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ORIGINAL PAPER

Genetic analysis and fine mapping of *RpsJS*, a novel resistance gene to *Phytophthora sojae* in soybean [*Glycine max* (L.) Merr.]

Jutao Sun · Lihong Li · Jinming Zhao · Jing Huang · Qiang Yan · Han Xing · Na Guo

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Abstract

Key message **We finely map a novel resistance gene (*RpsJS*) to *Phytophthora sojae* in soybean. *RpsJS* was mapped in 138.9 kb region, including three NBS-LRR type predicted genes, on chromosome 18.**

Abstract Phytophthora root rot (PRR) caused by *Phytophthora sojae* (*P. sojae*) has been reported in most soybean-growing regions throughout the world. Development of PRR resistance varieties is the most economical and environmentally safe method for controlling this disease. Chinese soybean line Nannong 10-1 is resistant to many *P. sojae* isolates, and shows different reaction types to *P. sojae* isolates as compared with those with known *Rps* (Resistance to *P. sojae*) genes, which suggests that the line may carry novel *Rps* genes or alleles. A mapping population of 231 F_2 individuals from the cross of Nannong 10-1 (Resistant, R) and 06-070583 (Susceptible, S) was used to map the *Rps* gene. The segregation fits a ratio of 3R:1S within F_2 plants, indicating that resistance in Nannong 10-1 is controlled by a single dominant gene (designated as *RpsJS*). The results showed that *RpsJS* was mapped on soybean chromosome 18 (molecular linkage group G, MLG G) flanked by SSR (simple repeat sequences) markers BARC-SOYSSR_18_1859 and SSRG60752K at a distance of 0.9 and 0.4 cm, respectively. Among the 14 genes annotated in this 138.9 kb region between the two markers, three genes (*Glyma18g51930*, *Glyma18g51950* and *Glyma18g51960*) are the nucleotide-binding site and a leucine-rich repeat (NBS-LRR) type gene, which may be involved in recognizing the presence of pathogens and ultimately conferring resistance. Based on marker-assisted resistance spectrum analyses of *RpsJS* and the mapping results, we inferred that *RpsJS* was a novel gene or a new allele at the *Rps4*, *Rps5* or *Rps6* loci.

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J. Sun and L. Li contributed equally to this study.

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J. Sun · L. Li · J. Zhao · J. Huang · Q. Yan · H. Xing (✉) · N. Guo (✉)
National Center for Soybean Improvement, National Key Laboratory of Crop Genetics and Germplasm Enhancement, Key Laboratory of Biology and Genetics and Breeding for Soybean, Ministry of Agriculture, Nanjing Agricultural University, Nanjing 210095, People's Republic of China
e-mail: xingyuan_2013@163.com; hanx@njau.edu.cn

N. Guo
e-mail: guona@njau.edu.cn

Introduction

Phytophthora root rot (PRR) caused by *P. sojae* has been reported in most soybean-growing regions throughout the world (Schmitthenner 1985). *P. sojae* is a soil-borne oomycete pathogen and can infect soybean plants of different growth stages throughout the growing season (Schmitthenner 1985). PRR is one of the most serious soybean diseases that caused consistent yield losses of 0.68×10^6–1.55×10^6 tonnes from 1996 to 2010 in the United States (Wrather 2013; Wrather and Koenning 2009). Utilization of resistance varieties is the most economical and environmentally safe method to control this disease (Dorrance et al. 2003). Two distinct types of host resistance have been described: (1) race-specific resistance conditioned by a single dominant *Rps* gene and (2) partial resistance conferred by multiple genes acting together (Sugimoto et al. 2012).

Molecular markers linked to *Rps* gene and quantitative trait loci (QTLs) of partial resistance to *P. sojae* in soybean have already been determined in different linkage populations. Among these studies, race-specific resistance is commonly evaluated by the hypocotyl inoculation method (Sun et al. 2011), and partial resistance by three different types of methods, including field evaluation (Han et al. 2008; Li et al. 2010; Weng et al. 2007), greenhouse inoculum layer (Han et al. 2008; Li et al. 2010) or tray test assay (Wang et al. 2010). A total of 45 QTLs of partial resistance, which were distributed on 15 molecular linkage groups (MLG) have been identified at the SoyBase website (http://soybase.org/).

To date, about 20 *Rps* genes/alleles on four chromosomes have been identified. *Rps1* (containing five alleles *Rps1a*, *Rps1b*, *Rps1c*, *Rps1d* and *Rps1k*), *Rps7*, *Rps9*, *RpsYu25*, *RpsYD29*, *RpsUN1* and the *Rps* gene in soybean cultivar 'Waseshiroge', have been mapped on chromosome 3 (MLG N) (Demirbas et al. 2001; Gao et al. 2005; Weng et al. 2001; Wu et al. 2011; Sun et al. 2011; Zhang et al. 2013; Lin et al. 2013; Sugimoto et al. 2011). *Rps2* and *RpsUN2* have been mapped on chromosome 16 (MLG J) (Demirbas et al. 2001; Lin et al. 2013). *Rps3* containing five alleles (*Rps3a*, *Rps3b* and *Rps3c*) has been mapped on chromosome 13 (MLG F), and is linked with *Rps8* (Gordon et al. 2006; Demirbas et al. 2001; Sandhu et al. 2005). *Rps4*, *Rps5* and *Rps6* are linked and located on chromosome 18 (MLG G) (Demirbas et al. 2001; Sandhu et al. 2004). Among these *Rps* genes/alleles, *RpsYD29* has been finely mapped (Zhang et al. 2013), and only *Rps1k* has been isolated (Gao et al. 2005). The BAC-cloned *Rps1k* region contains four coiled-coil (CC)-NBS-LRR type genes following fine mapping (Gao and Bhattacharyya 2008; Gao et al. 2005).

These cultivars or lines that carried *Rps* genes have effectively controlled PRR, and a few *Rps* genes, for example, *Rps1k*, have been widely used in commercial soybean varieties, which would reduce both crop losses and production expenses (Dorrance and Schmitthenner 2000). However, none of these *Rps* genes could control all of the races of *P. sojae*. Thus, continuous and enhanced utilization of *Rps* genes in soybean varieties against *P. sojae* races has created selection pressures for the evolution and new physiological races that can overcome resistance conferred by the known *Rps* genes (Dorrance et al. 2003). It is, therefore, urgent to identify and use new *Rps* genes in soybean breeding programs.

China has a large number of soybean germplasm resources, and many PRR resistance germplasm were identified in previous study (Sun et al. 2008; Wu et al. 2010). One hundred and eight soybean cultivars/lines were initially evaluated for their responses to *P. sojae* isolate JS08-12 (data not shown), and the soybean line Nannong 10-1 was identified as having race-specific to *P. Sojae* based on hypocotyl inoculations. The line may carry novel *Rps* genes or alleles. The objectives of this study were to further characterize the mode of its inheritance in line Nannong 10-1, finely map the *Rps* gene(s) on the soybean genome and predict the candidate gene(s).

Materials and methods

P. sojae isolates, host differential set and plant materials

A total of 15 *P. sojae* isolates (Supplementary Table 1), containing different virulence formula, were obtained from the Key Laboratory of Monitoring and Management of Plant Diseases and Insects, Ministry of Agriculture, Nanjing Agricultural University, China. They were used in the phenotype test.

The soybean line Nannong 10-1 and 06-070583 (Supplementary Fig. 1) were obtained from National Center for Soybean Improvement, Nanjing city, Jiangsu province, China. To determine the reaction pattern of Nannong 10-1, we used a differential set of cultivars/genotypes, each one containing one known resistance gene. They were Harlon (*Rps1a*), Harosoy13XX (*Rps1b*), Williams79 (*Rps1c*), PI103091 (*Rps1d*), Williams82 (*Rps1k*), L76-988 (*Rps2*), L83-570 (*Rps3a*), PRX146-36 (*Rps3b*), PRX145-48 (*Rps3c*), L85-2,352 (*Rps4*), L85-3,059 (*Rps5*), Harosoy62XX (*Rps6*) and Harosoy (*Rps7*). The variety Williams (*rps*) was included as the susceptible standard to indicate the success of inoculation.

The mapping populations consist of 231 F_2 individuals derived from Nannong 10-1 (P_1) × 06-070583 (P_2). F_1 seeds from the cross between Nannong 10-1 and 06-070583 were self-pollinated to produce the population of F_2 plants. Each F_2 plant (for genotype evaluation) from the cross was self-bred to form the $F_{2:3}$ families for phenotype evaluation.

Pathotype evaluation of isolates and disease evaluations of populations

The pathotypes of the isolates were determined using the modified hypocotyl inoculation technique (Sun et al. 2011). For each isolate inoculation, 12 seeds of each differential were planted in a 10-cm diameter plastic pot filled with vermiculite. After 10 day, mycelia from 7-day-old cultures grown on V8 juice agar medium (10 % Campbell's V8 vegetable juice, 0.02 % $CaCO_3$, 1.5 % Bacto-agar) were inoculated onto an incision in the hypocotyl of the seedling when the cotyledons were fully opened. After inoculation, the seedlings were placed in a mist chamber (90 % relative humidity) at 25 °C for 2 days and then transferred to the greenhouse at 25 °C with a 14-h light, and 10-h dark cycle.

Table 1 Segregation analysis of resistance to JS08-12 in cross of Nannong 10-1 × 06-070583

Cross or parent	No. of plant			Chi squared tests		
	Resistance	Segregation	Susceptibility	Expected ratio	χ^2	*P*
Nannong 10-1 (P_1)	40	0	0			
F_1	28	0	0			
F_2	98	0	30	3:1	0.17	0.77
$F_{2:3}$	60	118	53	1:2:1	0.53	0.68
06-070583 (P_2)	0	0	40			

Reactions of the seedling were evaluated 5 days after inoculation. Reactions were recorded as R (resistant, seedling alive with no expanding lesion), or S (susceptible, seedling dead with brown hypocotyls).

To test the phenotypes of the population, the *P. sojae* isolate JS08-12 (virulence formula is *1a, 1b, 1c, 1d, 1 k, 2, 3a, 3b, 3c, 4, 5, 6* and *7*) was used to inoculate Nannong 10-1, 06-070583, and 30 individual F_3 seedlings per $F_{2:3}$ family by the same technique as above. The soybean line Nannong 10-1 is recorded as R with 100 % seedling alive, and 06-070583 is S with 100 % dead (Table 1). So each $F_{2:3}$ family had 30 plants scored, either all R, all S, or H (heterozygous, a combination of both). For all experiments, plants were grown and maintained under greenhouse conditions at the Nanjing Agricultural University facilities.

DNA preparation and pooling for bulk segregation analysis

The genomic DNA was extracted from young leaves of soybean (P_1, P_2 and F_2 plants) by CTAB (cetyl trimethylammonium bromide) method with minor modifications (Allen et al. 2006). Resistant and susceptible bulks for the bulk segregation analysis (BSA) were prepared from DNA samples of either ten homozygous-resistant or ten homozygous-susceptible F_2 individuals (Michelmore et al. 1991). DNA bulks were prepared by pooling equal amounts of DNA (1 µg) from each of selected individuals. The final concentration of each bulk was adjusted to 50 ng/µl.

SSR marker and analysis

Initially, 600 SSR primers were synthesized and obtained from SoyBase (http://soybase.org/), which were screened for polymorphisms among parents and the two DNA bulks. 158 SSR markers were polymorphic between the two parents and two markers were polymorphic between the two bulks. These two polymorphic markers were further used for genotyping of the progenies. After the preliminary genetic mapping of *Rps* gene region, additional SSR markers were obtained from Song et al. (2010) and new markers were developed to fine mapping of the gene. We developed new SSR markers between BARCSOYSSR_18_1859 and BARCSOYSSR_18_1864 based on Williams 82 sequence (Song et al. 2010). The new markers were designed with Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, CA, USA) with default parameters according to the Williams 82 reference sequence (http://www.phytozome.net/), in which the SSR were identified using the SSRHunter software (Li and Wan 2005). PCR was conducted according to Sun et al. (2011). Briefly, each PCR reaction contained 50 ng genomic DNA, 0.2 mmol/l of dNTPs, 1 U *Taq* DNA polymerase, 2 µl of 10× PCR buffer containing 15 mmol/l $MgCl_2$, 0.25 µmol/l of each primer in a total volume of 25 µl. PCR was performed in a Peltier thermal cycler (PTC-225), at 95 °C for 3 min, followed by 30 cycles of denaturing at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min, with final incubation at 72 °C for 5 min before cooling to 4 °C. The amplified products were separated by electrophoresis through 8 % non-denaturing polyacrylamide gels and then visualized through silver staining. SSR primers were synthesized by Invitrogen Biotech Ltd. Co. (Shanghai, China).

Marker-assisted resistance spectrum (MARS) analyses of *RpsJS*

We use MARS analysis method to determine the resistance of *RpsJS*, and to distinguish it from previously identified *Rps* genes. The method was performed according to Lin et al. (2013). Eight homozygous resistance and segregating lines were selected from the 231 $F_{2:3}$ families on the basis of the co-segregated markers (BARCSOYSSR_18_1861). These selected families were evaluated with the 15 isolates of *P. sojae* used in this study.

Data analysis and linkage map construction

The Chisquare (χ^2) analysis was performed to test the phenotypic data for a goodness-of-fit to the Mendelian segregation ratio. The selected SSR markers in the mapping population were tested, also. A genetic linkage map of the *Rps* genes was constructed with the Joinmap 4.0 linkage analysis software (Van Ooijen 2006). Linkage groups were determined using a log-likelihood (LOD) threshold of 3.0.

Results

Inheritance of resistance to *P. sojae* JS08-12

The segregation ratio was investigated by the individual plants of F_1, F_2 and $F_{2:3}$ populations derived from the cross of Nannong 10-1 × 06-070583. All F_1 plants exhibited resistant phenotype. The segregating ratio of F_2 and $F_{2:3}$ fit well with the Mendelian 3R:1S ratio and 1R:2H:1S ratio, respectively ($\chi^2_{3:1} = 0.17 < \chi^2_{0.05} = 3.84$, $P > 0.50$, $\chi^2_{1:2:1} = 0.53 < \chi^2_{0.05} = 5.99$, $P > 0.50$) (Table 1). These results suggested that the resistance to JS08-12 is controlled by a single dominant gene, which is temporarily designated *RpsJS*.

Mapping *RpsJS* gene with SSR markers

Using the BSA method, two SSR markers, Satt472 and Sat_117 on chromosome 18 (MLG G), showed polymorphisms between Nannong 10-1 and 06-070583, as well as between the resistant bulk and the susceptible bulk. Linkage analysis revealed that *RpsJS* was linked to these two SSR markers and located 'below' them (Fig. 1a). Thus, soybean SSR markers 'below' Sat_117 (Gm18: 58,795,539) were selected from the BARCSOYSSR_1.0 database developed by Song et al. (2010), and their polymorphisms between Nannong 10-1 and 06-070583 were evaluated. Linkage analysis further revealed that *RpsJS* was located between BARCSOYSSR_18_1859 (18_1859) and BARCSOYSSR_18_1864 (18_1864) (Fig. 1a).

A 254 kb DNA segment of soybean chromosome 18 between BARCSOYSSR_18_1859 and BARCSOYSSR_18_1864 was identified using a 'BLAST genome' search in the Phytozome soybean genome database. Within this region, 55 SSR loci were identified by SSRHunter. A total of 37 SSR loci were selected to design primers for fine mapping of *RpsJS*. Three SSR markers (Table 2), SSRG60684K, SSRG60718K and SSRG60752K, showed polymorphisms between Nannong 10-1 and 06-070583. Molecular analysis of the 231 $F_{2:3}$ families using a total of 13 polymorphic markers revealed that their segregation pattern fits the 1:2:1 ratio (Supplementary Table 2 and Supplementary Table 3). A genetic map consisting of the 13 SSR markers and *RpsJS* was constructed, and *RpsJS* was closely linked to the SSR markers BARCSOYSSR_18_1859 and SSRG60752K at a distance of 0.9 and 0.4 cm, respectively; and BARCSOYSSR_18_1861 (18_1861), SSRG60684K and SSRG60718K are co-segregated with *RpsJS* (Fig. 1a).

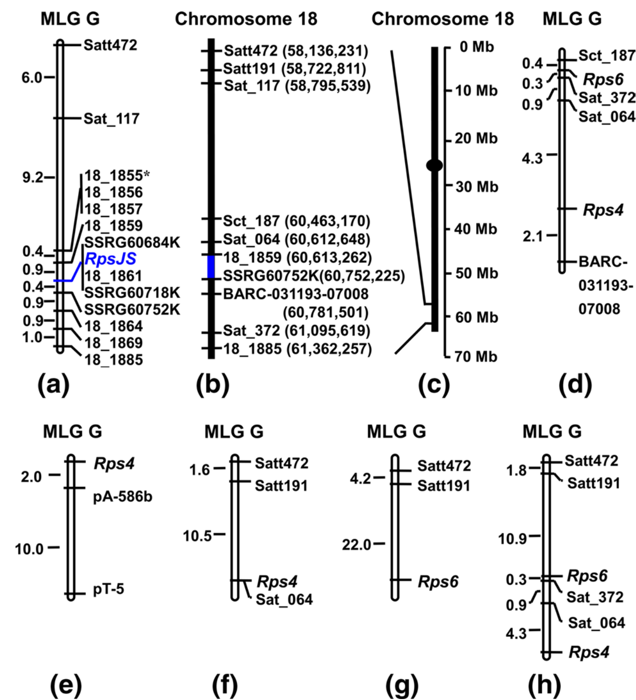

Fig. 1 Genetic and physical map of *RpsJS* and linked *Rps* genes on chromosome 18. **a** Genetic linkage map of *RpsJS* from this study. *Asterisk* abbreviation for 'BARCSOYSSR_18_1855'. **b** Physical position of SSR markers on chromosome 18 reported by Song et al. (2010). Numbers in brackets indicate the physical position of the markers in base pairs (bp). The blue shading shows the interval of *RpsJS*. **c** Physical position of the mapped region of *RpsJS* on chromosome 18 (Schmutz et al. 2010). The round circle indicates the position of centromeric region. **d** The linkage map of *Rps4* and *Rps6* was from Sugimoto et al. (2012). **e** The genetic map of *Rps4* was from Diers et al. (1992). **f** The genetic map of *Rps4* was from Sandhu et al. (2004). **g** The genetic map of *Rps6* was from Sandhu et al. (2004). **h** The genetic map of *Rps4* and *Rps6* was from Gordon et al. (2007)

MARS analyses

The reaction patterns of *RpsJS* and two parents to 15 isolates of *P. sojae* are shown in Table 3. Of the 15 isolates of *P. sojae* used in this study, *RpsJS* conferred resistance to 12 and susceptibility to 3 (Table 3). The $F_{2:3}$ families that did not contain *RpsJS*, along with recessive parent 06-070583 were susceptible to 15 isolates tested (Table 3). These results suggested that *RpsJS* may be the only *Rps* gene present in Nannong 10-1. Nannong 10-1 was susceptible to *P. sojae* strains Pm31 (virulence formula is *1a*, *1b*, *1c*, *1d*, *1k*, *2*, *3b*, *3c*, *4*, *5*, *6* and *7*) and Pmg (virulence formula is *1b*, *1d*, *2*, *3a*, *3b*, *4*, *5*, *6* and *7*), which implied the absence of 14 *Rps* genes (*Rps1-7*). So, we concluded that *RpsJS* is either a novel *Rps* gene or a novel allele.

Candidate gene prediction

Sequence analysis of the soybean genome showed that the physical distance of the region between markers BARCSOYSSR_18_1859 and SSRG60752K, which are at nucleotide positions 60,613,262 and 60,752,225, respectively, is approximately 138.9 kb. According to the soybean gene annotation database accessible at Phytozome

Table 2 List of PCR primer sequences of SSR markers linked to the *RpsJS* locus developed in this study

Marker name	Primer specificity	Primer sequence (5′→3′)	Tm (°C)	Core repeat motif	Nucleotide position on chromosome 18 (kb)
SSRG60684K[a]	Forward	CAATGTTTTACTTTGGAGC			
	Reverse	AAAATAAATACTAATGTAAAATGAT	55	TA	60684
SSRG60718K	Forward	TTTCTTAGCCTTGTACTTTC	55	AT	60718
	Reverse	CCAAAGGCTGTATCTGTT			
SSRG60752K	Forward	AACAACACCATCAACAAAACGAAA	55	AAG	60752
	Reverse	GTTCACGGACGAGGAAGATA			

[a] Markers SSRG60684K, SSRG60718K, and SSRG60752K were developed according the sequence between BARCSOYSSR_18_1859 and BARCSOYSSR_18_1864 on soybean chromosome 18 in the Phytozome database (http://www.phytozome.net)

Table 3 Marker-assisted resistance spectrum analysis (MARS) of *RpsJS* to isolates of *P. sojae*

P. sojae		$F_{2:3}$ families selected[a]		Parental lines		*Rps* genes on Gm18		
Isolate	Race	*RpsJS*	*rpsJS*	Nannong 10-1	06-070583	*Rps4*	*Rps5*	*Rps6*
P6497	2	S[b]	S	S[b]	S	R[b]	R	R
P7063	6	R	S	R	S	R	R	S
S16	17	R	S	R	S	R	R	S
S2	19	R	S	R	S	R	R	R
PNJ1	N/A[c]	R	S	R	S	S	R	S
Pmg	N/A	S	S	S	S	S	S	S
Pm28	N/A	R	S	R	S	R	S	S
Pm2	N/A	R	S	R	S	R	R	R
JS08-12	N/A	R	S	R	S	S	S	S
HeN08-35	N/A	R	S	R	S	S	S	S
HLJ08-17	N/A	R	S	R	S	S	S	R
H15	N/A	R	S	R	S	R	S	S
AH	N/A	R	S	R	S	S	S	R
P7071	N/A	R	S	R	S	R	R	S
Pm31	N/A	S	S	S	S	S	S	S

[a] The molecular markers for selections is BARCSOYSSR_18_1861 for *RpsJS*. The resistance pattern of *RpsJS* against a *P. sojae* isolate was evaluated by the average proportion of resistance progenies of all the selected eight homozygous $F_{2:3}$ families. The reaction of *rpsJS* was calculated from eight double recessive $F_{2:3}$ families

[b] The soybean differential and lines are recorded as resistant (R) if <25 % of plants were killed, intermediate resistant (I) between 25 and 70 %, and susceptible (S) if >70 %

[c] N/A: The pathotypes of these isolates do not fit any known race designation

(http://www.phytozome.net), 14 predicted genes exist within this region (Supplementary Table 4). The most common R proteins contain a nucleotide-binding site and a leucine-rich repeat (NBS-LRR) domain (Dangl and Jones 2001). Among these predicted genes, three (*Glyma18g51930, Glyma18g51950* and *Glyma18g51960*) are NBS-LRR type genes.

Discussion

In this study, a strong and broad-spectrum PRR resistance gene *RpsJS* (flanked by markers BARCSOYSSR_18_1859 and SSRG60752K, Gm18: 60,613,262–60,752,225) was identified from the soybean line Nannong10-1. This *Rps* gene was mapped to R gene rich regions on soybean chromosomes 18, three previously identified *Rps* genes (*Rps4*, *Rps5* and *Rps6*) have been mapped (Diers et al. 1992; Demirbas et al. 2001; Sandhu et al. 2004; Gordon et al. 2007; Grant et al. 2010). The RFLP marker pT-5 was linked to the *Rps5* locus (MLG G), but SSR markers linked to *Rps5* has not been found (Demirbas et al.2001). The *RpsJS* was located in the region of *Rps4* and *6* (Fig. 1). These results suggested *RpsJS* is likely an allele at the *Rps4*, *5* or *6* loci, or a novel gene at another locus tightly linked to *Rps4*, *5* and *6* loci.

P. sojae isolate JS08-12 is a strong virulent isolate and kills all host differentials (containing the known 13 single *Rps1-7* genes). To test the phenotypes of the population, the isolate JS08-12 was used to inoculate Nannong 10-1, 06-070583, F_1, F_2 and $F_{2:3}$ families using the hypocotyl inoculation technique. Nannong 10-1 have showed resistance to JS08-12. For both the F_2 and $F_{2:3}$ plants inoculated with JS08-12, the data fit well with the genetic model ratios of 3R:1S (F_2 individuals) and 1R:2H:1S ($F_{2:3}$ families). These indicated that *RpsJS* is a single dominant *Rps* gene and a distinct gene from the *Rps1-7* genes. *Rps4*, *5* and *6* have been mapped to the same region of chromosome 18 as *RpsJS*. However, based on MARS analysis, none of the previously described *Rps4*, *5* and *6* alleles showed a pattern identical to *RpsJS* (Table 3). For example, *RpsJS* and Nannong 10-1 were resistant to *P. sojae* strains JS08-12 and HeN08-35, while *Rps4*, *5* and *6* were susceptible, which implied that *RpsJS* is a distinct gene from the *Rps4*, *5* and *6*. Together these data suggested that *RpsJS* was a novel gene or a new allele at the *Rps4*, *5* or *6* loci.

Although new races of *P. sojae* appear with the release of resistant cultivars, the use of genetic resistance still remains the most effective strategy to reduce losses caused by the pathogen. Thus, it is necessary to identify new *Rps* genes and develop molecular markers to genes of interest for marker-assisted selection (MAS). The soybean line Nannong10-1 may be useful as sources of resistance for breeding new cultivars adapted to other parts of China or other countries where PRR is a problem. The SSR markers BARCSOYSSR_18_1861, SSRG60684K and SSRG60718K co-segregated with *RpsJS*, and these markers will be convenient tools for MAS of *RpsJS* in breeding programs.

About 20 *Rps* genes/alleles have been mapped to four chromosomes in soybean, but only one *Rps* gene (*Rps1k*) has been isolated. Unfortunately, the physical location of *Rps1k* gene corresponding to the Williams 82 (carries *Rps1k*) reference genome remains unknown (Lin et al. 2013), which in part limited the gene use in breeding programs. We have constructed a high-resolution map of a chromosomal region controlling the PRR resistance for the eventual cloning of the *Rps* gene. The future cloning and functional elucidation of this *Rps* gene is expected to provide fundamental knowledge useful in efforts to improve the disease resistance trait of soybean and other crops. Also, fine mapping and characterization of *Rps* genes provide a special model to study the interaction of soybean and *P. sojae*.

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

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