fisher's protected least significant difference (LSD) was calculated with an  $\alpha = 0.05$  using SAS enterprise guide.

## RESULTS

### Presence of a Putative Novel *Rps* Gene or Allele in PI 399036

Each individual *P. sojae* isolate as well as the mixed inoculum was virulent on Sloan, AR2, and AR3 but not on PI 399036. When *P. sojae* isolates were combined, the mixed inoculum killed over 70% of the plants of the individual differential lines, except the line carrying *Rps8*, indicating that the PI 399036 may carry either *Rps8* and/or a novel *Rps* gene not overcome by the selected isolates of the pathogen.

When the RILs were inoculated with a combination of all three isolates, the lines segregated in a 1:1 ratio for resistant:susceptible (consistent with a single gene segregation) in both the AX20925 ( $\chi^2_{df=1} = 2.914, p = 0.088$ ) and the AX20931 populations ( $\chi^2_{df=1} = 0.177, p = 0.674$ ). This segregation indicated the presence of a novel *Rps* gene or allele that putatively mapped to the *Rps4/6* region in the lower arm of chromosome 18 in both RIL populations (data not shown). On the basis of this initial mapping result and molecular marker data, it was evident that the putative *Rps* gene or allele was not *Rps8* that was mapped to chromosome 13 (Sandhu et al., 2005).

### Evaluation of Phytophthora Root Rot in the Two Recombinant Inbred Populations

None of the checks or parental lines showed complete root resistance. Although PI 399036 showed complete resistance to the hypocotyl inoculations, it did not show complete root resistance. PI 399036 and Conrad showed significantly less %RR than the checks and the parents AR2 and AR3 ( $p < 0.05$ ) (Table 1). There was no significant difference of %RR between the PI line and Conrad.

The mixed inoculum caused a range of root rot severity in both RIL populations and exhibited transgressive segregation. The mean %DRL for the AX20925 and AX20931 populations were similar, 70% and 70.7%, and ranged from 37 to 99% and 30 to 99%, respectively (Table 1, Fig. 1). Likewise, the mean %DSA for both populations was similar (68.0 vs. 68.6%) and ranged from 36 to 97% in the AX20925 population and 31 to 98% in the AX20931 population (Table 1, Fig. 2). For %RR, the AX20931 population appeared to have a higher percentage of susceptible lines than the recurrent parent AR3 as shown by the distribution of the values (Figs. 1 and 2).

### Corrected Dry Weights in the AX20925 and AX20931 Recombinant Inbred Line Populations

The common parent PI 399036 showed a mean corrected dry root weight (CDRW) of 0.82, while that of AR2 was 0.43 and for AR3 it was 0.44 (Table 1). The positive check for PR Conrad, and negative check for PR Sloan, showed mean CDRW of 0.72 and 0.29, respectively ( $p < 0.05$ ). The CDRW of PI 399036 was significantly ( $p < 0.05$ )

**Table 1. Descriptive statistics of corrected dry weights and percent root rot for parental lines, PI 399036, AR2, and AR3, positive ('Conrad') and negative ('Sloan') control checks for partial resistance, and two recombinant inbred line populations, AX20925 and AX20931.**

Line	Corrected dry weight		Percentage of root rot		
	CDRW†	CDSW‡	%DRL§	%DSA¶	
PI 399036	0.82 ±0.02 A¶¶	0.86 ±0.02 A	36.4 ±4.5 D	42.4 ±2.3 D	
AR2	0.43 ±0.02 C	0.64 ±0.01 C	64.1 ±2.9 B	67 ±2.5 B	
AR3	0.44 ±0.03 C	0.65 ±0.01 C	61 ±2.3 C	58.3 ±3.1 C	
Conrad#	0.72 ±0.02 B	0.75 ±0.02 B	38.8 ±3.2 D	42.9 ±2.1 D	
Sloan††	0.29 ±0.03 D	0.32 ±0.02 D	73.1 ±3.1 A	78.6 ±4.2 A	
Population (n‡‡)					
AX20925 (232)	mean ±SD§§	0.71 ±0.23	0.87 ±0.24	70 ±11.06	68 ±11
	range	0.12–1.46	0.12–1.48	37–99	36–97
AX20931 (277)	mean ±SD	0.69 ±0.27	0.87 ±0.21	70.7 ±14.35	68.6 ±14.27
	range	0.14–1.55	0.12–1.43	30–99	31–98

† Corrected dry root weight.

‡ Corrected dry shoot weight.

§ Percentage of diseased root length.

¶ Percentage of diseased root surface area.

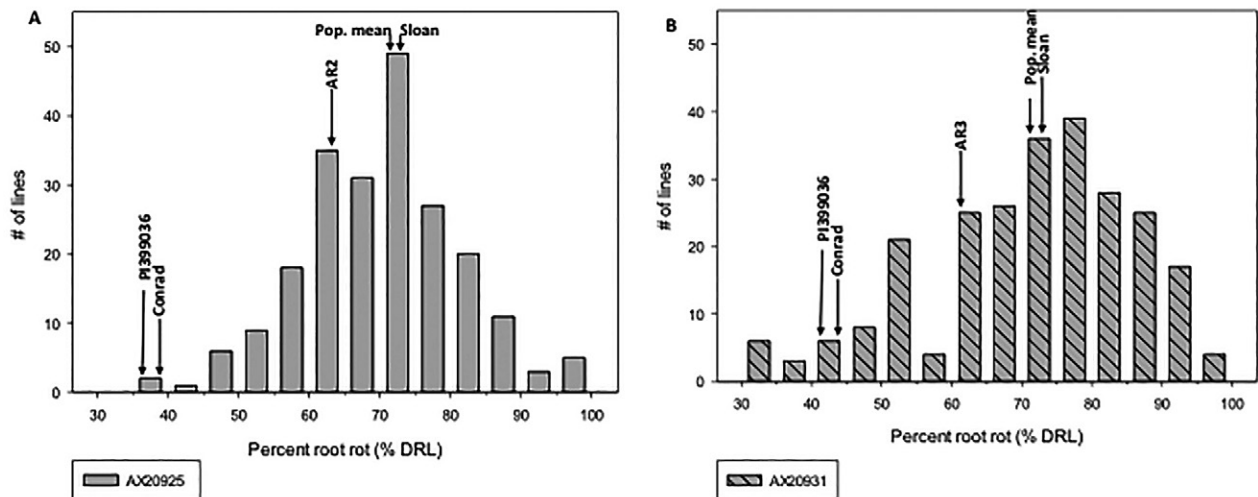
# High partial resistance.

†† Low partial resistance.

‡‡ Number of lines.

§§ Standard deviation.

¶¶ Values with the same letter are not statistically significant at an LSD of  $P < 0.05$ .



**Fig. 1. Frequency distribution of root rot calculated as a percentage of total diseased root length (%DRL) in two recombinant inbred line populations, (A) AX20925 (PI 399036 × AR2) and (B) AX20931 (PI 399036 × AR3). Root images were analyzed using WinRhizo software to obtain root parameters. 'Conrad' (high partial resistance) and 'Sloan' (low partial resistance) were used as checks for partial resistance.**

greater than that of Conrad, Sloan, AR2, and AR3. The different levels of PR in AR2 and AR3 were not statistically significant. However, AR2 and AR3 showed significantly ( $p < 0.05$ ) higher levels of PR than Sloan (Table 1). Corrected dry root weight for AX20925 population ranged from 0.12 to 1.46, while that of AX20931 ranged from 0.14 to 1.55 (Fig. 3). Individual lines of both populations were normally distributed, with a mean CDRW of 0.71 for AX20925 and 0.69 for AX20931.

Similar results also were observed for the corrected dry shoot weights (CDSW) for both RIL populations. The CDSW for the AX20925 population ranged from

0.12 to 1.48, while the range for AX20931 was from 0.12 to 1.43 (Fig. 4). The mean CDSW of both RILs was 0.87. The common parent PI 399036 showed a mean CDSW of 0.86, while the mean of AR2 and AR3 were 0.64 and 0.65, respectively (Table 1). Mean CDSWs for Conrad and Sloan were 0.75 and 0.32, respectively. The CDSW of PI 399036 was significantly ( $p < 0.05$ ) greater than that of PR checks and the two parental lines. The CDSWs of AR2 and AR3 were not significantly different from each other, but were significantly ( $p < 0.05$ ) greater than that of Sloan (Table 1). Both RIL populations showed transgressive segregation for CDW.

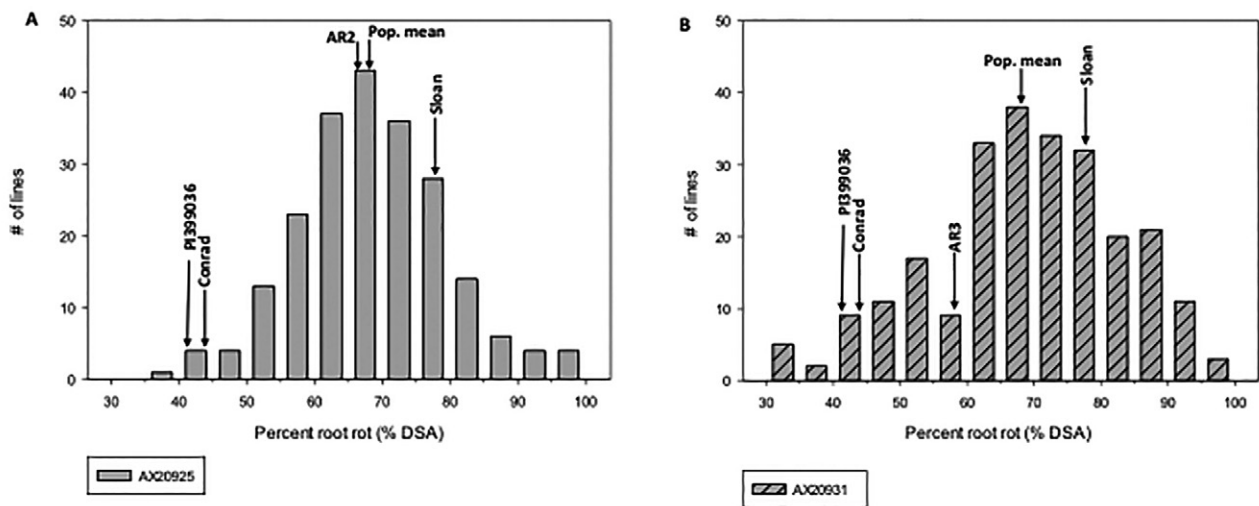


Fig. 2. Frequency distribution of root rot calculated as a percentage of total diseased root surface area (%DSA) in two recombinant inbred line populations, (A) AX20925 (PI 399036 × AR2) and (B) AX20931 (PI 399036 × AR3). Root images were analyzed using WinRhizo software to obtain root parameters. ‘Conrad’ and ‘Sloan’ were used as checks for high and low partial resistance, respectively.

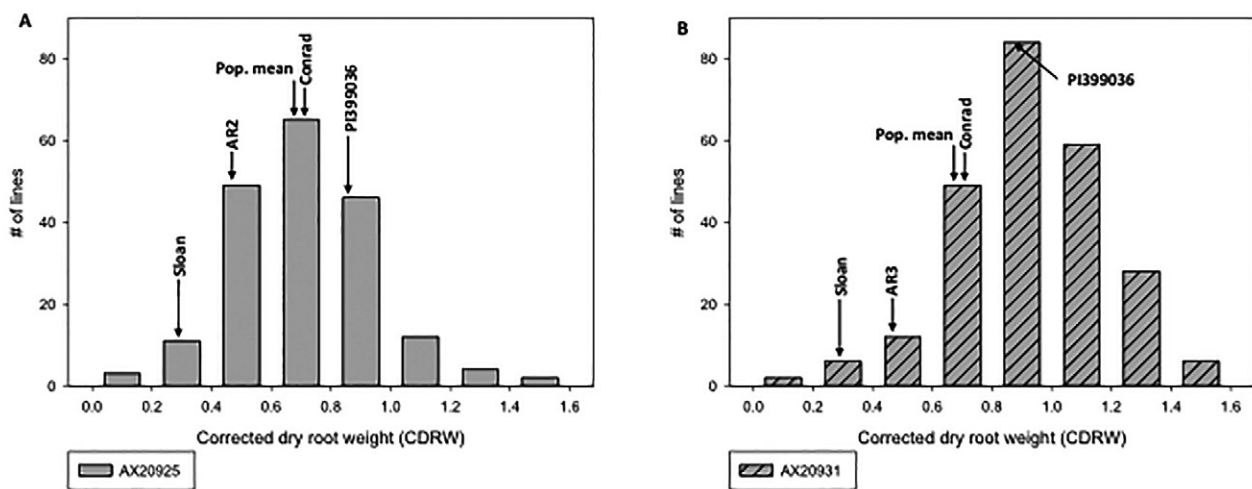


Fig. 3. Frequency distribution of corrected dry root weight (CDRW) in two recombinant inbred line populations, (A) AX20925 (PI 399036 × AR2) and (B) AX20931 (PI 399036 × AR3). ‘Conrad’ and ‘Sloan’ were used as checks for high and low partial resistance, respectively.

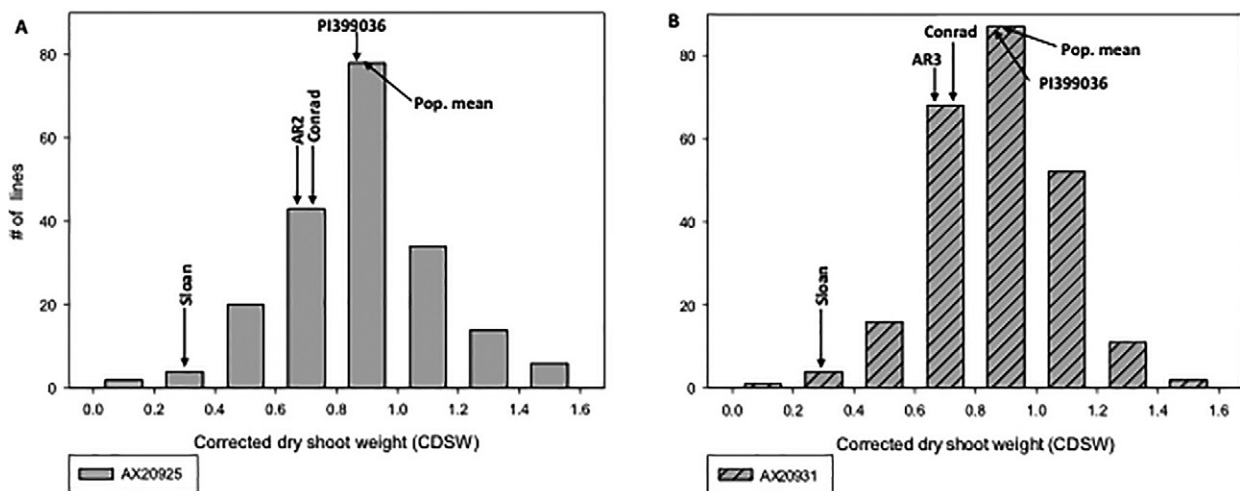


Fig. 4. Frequency distribution of corrected dry shoot weight (CDSW) in two recombinant inbred line populations, (A) AX20925 (PI 399036 × AR2) and (B) AX20931 (PI 399036 × AR3). ‘Conrad’ and ‘Sloan’ were used as checks for high and low partial resistance, respectively.

## Correlation between Disease Assessment Parameters

Percent root rot data were negatively correlated with CDW (Table 2). The correlation coefficients between the CDW and %RR of the control phenotypes PI 399036, AR2, AR3, Conrad, and Sloan were significantly different ( $p < 0.05$ ) (Table 2). Correlations of the different disease assessment parameters (CDW and %RR) varied between the two RIL populations (Table 2). Corrected dry root weight was significantly ( $p < 0.05$ ), associated with CDSW in both RIL populations. The correlation between CDRW and CDSW was lower for the AX20931 population ( $r = 0.345$ ,  $p < 0.05$ ) than the AX20925 population ( $r = 0.575$ ,  $p < 0.05$ ). The correlation between %DRL and %DSA was significantly high in both RIL populations ( $p < 0.05$ ). A significant ( $p < 0.05$ ) yet low correlation between CDW and %RR was observed in the AX20925 population (Table 2). However, no significant correlation was detected between the same variables (CDW and %RR) in the AX20931 population.

## Marker Genotyping and Linkage Maps

A total of 597 polymorphic SNPs were used to develop whole genome maps of the AX20925 population, and 507 polymorphic SNPs were used for AX20931. Of these markers, 402 SNPs were mapped to the AX20925 population and 468 SNP markers were mapped to the AX20931 population. The number of markers mapped in each linkage group ranged from 6 to 27 in the AX20925 population and 9 to 37 in the AX20931 population. Molecular marker density was not sufficient in some of the highly recombinant regions of the genome leading to the formation of 21 linkage groups in the AX20925 population and 23 linkage groups in the AX20931 population. Marker densities for AX20925 and AX20931 populations were 7.01 and 6.46 cM per marker, respectively. Details of linkage maps for both RIL populations are shown in the supplemental file.

## Quantitative Trait Loci Identified in AX20925

Corrected dry weights and %RR data showed a normal distribution, indicating the presence of a quantitative trait. Although PI 399036 did not show complete root resistance, it did have an incompatible reaction to *P. sojae* inoculum with the hypocotyl inoculation. This indicates the possible presence of a major gene. As the presence of a major gene could mask effects of QTL for PR, data for lines containing the novel *Rps* gene or allele identified through the hypocotyl inoculation results were eliminated from the analysis. For the AX20925 population, the number of lines eliminated from data analysis for PR was 103. Inclusive composite mapping identified a total of six QTL, one significant QTL with major effects and five QTL with minor effects, associated with PR to *P. sojae*. All four disease-assessment parameters identified

**Table 2. Correlation coefficients of corrected dry weights and two root rot parameters in two recombinant inbred line populations and checks.**

		CDSW†	CDRW‡	%DSA§
CDRW	Checks¶	0.976*		
	AX20925	0.575*		
	AX20931	0.345*		
%DSA	Checks	-0.931*	-0.886*	
	AX20925	-0.155*	-0.157*	
	AX20931	-0.099	-0.051	
%DRL#	Checks	-0.962*	-0.994*	0.899*
	AX20925	-0.138	-0.171*	0.989*
	AX20931	-0.103	-0.052	0.983*

\* Significant interactions at  $P = 0.05$ . Negative sign indicates a negative correlation between two variables.

† Corrected dry shoot weight.

‡ Corrected dry root weight.

§ Percentage of diseased root surface area.

# Percentage of diseased root length.

¶ Parental lines (PI 399036, AR2, AR3), 'Conrad', and 'Sloan'.

one overlapping QTL on the same genomic region on chromosome 2 (Tables 3 and 4, Fig. 5). The QTL identified with CDRW and %RR on chromosome 2 accounted for 11 to 14% of the total phenotypic variation in this population (Table 3). The overlapping QTL, identified by CDSW on the same QTL region, explained only 5.6% of the total phenotypic variation of the population. CDW and %RR showed negative and positive additive effects for this QTL, respectively, and the resistance alleles for this trait were contributed by parent AR2.

Quantitative trait loci with minor effects identified with chromosome-wide LOD thresholds were located on chromosomes 3, 6, 12, 15, and 19 and explained 5.5 to 8.9% of the phenotypic variation in this population (Table 4). Minor QTL on chromosomes 3, 6, 12, and 19 were identified by both %DRL and %DSA. Plant introduction 399036 contributed the favorable alleles for 3 of the 5 minor QTL (Table 4). No epistatic interactions were observed between any of the QTL, nor a major QTL detected on chromosome 18, where the putatively novel *Rps* gene or allele was mapped using the phenotypic data of the hypocotyl inoculations. This study identified nine RILs that harbor the QTL for PR identified on chromosome 2 by ICIM in the AX20925 population.

## Quantitative Trait Loci Identified in AX20931

In the AX20931 population, 135 lines showing resistance when the hypocotyl test was used were eliminated from data analysis. Two QTL located on chromosomes 9 and 17 were detected by ICIM with a genomewide LOD threshold of 3.22 (Table 3, Fig. 6). Together these two QTL explained 31% of the total phenotypic variation in this population using CDRW. Quantitative trait loci identified on chromosome 9 individually explained 21 to 30% of the total phenotypic variation in this population. The same



**Table 3. Quantitative trait loci identified in the AX20925 (PI 399036 × AR2) and AX20931 (PI 399036 × AR3) populations by inclusive composite interval mapping with a genomewide logarithm of the odds threshold of 3.03 and 3.22, respectively.**

Trait	Population	Chr.†	Map position cM‡	Left marker	Right marker	LOD§	PVE¶	Add#	R2	Contributor
CDRW††	AX20925	2	132	BARC-019805-04379	BARC-042881-08448	3.23	12.09	-0.11	11.3	AR2
	AX20931	9	54	BARC-017625-02635	BARC-055533-13402	8.46	29.92	0.11		PI 399036
	AX20931	17	33	BARC-058841-15463	BARC-052295-11407	3.93	12.72	0.07	30.7	PI 399036
CDSW‡‡	AX20931	9	54	BARC-055533-13402	BARC-007999-00186	5.42	21	0.1	19.9	PI 399036
%DRL§§	AX20925	2	133	BARC-019805-04379	BARC-042881-08448	3.91	13.3	4.20	12.6	AR2
%DSA¶¶	AX20925	2	133	BARC-019805-04379	BARC-042881-08448	4.32	14.63	4.39	13.9	AR2

† Chromosome.

‡ Based on soybean consensus map 4.0 (Hyten et al., 2010).

§ Logarithm of the odds.

¶ Phenotypic variance explained by the quantitative trait loci at the current scanning position.

# Additive effects. Negative sign for corrected dry weights indicates that AR2 contributed the favorable alleles. Negative sign for percentage of root rot indicates that parent PI 399036 contributed the favorable alleles.

†† Corrected dry root weight.

‡‡ Percentage of diseased root length.

§§ Percentage of diseased root length.

¶¶ Percentage of diseased root surface area.

**Table 4. Minor effect quantitative trait loci identified in the AX20925 (PI 399036 × AR2) population by inclusive composite interval mapping with chromosomewide logarithm of odds thresholds. Underlined = nearest marker.**

Trait	Chr.†	Map position cM‡	Left marker	Right marker	LOD§	PVE¶	Add#	Contributor
CDSW††	2	134	<u>BARC-906743-01012</u>	BARC-019805-04379	1.5 (1.5)	5.6	-0.07	AR2
	12	49	<u>BARC-019775-04370</u>	BARC-025943-05179	1.5 (1.5)	5.7	-0.07	AR2
%DRL‡‡	3	45	<u>BARC-050433-09624</u>	BARC-010179-00543	1.93 (1.6)	6.74	-2.73	PI 399036
	6	103	BARC-062515-17881	<u>BARC-040475-07751</u>	1.75 (1.5)	5.50	2.50	AR2
	15	25	BARC-054257-12408	<u>BARC-028907-06042</u>	1.56 (1.5)	7.32	-3.30	PI 399036
	19	86	<u>BARC-064609-18739</u>	BARC-039977-07624	1.89 (1.7)	8.48	-3.13	PI 399036
%DSA§§	3	45	<u>BARC-050433-09624</u>	BARC-010179-00543	1.99 (1.6)	6.88	-2.75	PI 399036
	6	103	BARC-062515-17881	<u>BARC-040475-07751</u>	1.51 (1.5)	4.66	2.30	AR2
	15	25	BARC-054257-12408	<u>BARC-028907-06042</u>	1.60 (1.5)	6.65	-3.19	PI 399036
	19	86	<u>BARC-064609-18739</u>	BARC-039977-07624	1.93 (1.7)	8.89	-3.19	PI 399036

† Chromosome.

‡ Based on soybean consensus map 4.0 (Hyten et al., 2010).

§ Logarithm of the odds. Chromosomewide LOD threshold is indicated within parentheses.

¶ Phenotypic variance explained by the quantitative trait loci at the current scanning position.

# Additive effects. Negative sign for corrected dry weights indicate that AR2 contributed the favorable alleles. Negative sign for percentage of root rot indicate that parent PI 399036 contributed the favorable alleles.

†† Corrected dry shoot weight.

‡‡ Percentage of diseased root length.

§§ Percentage of diseased root surface area.

QTL was detected by CDRW in the AX20925 population with the entire dataset, which included lines carrying the novel *Rps* gene or allele, but was not detected with the edited dataset lacking data for the resistance lines (data not shown). No major QTL was detected on chromosome 18 and no epistatic interactions were observed between any of the QTL. Nine QTL with minor effects were also identified on chromosomes 2, 5, 7, 8, 9, 13, 14, 15, and 20 with chromosomewide LOD thresholds (Table 5).

None of the QTL were detected by all four disease assessment parameters. The QTL on chromosome 9 and 17 were the only QTL commonly identified by CDRW and

CDSW. Both QTL identified by CDW show positive additive effects, and the resistance alleles were contributed by PI 399036. Three minor QTL were identified by CDRW on chromosomes 13, 14, and 15, which individually accounted for 5 to 6% of the phenotypic variation in this population, and PI 399036 contributed the favorable alleles for two of the three minor QTL identified by CDRW. One minor QTL was identified by CDSW with negative additive effects. The parent AR3 contributed the resistance alleles to this QTL. The same minor QTLs (except the QTL on chromosome 5 identified only by %DSA) were identified by %RR. One RIL was identified in the AX20931 population



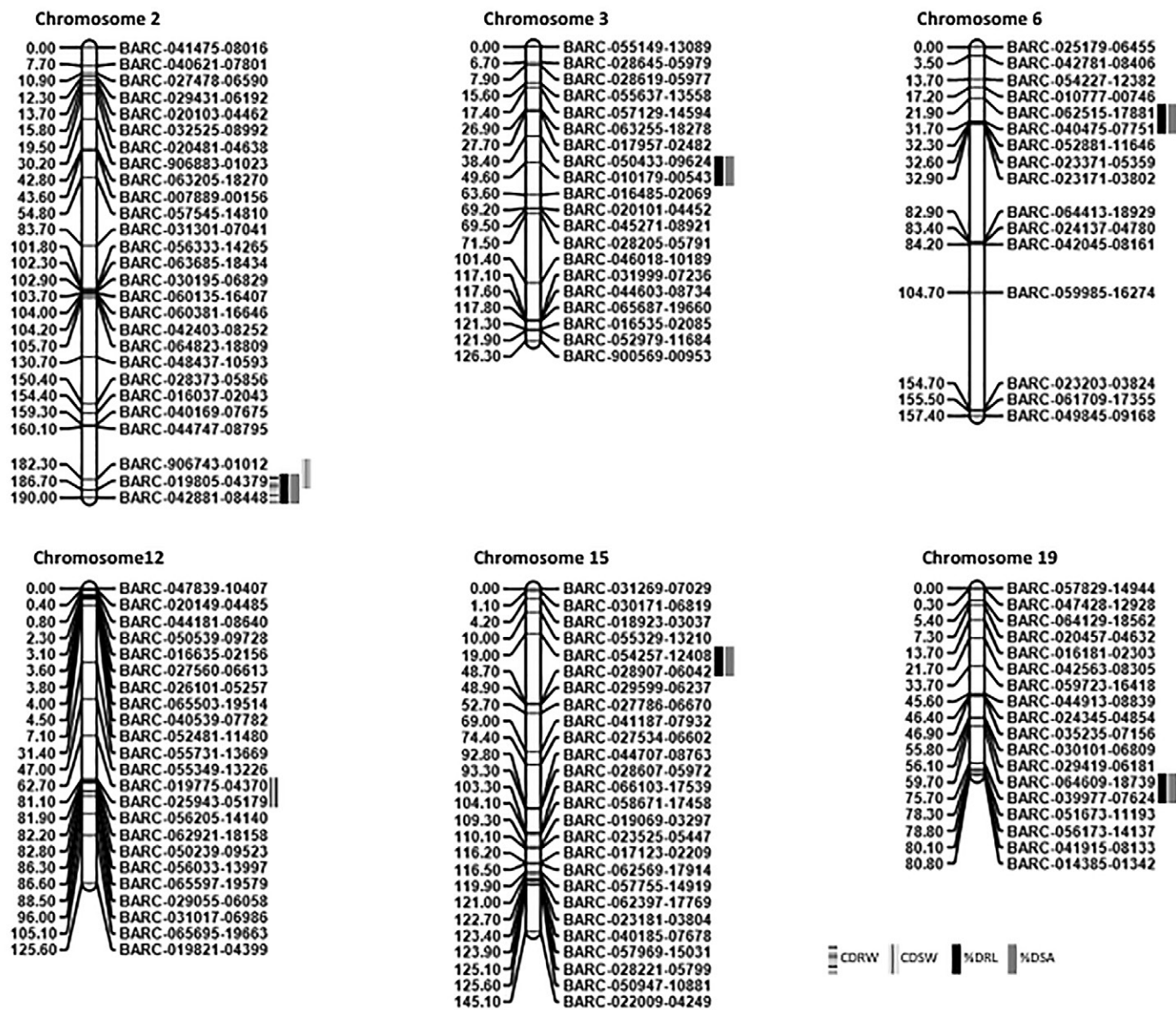


Fig. 5. Quantitative trait loci identified in the AX20925 (PI 399036 × AR2) recombinant inbred line population using corrected dry weights and percentage of root rot data using inclusive composite interval mapping. CDRW, corrected dry root weight; CDSW, corrected dry shoot weight; %DRL, percentage of total diseased root length; %DSA, percentage of total diseased root surface.

carrying both putative PR QTL identified by CDW using ICIM with a genomewide LOD threshold of 3.22.

## DISCUSSION

The soybean PI 399036 was previously identified as a source of resistance with at least three *Rps* genes (Gordon et al., 2007a,b). To identify single loci, it is necessary to combine inoculum to bypass all major *Rps* genes except the targeted *Rps* gene present in a soybean line. The same is true for characterizing PR, as the presence of an effective *Rps* gene will mask the presence of PR. This study investigated the possibility of characterizing PR in two soybean RIL populations by combining three *P. sojae* isolates that differ in pathotype into a single source of inoculum. The hypocotyl inoculation method was applied to identify any novel *Rps* genes, and CDRW was used to identify QTL for PR to *P. sojae*.

Corrected dry weights of each of the parental lines clearly differentiated high PR levels of PI 399036 compared with low PR parental lines AR2 and AR3 (Table 1). Similar results differentiating the high PR parent and the low PR parents were observed for the measurements of diseased root length and root area expressed in percentage. Significant levels were more variable for the data expressed in percentages than for the dry weights. The trend, however, was similar between the two groups of traits. Matthiesen et al. (2016) showed that using inoculum comprised of a combination of *P. sojae* isolates with different pathotypes was an effective and time-conserving method of screening for novel *Rps* resistance genes using the hypocotyl inoculation method. We modified the rice inoculation method and showed that the modified rice-based inoculum containing a mixture of isolates used in

**Table 5. Additional quantitative trait loci identified the AX20931 (PI 399036 × AR3) population by inclusive composite interval mapping with a chromosome-wide LOD thresholds. Underlined = nearest marker.**

Trait	Chr.†	Map position cM‡	Left Marker	Right Marker	LOD§	PVE¶	Add#	Contributor
CDRW††	13	92	<u>BARC-061571-17276</u>	BARC-013325-00484	1.79 (1.6)	5.68	-0.05	AR3
	14	45	BARC-050249-09527	<u>BARC-064873-18956</u>	1.82 (1.6)	5.85	0.05	PI 399036
	15	16	<u>BARC-055329-13210</u>	BARC-062899-18147	1.60 (1.5)	4.60	0.04	PI 399036
CDSW‡‡	9	8	<u>BARC-007972-00189</u>	BARC-051275-11075	2.02 (1.8)	7.07	-0.06	AR3
	17	38	BARC-058841-15463	<u>BARC-052295-11407</u>	2.21 (1.7)	7.63	0.06	PI 399036
%DRL§§	2	28	<u>BARC-065787-19749</u>	BARC-056237-14178	2.53 (2.0)	11.40	-5.07	PI 399036
	7	17	<u>BARC-029825-06442</u>	BARC-042815-08424	1.57 (1.5)	11.65	-4.86	PI 399036
	8	98	<u>BARC-060405-16674</u>	BARC-057257-14650	2.07 (1.8)	14.45	5.71	AR3
	20	3	<u>BARC-042281-08231</u>	BARC-057033-14543	2.09 (1.7)	7.40	-3.91	PI 399036
%DSA¶¶	2	28	<u>BARC-065787-19749</u>	BARC-056237-14178	2.32 (2.0)	10.72	-4.89	PI 399036
	5	51	BARC-031361-07059	<u>BARC-018011-02495</u>	1.93 (1.6)	7.86	-4.06	PI 399036
	7	17	<u>BARC-029825-06442</u>	BARC-042815-08424	1.51 (1.5)	12.37	-4.92	PI 399036
	8	98	<u>BARC-060405-16674</u>	BARC-057257-14650	1.89 (1.8)	15.93	5.92	AR3
	20	3	<u>BARC-042281-08231</u>	BARC-057033-14543	1.72 (1.7)	6.75	-3.67	PI 399036

† Chromosome.

‡ Based on soybean consensus map 4.0 (Hyten et al., 2010).

§ Logarithm of the odds. Chromosome-wide LOD threshold is indicated within parentheses.

¶ Phenotypic variance explained by the quantitative trait loci at the current scanning position.

# Additive effects. Negative sign for corrected dry weights indicate that AR2 contributed the favorable alleles. Negative sign for percent root rot indicate that parent PI 399036 contributed the favorable alleles.

†† Corrected dry root weight.

‡‡ Corrected dry shoot weight.

§§ Percentage of diseased root length.

¶¶ Percentage of diseased root surface area.

a layer test could efficiently characterize the resistance in PI 399036. We identified a novel *Rps* gene or allele and PR QTL in two RIL soybean populations derived from crosses with the accession PI 399036.

Results of the parental line inoculations, with a mixture of three *P. sojae* isolates virulent on *Rps1-7* using the hypocotyl inoculation method, confirmed previous reports (Gordon et al. 2007 a,b) that PI 399036 contained multiple *Rps* genes. The results also showed that at least one novel *Rps* gene or allele was also present in this line. The initial mapping results indicated this novel gene maps to the *Rps4/6* region on chromosome 18 using phenotypic data with the hypocotyl inoculation method in both RIL populations (data not shown). However, PI 399036 failed to confer complete root resistance. It is possible that the mixed inoculum caused more disease pressure on the roots than on the hypocotyl or that the novel *Rps* gene or allele only provides a weak form of root resistance in contrast to previously identified *Rps* genes. Current research underway to explore this gene region will shed light on the possible molecular mechanisms underlying the form of resistance provided by this novel *Rps* gene or allele. Detailed mapping of this gene or allele is in progress and data will be published elsewhere.

Inclusive composite interval mapping was chosen for this study as it has shown to have higher QTL detection power with a low false detection rate. At the same time

the estimates of the QTL effects are reported to be “less biased” with ICIM (Li et al., 2007). As indicated, data for lines exhibiting complete root resistance were eliminated from the final PR analysis as the presence of a major gene could mask minor QTL effects. We did not detect a major QTL on chromosome 18, indicating the QTL identified in this study are in fact putative QTL for PR.

Using ICIM, we identified a total of three significant QTL on chromosomes 2, 9, and 17 that were associated with PR to *P. sojae* in our two RIL populations using the modified rice-based method with a mixed inoculum containing three *P. sojae* isolates. Additionally, 14 minor effect QTL were also identified on 13 chromosomes. None of the QTL identified in this study are colocalized with previously reported PR QTL on the same chromosomes (Burnham et al., 2003; Han et al., 2008; Weng et al., 2007; Wang et al., 2010, 2012; Tucker et al., 2010; Li et al., 2010; Wu et al., 2011; Nguyen et al., 2012).

Several reasons may have contributed to the identification of PR QTL that differ from previously published PR QTL. Our study used a combination of three *P. sojae* isolates that differed in pathotypes from those used in previous work; the germplasm we evaluated has not been used in any previously published studies to map QTL for PR to PRR; the RIL populations were evaluated using a different disease screening protocol, the rice-based method; and disease assessment was done using four different disease



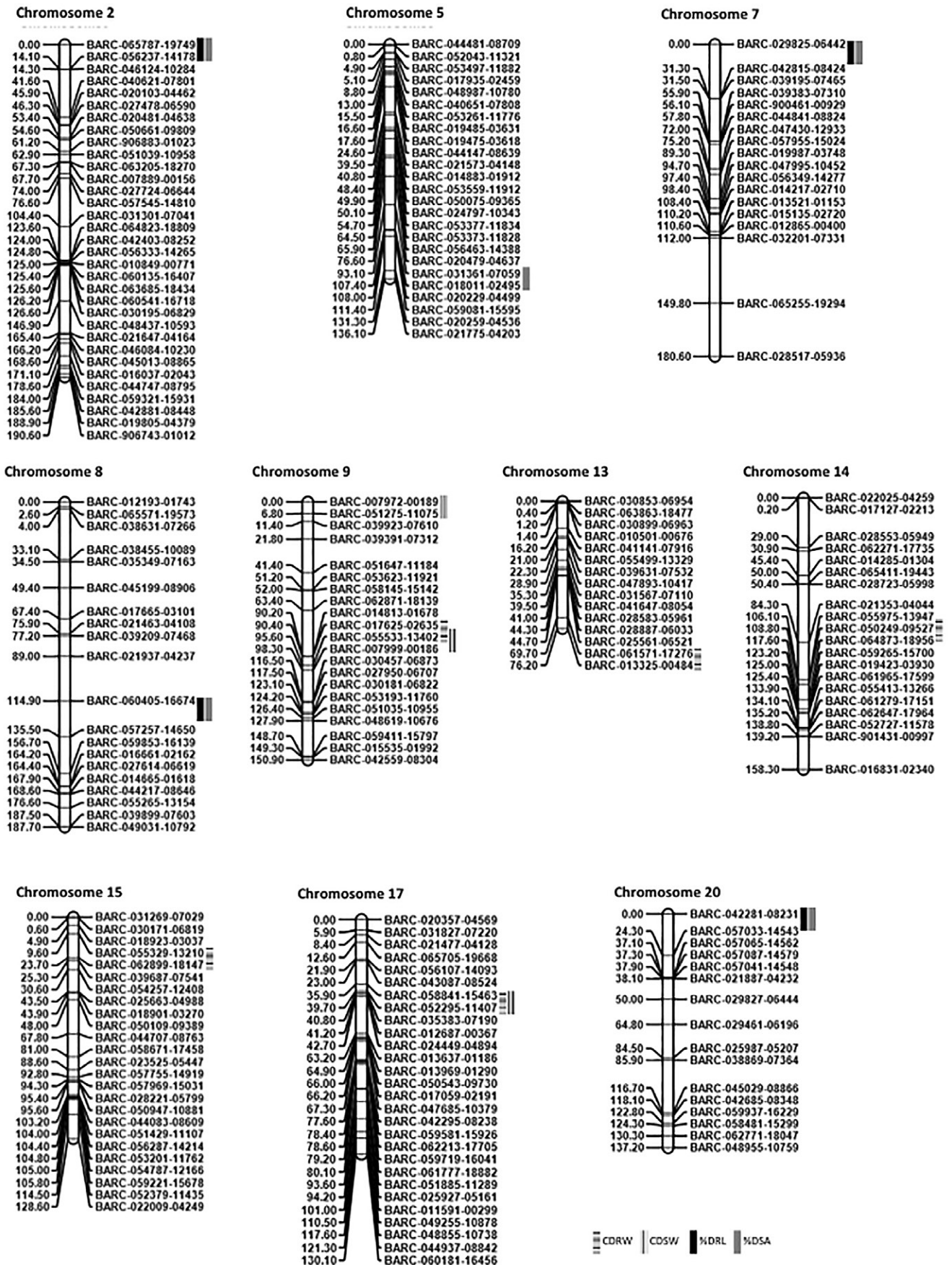


Fig. 6. Quantitative trait loci identified in the AX20931 (PI 399036 × AR3) recombinant inbred line population using corrected dry weights and percentage of root rot data using inclusive composite interval mapping. CDRW, corrected dry root weight; CDSW, corrected dry shoot weight; %DRL, percentage of total diseased root length; %DSA, percentage of total diseased root surface.

parameters. Studies have shown that result outcomes can differ depending on the isolates, germplasm, and phenotyping protocols used. In addition, environmental conditions for each of the studies also contribute to variation in results. These considerations suggest that the identified QTL can be isolate and germplasm specific and may vary on the basis of the phenotyping method used to characterize PR as well as the environment in which the characterization was done (Wang et al., 1994; Han et al., 2008; Marcel et al., 2008; Li et al., 2010; St. Clair 2010; Wang et al., 2010, 2012). It is possible that to validate PR QTL results it might be necessary to reevaluate the different germplasm sources under similar environmental conditions using similar screening tests to estimate phenotypic variation.

In our study the QTL on chromosome 2 associated with marker BARC-019805-04379 was identified by all four disease assessment parameters in the AX20925 RIL population. A major effect QTL was also identified on chromosome 9 in the AX20931 population, which accounted for 21 to 30% of the phenotypic variation. However, the presence of a major gene on chromosome 2, 9, or 17 was not detected in the analysis of the hypocotyl inoculation data (data not shown). Therefore, the three QTL could potentially be novel QTL for PR to PRR rather than major *Rps* genes. Identification of the same QTL on chromosome 2 by all four disease assessment methods in this study clearly shows that the four disease assessment parameters we used are useful for detecting QTL associated with PR to PRR. However, no consistent correlation between CDW and %RR was observed. A previous study by Stewart and Robertson (2012) demonstrated that CDRW is negatively and significantly correlated with root rot severity ratings. We tested three additional parameters, CDSW, %DRL, and %DSA, to determine if they are correlated with CDRW. Even though we did observe significant correlation between CDW and %RR among the parental lines, the association was not always detected within the populations. A significant correlation between CDW with %RR may have led to the identification of the same QTL on chromosome 2 in the AX20925 population. Conversely, in the AX20931 population low correlation occurred between CDW and %RR, and different QTL were identified. Corrected dry shoot weight showed significant but moderate to low association with CDRW. Corrected dry shoot weight and %RR could also be influenced by a number of factors leading to the identification of other loci. At the same time, use of multiple isolates and the level of heterozygosity in the F<sub>5,7</sub> generation compared with a more advanced inbred generation could have also contributed to inconsistencies in detecting QTL with different disease assessment parameters. Hence, we hypothesize that CDSW and %RR are presumably not only segregating for genes for PR. Taking into consideration the poor correlation of %RR with PR in the AX20931 population, we cannot be certain that the

minor effect QTL identified by %RR in this population are PR QTL. Therefore, we have not reported the QTL identified through %RR in the AX20931 as PR QTL. Our results suggest that reporting QTL using different disease assessment parameters and correlation among the disease assessments parameters must be done with caution.

A total of 10 RILs were identified in this study that can be useful in future breeding efforts to develop improved *P. sojae*-resistant commercial varieties. Currently, breeding efforts are in progress to develop germplasm lines with the different combinations of the QTL identified in this research.

## Acknowledgments

The authors wish to thank Dr. Perry Cregan, Soybean Genomics and Improvement Lab, Beltsville Agricultural Research Center, West, USDA, ARS Beltsville, MD, for conducting the SNP genotyping; Dr. Anne Dorrance for providing one of the isolates used in this study and for helpful discussions and critical reviews of drafts of the manuscript; Dr. Brett Tyler for providing one of the *P. sojae* isolates used in this study; Miss Ashley Greff for technical assistance; and the Iowa Soybean Association for the generous funding of this project.

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