Tightly linked \( \textit{Rps12} \) and \( \textit{Rps13} \) genes provide broad-spectrum \textit{Phytophthora} resistance in soybean

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The \textit{Phytophthora} root and stem rot is a serious disease in soybean. It is caused by the oomycete pathogen \textit{Phytophthora sojae}. Growing \textit{Phytophthora} resistant cultivars is the major method of controlling this disease. Resistance is race- or gene-specific; a single gene confers immunity against only a subset of the \textit{P. sojae} isolates. Unfortunately, rapid evolution of new \textit{Phytophthora sojae} virulent pathotypes limits the effectiveness of an \( \textit{Rps} \) ("resistance to \textit{Phytophthora sojae}") gene to 8–15 years. The current study was designed to investigate the effectiveness of \( \textit{Rps12} \) against a set of \( \textit{P. sojae} \) isolates using recombinant inbred lines (RILs) that contain recombination break points in the \textit{Rps12} region. Our study revealed a unique \( \textit{Rps} \) gene linked to the \textit{Rps12} locus. We named this novel gene as \( \textit{Rps13} \) that confers resistance against \( \textit{P. sojae} \) isolate V13, which is virulent to recombinants that contains \( \textit{Rps12} \) but lack \( \textit{Rps13} \). The genetic distance between the two \( \textit{Rps} \) genes is 4 cM. Our study revealed that two tightly linked functional \( \textit{Rps} \) genes with distinct race-specificity provide broad-spectrum resistance in soybean. We report here the molecular markers for incorporating the broad-spectrum \textit{Phytophthora} resistance conferred by the two \( \textit{Rps} \) genes in commercial soybean cultivars.

Soybean \( \textit{Glycine max} \) (L.) Merr.) is one of the main oilseed crops produced and consumed worldwide and is among the world’s five utmost significant food crops\(^1\). Its production is limited by several soybean diseases, with an average annual yield loss of 11% in the United States\(^2\). \textit{Phytophthora} root and stem and root rot (PRS) disease is in the top five diseases that cause severe annual yield losses in soybean\(^1\). The annual crop damage from PSR between 2003 and 2005 averaged about $251.6 million\(^3,4\). From 2010 to 2014, in 28 US states and Ontario, Canada, PSR caused an estimated annual yield loss of $403 millions\(^3\). The PSR disease in soybean is caused by the soil-borne oomycete \textit{Phytophthora sojae}\(^34\), and soybean plants infected with \textit{P. sojae} are more susceptible to infection by other soil-borne pathogens.

Oomycete pathogens are challenging to control and most fungicides are ineffective because the \textit{P. sojae} infected roots are difficult to treat effectively with chemicals. Another difficulty is that many oomycetes appear to have extraordinary genetic flexibility, enabling them to adapt to and overcome rapidly the chemical control measures as well as host resistance mechanisms\(^5,6\). Methods employed to control PRS include fungicide applications\(^6\), planting resistant cultivars\(^6,11\), improvement in soil drainage\(^7\), modification of tillage practices\(^12\), and application of calcium-containing compounds\(^13,14\). The most effective way to reduce PRS damage is planting \textit{Phytophthora} resistant soybean cultivars\(^11\).

Single dominant \( \textit{Rps} \) genes confer resistance against \( \textit{P. sojae} \) isolates that carry the cognate avirulence (\textit{Avr}) gene. Soybean \( \textit{Rps} \) genes activate effector-triggered immune responses\(^7,15\), as in other pathosystems\(^16\). More than 30 \( \textit{Rps} \) genes/alleles have been mapped to nine chromosomes, including the newly identified \( \textit{Rps1} \) genes, \( \textit{RpsGZ} \) and \( \textit{RpsX} \)\(^17,18\). The \( \textit{Rps1} \) locus contains five functional alleles (\( \textit{Rps1a}, \textit{1b}, \textit{1c}, \textit{1d}, \textit{1k} \))\(^19–21\), and the \( \textit{Rps3} \) locus contains three (\( \textit{Rps3a}, \textit{3b}, \textit{3c} \))\(^17,22\). The \( \textit{Rps} \) genes mapped to \textit{Chromosome 3} include \( \textit{Rps1}, \textit{Rps7}, \textit{Rps9}, \textit{RpsYu25}, \textit{RpsYD29}, \textit{RpsYD25}, \textit{RpsUN1}, \textit{Rps17}, \textit{RpsWY14},\textit{RpsWY16},\textit{RpsWY25}, \textit{RpsWY28} \). While \( \textit{Rps2} \) gene and \( \textit{RpsUN2} \) have been mapped to \textit{Chromosome 1}\(^23,24,25\), and the three \( \textit{Rps3} \) alleles, \( \textit{Rps3a}, \textit{Rps3b}, \textit{Rps3c} \) along with \( \textit{Rps8} \) and \( \textit{RpsSN10} \) to \textit{Chromosome 13}\(^22,24,31–33\). The \( \textit{Rps4}, \textit{Rps5}, \textit{Rps6}, \textit{Rps12}, \textit{and RpsIS} \) genes are tightly linked and are located on the lower arm of \textit{Chromosome 18}\(^27,28,31–33\). \( \textit{Rps10} \) has been mapped to \textit{Chromosome 17}\(^25\), \( \textit{RpsYB30} \), and \( \textit{RpsZS18} \) to \textit{Chromosome 19}\(^27\) and \textit{Chromosome 27}\(^27\), respectively, and \( \textit{Rps11} \) to \textit{Chromosome 7}\(^38\).

\( \textit{P. sojae} \) isolates evolve rapidly to overcome the introduced resistance genes in commercial cultivars, especially under the monoculture scenario. Over 200 known pathotypes of this pathogen have been reported and the number is ever growing presumably due to selection pressure on the \( \textit{P. sojae} \) population for new pathotypes that can overcome the newly introduced \( \textit{Rps} \) genes. The rapid evolution of new \( \textit{P. sojae} \) virulent pathotypes limits the

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effectiveness of an Rps gene to 8–15 years. For example, a survey on pathotype changes in the population of Phytophthora sojae over several decades showed that while 6% of the pathotypes could defeat the Rps1c gene from 1991 to 1994, it was 57% by 2004. While in 1994, no pathotype could defeat Rps1k, the number of pathotypes increased to 12% in 2004, and to 41% in 2015. The number of pathotypes that defeat both Rps1c and Rps1k increased from none to 31% between 1994 and 2015. With increased complexity of Phytophthora sojae pathotypes, new strategies for managing this pathogen are needed. The use of resistant cultivars is the most cost-effective and environmentally safe method to control this disease. Henceforth, there is a constant need for novel Rps (“resistance to Phytophthora sojae”) genes to manage the disease effectively.

It was suggested that plant introduction (PI) line, PI399036 contains multiple Rps genes. An Rps gene, Rps12, from this PI line was mapped to a 5.4 cM region between the simple sequence repeat (SSR) marker BARC-Rps12 and the NBSRps4/6-130/533 sequence. To determine the utility of this pathogen are needed. The use of resistant cultivars is the most cost-effective and environmentally safe method to control this disease. Henceforth, there is a constant need for novel Rps (“resistance to Phytophthora sojae”) genes to manage the disease effectively.

### Materials and methods

**Plant genetic material.** The AX20925 RIL population used in this study was developed by crossing PI399036 with the germplasm line AR2. This population was used earlier to map Rps12. The individual F2 generation by applying the single-seed descent method. In this study, 120 F2 families (recombinant inbred lines, RILs) were phenotyped for responses to a set of Phytophthora sojae isolates. We utilized a set of recombinant inbred lines (RILs) containing recombination break points in the Rps12 region and lacking functional Rps genes. Rps12, linked tightly to Rps13 on Chromosome 18. The Rps12 and Rps13 genes together provide broad-spectrum Phytophthora resistance in soybean.

**Phytophthora sojae isolates.** Phytophthora sojae isolates R17 (vir 1b, 1d, 3a, 3b, 3c, 4, 5, 6), Val 12-11 (vir 1a, 1b, 1c, 1d, 1k, 2, 3a, 3b, 3c, 4, 5, 6, and 7) genes. Of these 120 RILs, 60 were homozygous resistant, and 60 were homozygous susceptible. The 120 RILs were used in molecular mapping of the Rps13 gene.

**Evaluation of genetic materials for Phytophthora resistance.** Hypocotyls of 7-day-old seedlings of 120 RILs, the parents PI399036 and AR2 along with 14 differential lines carrying Rps1a, Rps1b, Rps1c, Rps1d, Rps1k, Rps2, Rps3a, Rps3b, Rps3c, Rps4, Rps5, Rps6, Rps7, and Rps8 genes and the susceptible cultivar ‘Sloan’ with
ment was conducted three times. Plants were rated seven days after inoculation as either R (resistant, < 30% seedling death) or S (susceptible, ≥ 70% seedling death). Inocula were prepared using a modified version of the protocol described by Dorrance et al. (2008)\(^42\). The macerated R17 and Val 12–11 cultures were mixed in equal proportion to prepare the mixed inoculum\(^45\) that is virulent to soybean cultivars carrying the Rps13 gene. Among \(Rps1\) to 7 loci and partially virulent to lines carrying \(Rps8\). \(P. sojae\) strain V13 was also used as a separate inoculum as it is virulent to soybean lines carrying any of \(Rps1a, 1c, 1d, 4, 7, 12\) genes.

Table 2. SSR markers linked to known \(Rps\) regions.

| \(Rps\) gene | Linked SSR markers | Chromosome | Molecular linkage group |
|-------------|--------------------|------------|------------------------|
| \(Rps1\) a, b, c, d, k | Sat152, Sat_186, Satt631, Satt683, Satt159, Satt530 & Satt009 | 3 | N (Gordon et al.\(^{40}\); Sugimoto et al.\(^{44}\); Wu et al.\(^{38}\); Sun et al.\(^{27}\); Lin et al.\(^{29}\)) |
| \(Rps2\) | Sat_144 & Satt440 | 16 | J (Gordon et al.\(^{40}\)) |
| \(Rps3\) a, b, c | Satt335 & Satt510 | 13 | F (Gordon et al.\(^{40}\)) |
| \(Rps4\) | Sat_064 | 18 | G (Sandhu et al.\(^{31}\); Sahoo et al.\(^{37}\)) |
| \(Rps6\) | Sat_064 | 18 | G (Sandhu et al.\(^{31}\); Sahoo et al.\(^{37}\)) |
| \(Rps7\) | Satt631, Satt683, Satt152, Satt530 & Satt009 | 3 | N (Gordon et al.\(^{40}\); Sugimoto et al.\(^{44}\); Sun et al.\(^{27}\)) |
| \(Rps8\) | Satt663 | 13 | F (Gordon et al.\(^{40}\)) |
| \(Rps9\) | Satt631 & Satt186 | 3 | N (Wu et al.\(^{38}\); Lin et al.\(^{29}\)) |
| \(Rps10\) | Sattwd15-24, Sattwd15-25 & Sattwd15-47 | 17 | D2 (Zhang et al.\(^{39}\)) |
| \(Rps11\) | SSR_07_0286, SSR_07_0300 & SSR_07_0295 | 7 | M (Ping et al.\(^{39}\)) |
| \(Rps12\) | BARCSOYSSR_18_1840 & Sat_064 | 18 | G (Sahoo et al.\(^{37}\)) |
| \(UN1\) | Satt159 & SSR_03_0250 | 3 | N (Lin et al.\(^{37}\)) |
| \(RpsUN2\) | SSR_16_1275 & Sat_144 | 16 | J (Lin et al.\(^{37}\)) |
| \(RpsYn25\) | Sat186 & Satt152 | 3 | N (Sun et al.\(^{27}\); Lin et al.\(^{29}\)) |
| \(YD29\) | SattWM82-50 & Satt1kb | 3 | N (Zhang et al.\(^{43}\)) |
| \(RpsWY\) | Satt631 & Satt152 | 3 | N (Cheng et al.\(^{43}\)) |
| \(RpsZ\) | Satt631, Sat186 & Satt009 | 3 | N (Sugimoto et al.\(^{44}\)) |
| \(RpsIS\) | SSRG60684K & BARCSOYSSR_18_1861 | 18 | G (Sun et al.\(^{44}\)) |

no known \(Rps\) genes\(^{17,42–44}\) were inoculated using the wounded-hypocotyl inoculation technique\(^{45,46}\). The experiment was conducted three times. Plants were rated seven days after inoculation as either R (resistant, < 30% seedling death) or S (susceptible, ≥ 70% seedling death). Inocula were prepared using a modified version of the protocol described by Dorrance et al. (2008)\(^{45}\). The macerated R17 and Val 12–11 cultures were mixed in equal proportion to prepare the mixed inoculum\(^45\) that is virulent to soybean cultivars carrying \(Rps\) genes mapped to \(Rps1\) to 7 loci and partially virulent to lines carrying \(Rps8\). \(P. sojae\) strain V13 was also used as a separate inoculum as it is virulent to soybean lines carrying any of \(Rps1a, 1c, 1d, 4, 7, 12\) genes.

DNA preparation, bulked segregant analysis (BSA). Before inoculation, one unifoliate leaf from each of 11 random plants of individual RIL was collected, bulked and frozen in liquid nitrogen, and stored at −80 °C. The genomic DNA was extracted from the bulked leaf samples using the CTAB (cetyl trimethylammonium bromide) method\(^{47}\). The identified SSR markers linked to \(Rps12\) locus\(^{17}\) were used to conduct BSA for the \(Rps13\) region on pooled DNA samples of 10 homozygous resistant RILs (Resistant Bulk) or ten susceptible RILs (Resistant Bulk) or ten susceptible RILs (Susceptible Bulk)17,\(^{48}\). In BSA48, a polymorphic molecular marker linked tightly to a target locus shows its allelic segregation either in coupling or repulsion phase linkage with alleles of the target locus. In BSA assays, the markers that are not linked to the target locus show heterozygosity due to recombination of the marker alleles with alleles of the target locus.

PacBio long-read sequencing and development of sequence-based polymorphic (SBP) molecular markers. A ~ 50 genome equivalents genome sequence of the PI399036 and AR2 was obtained by PacBio long-read sequencing at the DNA Facility, Iowa State University. The bowtie program was run to identify single nucleotide polymorphisms (SNPs) between genomes of the resistant (PI399046) and susceptible (AR2) lines by mapping sequence reads onto the 8 Mbp region spanning the 53–61 Mbp physical locations on Chromosome 18 containing \(Rps12\) and \(Rps13\). Over 26,000 putative SNPs were identified. We used SNPs of the putative \(Rps13\) region to develop necessary SBP markers according to Sahu et al.\(^{49}\) for mapping the \(Rps13\) gene. Among the identified SNPs, we looked for the ones that are polymorphic for restriction endonucleases. Polymerase chain termination reaction (PCR) amplicons of approximately 200 nucleotides DNA containing variations for restriction endonuclease sites between PI399036 and AR2 were considered as putative SBP markers. Finally, primers for PCR amplification were designed in such a way that one can easily distinguish the haplotype-specific restriction fragment length polymorphisms by separating the restriction enzyme digested PCR products on a 4% (w/v) agarose gel\(^{49}\). Seventeen SBP markers were identified for the \(Rps12-Rps13\) region (Table S1).

Simple sequence repeats (SSR) and SBP markers were used to construct a linkage map of the genomic region carrying the putative novel \(Rps13\) gene. Molecular markers based on previously reported NBS\(Rps4/6\) sequence and SSR markers\(^{17}\), and newly developed SBP markers were used in mapping the \(Rps13\) gene (Table S1). SSR markers linked to \(Rps1S\) were also used in mapping the \(Rps13\) region\(^{34}\). Eleven polymorphic SSR markers, two previously reported NBS\(Rps4/6\) molecular markers along with the newly developed five SBP markers were used to map the \(Rps13\) gene\(^{17}\) (Tables S1, S2).
Screening RILs and parental lines for the presence of known Rps genes. Twenty-three SSR markers linked to the Rps1, 2, 3, 7, 8, 9, 10, 11, Yu25, WY, Rps1?, RpsUN1, UN2, and YD29 loci were used to evaluate for possible polymorphisms between the AR2 (susceptible), and PI399036 (resistant) parents (Table 2) in order to identify RILs that carry SSR alleles specific to the P. sojae susceptible AR2 parent.

Linkage map construction and statistical analysis. The Chi-square (χ²) analysis was performed to check the phenotypic data for goodness-of-fit to a Mendelian segregation 1:1 ratio using Graphpad (http://www.graphpad.com/quickcalcs). Mapmaker version 3.0⁵⁰ and the Kosambi mapping function⁵¹ were used to calculate genetic distances in cM units from the recombination fractions between any given two loci. A logarithm of the odds (LOD) threshold was set as 3.0 to determine the linkages between studied loci. Mapmaker package uses the Lander-Green algorithm to calculate the “best” map order of loci⁵². The marker order was determined using the log-likelihood method⁵⁰. The linkage map of molecular markers and the Rps genes was drawn using MapChart 2.3.⁵³

The source of Rps12 and Rps13 genes. The PI399036 containing the two Rps genes, Rps12 and Rps13, is available from the USDA Soybean Germplasm Collection. The contact person for the seeds is Esther K Per-egrine (esther.peregine@ars.usda.gov), Assistant Soybean Curator, USDA/ARS Soybean Soybean Germplasm Collection, 1101 W. Peabody Dr., Rm. 180, National Soybean Research Center, Urbana, IL 61801, USA.

The segregating materials studied in this study were generated by author Silvia Cianzio and will be available from the Bhattacharyya lab, G319 Agronomy Hall, Iowa State University, Ames, IA 50011, USA. All plant collection methods were complied with relevant institutional, national, and international guidelines and legislation.

Results
Identification of putative RILs carrying the Rps12 gene. It was proposed that the Phytophthora resistant PI399036 line contains multiple Rps genes⁴⁶. Earlier we mapped Rps12 of this line using a mixture isolates that overcome most known Rps genes⁴⁷. To investigate the utility of Rps12 against a set of P. sojae isolates collected from Iowa soybean field, we looked for RILs that carry only Rps12. We have investigated 60 Phytophthora resistant RILs generated from the cross between PI399036 × AR2¹⁷ for SSR markers linked to the known Rps regions as described below. We used 23 SSR markers that were published earlier (Table 2). These include SSR markers Satt335 and Satt510 for Rps3 locus, Satt663 for Rps8 locus, Sat_144 and Satt440 for Rps2 locus, and Satt631, Satt152, Satt530, Satt009 and Satt186 for Rps1, 7, 9, Yu25, WY, and Rps1? Loci, Sattwd15-24, Sattwd15-25 and Sattwd15-47 for Rps10, SSR_07_0286, SSR_07_0300 for Rps11 and SSR_07_0295 for Rps1, Satt159 and SSR_03_0250 for RpsUN1, SSR_16_1275 and Sat144 for RpsUN2, and SattWM82-50 and Satt1kb for RpsUD29 locus ¹⁴,²⁷,³⁸,⁴⁰ (Table 2). The 23 SSR markers were investigated for polymorphisms between the resistant PI399036 and susceptible AR2 parents. Of the 23 SSR markers, 10 SSR markers were polymorphic between the two parents (Fig. 1) and applied initially in evaluating all 60 RILs homozygous for Rps12; and subsequently, 60 Phytophthora susceptible RILs (rps12rps12). The ten polymorphic SSR markers considered for this study include Satt510 for Rps3 locus, Satt663 for Rps8, Satt440 for Rps2 and Satt631, Satt152 and Satt009 for Rps1, 7, 9, Yu25, WY, and Rps1?, Sattwd15-24 for Rps10, SSR_07_0286 for Rps11, and Satt159 and SSR_03_0250 for RpsUN1 (Table S3). From screening of the 60 resistant RILs, we identified RILs 12 and 14 that carry AR2-specific SSR alleles for nine and eight SSR markers, respectively. For RIL12, SSR marker linked to Rps11 is heterozygous; and for RIL14, two SSR markers linked to Rps8 and Rps11 are heterozygous. These two lines were selected to determine the efficacy of Rps12 to a set of P. sojae isolates.

Identification of Rps13. We have obtained 17 P. sojae isolates from the Robertson lab, Iowa State University, collected earlier from the Iowa soybean fields. The isolates were characterized for their pathotypes by inoculating a set of 14 soybean lines that are considered to be differential lines for 14 individual Rps genes (Table 1). All these isolates were used to infect the differential cultivars and selected RIL12 and RIL14 and two parents, PI399036 and AR2. RIL12 and RIL14 contain Rps12 and confers resistance against the isolate mixture of R17 and Val 12-11⁰⁷. Surprisingly, RIL12 is not resistant against seven of the 17 new P. sojae isolates and Val 12-11 (Table 1). On the contrary, RIL14 is resistant against these seven isolates. Based on the genetic make-ups of RIL12 and RIL14 for molecular markers of the Rps12 region, we deduced that there is recombination breakpoint in between the NBSLRR533 and Sat_064 in RIL12. We hypothesized that there could be a novel Rps gene named Rps13 located in between Rps12 and telomere on Chromosome 18.

To further support our hypothesis that there is an Rps gene next to Rps12, we evaluated 60 Phytophthora resistant RILs (Rps12Rps12) and 60 Phytophthora susceptible RILs (rps12rps12) for molecular markers of the genomic region containing the Rps12 gene (Table S4). From molecular mapping of the 120 RILs, we were able to identify two additional RILs, RIL9 and RIL81, that carry recombination breakpoints in the Rps12 region, and were evaluated for their responses to 17 new P. sojae isolates, and mixture of R17 and Val 12-11 isolates. The RIL12 contains Rps12, but not the putative Rps13 region; whereas, RIL81 contains the putative Rps13 region but not Rps12. RIL9 contains the putative Rps13 region, but not Rps12. RILs that carry Rps12, but not Rps13, were susceptible to the P. sojae isolates, V13, IV 6b and Val 12-11, resistant to R17 (Table 3). On the contrary, RIL81 carrying Rps13 but not Rps12 was susceptible to P. sojae isolate R17 (Table 3). Our results established that Rps12 is overcome by several P. sojae isolates, against which Rps13 provides immunity.
Molecular mapping of the Rps13 gene. We determined the inheritance of the putative novel Rps13 gene by evaluating 120 RILs for segregation of Phytophthora resistance against an inoculum mixture of Val 12-11 and R17, which together are virulent on soybean lines carrying all Phytophthora resistance genes mapped to the Rps1 to 7 loci and partially virulent to lines carrying Rps8 along with V13 isolate which is virulent to soybean lines carrying Rps1a, 1c, 1d, 4, 7 and Rps12 (Figs. 2, 3, Table 3).
Analysis of *Rps* gene-linked SSR markers revealed that alleles of Satt009 and Satt510 markers specific to *Rps1c* and *Rps3a* alleles, respectively, are present in PI399036, but not in AR2. We hypothesized that most likely PI399036 contains *Rps1c* and *Rps3a*, in addition to *Rps12* and *Rps13*.

*P. sojae* isolate V13 overcomes the resistance conferred by *Rps1c*, but not *Rps3a*. We therefore classified the RILs into two groups based on Satt510: (i) The RILs which carry Satt510 allele specific to *rps3a* and AR2 parent; and (ii) RILs carry Satt510 allele specific to *Rps3a*. Both groups segregated for resistance to susceptibility in a 3:1 ratio, as observed for single Mendelian genes, following infection with *P. sojae* V13 isolate that overcomes the resistance governed by *Rps12* and *Rps1c*. This confirms that there is a novel *Rps* gene in PI399036.

To map the novel gene, the 120 RILs from the AX20925 population were infected with a mixture of *P. sojae* R17 and V13 isolates that together overcome all known *Rps* genes including *Rps12*, but not the novel *Rps13* gene. Both groups segregated for resistance to susceptibility in a 3:1 ratio, as observed for single Mendelian genes, following infection with *P. sojae* V13 isolate that overcomes the resistance governed by *Rps12* and *Rps1c*. This confirms that there is a novel *Rps* gene in PI399036.

Table 3. Response of four RILS and their parents to 23 *P. sojae* isolates along and a few *P. sojae* isolate mixtures. Plants were rated seven days after inoculation as either R (resistant, < 30% seedling death) or S (susceptible, ≥ 70% seedling death).

| *P. sojae* isolate | Sloan | PI399036 (*Rps12, Rps13*) | AR2 (*rps12, rps13*) | RIL12 (*Rps12, rps13*) | RIL81 (*rps12, Rps13*) | RIL14 (*Rps12, Rps13*) | RIL9 (*Rps12, rps13*) |
|-------------------|-------|--------------------------|-----------------------|------------------------|------------------------|------------------------|------------------------|
| R17 (vir 1b, 1d, 3a, 3b, 3c, 5, 6) | S     | R                        | S                     | R                      | S                      | R                      | R                      |
| Val 12.11 (vir 1a, 1b, 1c, 1d, 1k, 1k, 2, 4, 7) | S     | R                        | S                     | R                      | R                      | R                      | R                      |
| 1005-2.9 (vir 1a, 1b, 1c, 1k, 3b, 7) | S     | R                        | S                     | R                      | R                      | R                      | R                      |
| III 5.2b (vir 1a, 1b, 1c, 1d, 1k, 2, 4, 7) | S     | R                        | S                     | R                      | R                      | R                      | R                      |
| III 23.4b (vir 1a, 1c, 1d, 2, 3b, 3c, 4, 5, 7) | S     | R                        | S                     | R                      | R                      | R                      | S                      |
| IV 5.2 (vir 1c, 1d, 7) | S     | R                        | R                     | R                      | R                      | R                      | R                      |
| IV 6b (vir 1a, 1c, 1d, 7) | S     | R                        | S                     | R                      | R                      | R                      | S                      |
| IV 10 (vir 1a, 1c, 1d, 7) | S     | R                        | S                     | R                      | R                      | R                      | R                      |
| IV 12.2a (vir 2, 4, 7) | S     | R                        | S                     | R                      | R                      | R                      | R                      |
| IV 13.4a (vir 7) | S     | R                        | S                     | R                      | R                      | R                      | R                      |
| IV 23.3 (vir 1a, 1c, 1d, 2, 7) | S     | S                        | R                     | R                      | R                      | R                      | S                      |
| V 13 (vir 1a, 1c, 1d, 4, 7) | S     | S                        | S                     | S                      | R                      | R                      | S                      |
| VI 2.5b (vir 1a, 1c, 1d, 7) | S     | R                        | S                     | R                      | R                      | R                      | R                      |
| VI 12.1a (vir 6, 7) | S     | S                        | S                     | R                      | R                      | R                      | R                      |
| VI 12.2b (vir 1d, 3a, 4, 5, 6, 7) | S     | S                        | S                     | S                      | R                      | R                      | S                      |
| V 15 (vir 1d, 7) | S     | S                        | S                     | R                      | R                      | R                      | R                      |
| V 17 (vir 7) | S     | S                        | S                     | R                      | R                      | R                      | R                      |
| VI 20 (vir 1c, 2, 7) | S     | R                        | S                     | R                      | R                      | R                      | R                      |
| VI 23.3b (vir 1d, 7) | S     | S                        | R                     | R                      | R                      | R                      | R                      |
| S 5-5 (vir 7) | S     | R                        | S                     | R                      | R                      | R                      | R                      |
| P7074 (vir 1b, 1d, 2, 3a, 3b, 3c, 4, 5, 6, 7, 8) | S     | S                        | S                     | R                      | S                      | R                      | R                      |
| PR1 (vir 7) | S     | S                        | S                     | R                      | R                      | R                      | R                      |
| PR6 (vir 7) | S     | S                        | S                     | R                      | R                      | R                      | R                      |
| III 2.5b + R17 + V13 (vir 1a, 1b, 1c, 1d, 1k, 1k, 2, 3a, 3b, 5, 7, 8) | S     | R                        | S                     | S                      | R                      | R                      | S                      |
| 1005-2.9 + V12.3b + R17 (vir 1d, 2, 3b, 7) | S     | R                        | S                     | R                      | R                      | R                      | R                      |
| R17 + Val 12-11 (vir 1a, 1b, 1c, 1d, 1k, 2, 3a, 3b, 3c, 4, 5, 6, 7) | S     | S                        | R                     | S                      | R                      | R                      | R                      |

Analysis of *Rps* gene-linked SSR markers revealed that alleles of Satt009 and Satt510 markers specific to *Rps1c* and *Rps3a* alleles, respectively, are present in PI399036, but not in AR2. We hypothesized that most likely PI399036 contains *Rps1c* and *Rps3a*, in addition to *Rps12* and *Rps13*. *P. sojae* isolate V13 overcomes the resistance conferred by *Rps1c*, but not *Rps3a*. We therefore classified the RILs into two groups based on Satt510: (i) The RILs which carry Satt510 allele specific to *rps3a* and AR2 parent; and (ii) RILs carry Satt510 allele specific to *Rps3a*. Both groups segregated for resistance to susceptibility in a 3:1 ratio, as observed for single Mendelian genes, following infection with *P. sojae* V13 isolate that overcomes the resistance governed by *Rps12* and *Rps1c*. This confirms that there is a novel *Rps* gene in PI399036.

To map the novel gene, the 120 RILs from the AX20925 population were infected with a mixture of *P. sojae* R17 and V13 isolates that together overcome all known *Rps* genes including *Rps12*, but not the novel *Rps13* gene. Of the 120 RILs, 52 RILs showed resistance against the isolate mix and 67 showed susceptibility. The observed segregating 0.867:0:0.08:1.117 genotypic ratio of resistance to susceptibility among the 120 RILs fits to the expected 0.984:0.032:0.984::RR:Rr:rr ratio, where R is *Rps13* and r is *rps13* for single gene segregation among the RILs in F2 generation with an estimated 98.4% of the genes homozygous ($\chi^2 = 0.104$).

We conducted bulked segregant analysis (BSA) to identify molecular markers linked to the novel *Rps13* resistance gene and confirm that *Rps13* is mapped next to *Rps12*. In this BSA study, we used SSR markers of the *Rps12* region to test our hypothesis that *Rps13* is linked to *Rps12*. The results of BSA suggested that indeed the
The gene is co-segregated with the markers mapped in between \textit{Rps12} and telomere. To develop a high-resolution map of the \textit{Rps13} region, we investigated 19 putative SBP markers for polymorphisms. Five of the 19 putative SBP markers are polymorphic between resistant and susceptible parents and were used for mapping the \textit{Rps12-Rps13} region (Fig. 4). The \textit{Rps13} gene co-segregated with the Sat\_064, BARCOSOYSSR\_18\_1859, and BARCOSOYSSR\_18\_1860 markers. The genetic distance between \textit{Rps12} and \textit{Rps13} genes is 4 cM (Fig. 5, Table S4).

To identify homologues of the candidate \textit{Rps13} genes, we investigated the annotated soybean genes in the 92.7 kb \textit{Rps13} region between the two markers, SBP56.32 and BARCOSOYSSR\_18\_1861, in the Williams 82 genome sequence located at the soybean genome browser (SoyBase; https://www.soybase.org)\textsuperscript{53}. Sixteen genes including an NB-ARC domain-containing disease resistance-like gene, \textit{Glyma.18g283200}, are present in this region (Table S5). Williams 82 does not carry the \textit{Rps13} gene.

**Discussion**

This study was designed to investigate the usefulness of the \textit{Phytophthora} resistance governed by the \textit{Rps12} gene\textsuperscript{17}. In mapping \textit{Rps12}, we had to use a mixture of \textit{P. sojae} isolates to mask the effect of previously known \textit{Rps} genes that were in the PI399036 line, the source of \textit{Rps12}\textsuperscript{17}. To determine the utility of \textit{Rps12} genes against a set of uncharacterized \textit{P. sojae} isolates, we must identify an RIL that contains only \textit{Rps12}. We therefor first examined a set of 60 \textit{Phytophthora} resistant RILs for possible absence of other known \textit{Rps} genes by studying the

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**Figure 2.** Two RILs differing alleles at the linked \textit{Rps12} and \textit{Rps13} loci showed distinct responses to \textit{P. sojae} V13 isolate and the mixture of R17 and Val12-11 isolates. \textit{P. sojae} isolate V13 failed to defeat resistance mediated by \textit{Rps13} gene but could overcome that by \textit{Rps12}; whereas, the mixture of R17 and Val12-11 isolates could overcome \textit{Rps13}, but not \textit{Rps12}.
...polymorphisms of Rps gene-linked SSR markers. Linked SSR markers co-evolved with linked Rps genes and SSR alleles can be used to predict alleles of the linked Rps genes.

A total of 210,990 SSRs were identified from the soybean genome. Of these, 61,458 SSRs contain repeat units of di-, tri-, and tetranucleotide with (AT)n, (ATT)n and (AAAT)n as the most abundant motifs54. A genetic linkage map consisting of 20 linkage groups with approximately 1500 SNP, 1000 SSR markers, 700 RFLP, and 73 RAPD markers and 46 classical trait loci is available in soybean55–57. Information of genetic markers has been used to map Rps1, Rps2, Rps3, Rps4, Rps5, Rps6, Rps7, and Rps8 loci to Chromosomes 3, 16, 13, 18 and 13, respectively24,25,31,32,35,57–59. While the Rps4 locus was mapped close to the Rps6 region, the Rps8 locus mapped close to the Rps3 region31,32,35. The RFLP marker pT-5 was shown to be linked to the Rps5 locus36. SSR markers mapped to the Rps5 locus are yet to be identified36. Thus, SSR markers linked to each Rps gene except Rps5 have been reported31.

In this study, we selected 23 SSR markers that have been shown to be linked to most of the reported Rps genes (Table 2). Out of 21 SSR markers, 10 were polymorphic between the parents, PI399036 and AR2 (Fig. 1). These 10 SSR markers were applied in evaluating all 60 RILs homozygous for Rps12. These polymorphic markers included Satt510 linked to the Rps3 locus, Satt663 to Rps8, Satt440 to Rps2, and Satt631, Satt152 and Satt009 to Rps1, 7, 9, Yu25, WY, Rps1?, Sattwd15-24 to Rps10, Satt159 and SSR_03_0250 to Rps1, 7, 9, Yu25, WY, Rps1?, Sattwd15-24 to Rps10, Satt159 and SSR_03_0250 to RpsUN1. PI399036, the source of Rps12, exhibited the alleles of the Satt009 and Satt510 linked to linked to PI399036, the source of Rps12...
Figure 4. Identification of sequence-based polymorphic (SBP) markers linked to \textit{Rps12} and \textit{Rps13} genes. \textit{AR2} susceptible parent \textit{AR2}; \textit{PI} resistant parent PI399036; Undigested (UD) and digested (as marked with respective restriction enzymes) PCR products for the SBP markers: SBP57.31, SBP56.59, SBP51.3, SBP55.61400, SBP57.21, SBP55.611380, SBP50.9, and SBP55.59. Primers and enzymes used for SBP markers are presented in Table S1.
major classes of flowering plants, dicots, and monocots. Clustering of R genes at a single locus is a well-reported, and many R genes are clustered in plant genomes, including soybean,60 common bean,61 Arabidopsis,62-64 Brassicaceae,62 wild potato,65 tomato,66,67 coffee trees,68, wheat,69 and rice.70,71 The clustered distribution of R-genes provides a reservoir of genetic variation from which new pathogen specificity can evolve through gene duplication, ectopic recombination, unequal crossing-over and diversifying selection.72 These clusters frequently comprise tandem arrays of genes that regulate resistance to multiple pathogens and to multiple variants of a single pathogen. The clusters may be tight with a little intervening sequence as 20 kb between two functional Rps1-k genes in soybean,73 the RPP5 cluster in Arabidopsis thaliana spans 91 kb,64 or be spread over several megabases as the Resistance Gene Candidate2 (RGC2) locus in lettuce (Lactuca sativa).74 In rice, also Chromosome 11 is highly enriched in R-genes, mostly in clusters; up to 201 loci encode the domains of NBS-LRR and LRR—receptor-like kinase (LRR-RK) or wall-associated serine/threonine protein kinase (WAK).75

The Rps12-Rps13 region is rich in Rps genes. As of now, Rps4, 6, 12, 13 and JS are mapped to the same genomic region spanning probably less than 5 cM in different soybean haplotypes (7,23,35 this work). Earlier we demonstrated that Rps4 and Rps6 are allelic and Rps4 co-segregates with Sat_064.35 Therefore, most likely Rps13 is allele to Rps4 and Rps6. The PI399036, the donor of Rps12 and Rps13, does not carry Rps4 or Rps6 and therefore Rps13 is distinct from the two Rps genes17, this study.

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**Figure 5.** Linkage and physical map of the Rps4/6/12/13/JS region. (A) Genetic map of the Rps12-Rps13-RpsJS region. SSR and SBP markers are shown on the right side of the map and corresponding genetic distances between two adjacent loci in centi-Morgan (cM) on the left side of the map. Rps13 gene is mapped between Rps12 and RpsJS and tightly linked to Sat_064, BARCOSYSSR_18_1859 and BARCOSYSSR_18_1860 SSR markers. The placement of Rps4, 6, and JS on the map is based published work.25,38 (B) The physical map positions of the SSR and SBP markers are based on the cultivar 'Williams 82' genome sequence (http://soybase.org). The physical distances between adjacent loci are presented in kilobases DNA (shown on the left side of the map).
The Rps13 locus is very close to the RpsJS locus (Fig. 5). Rps13 co-segregates with Sat_064, BARCOSYSSR_18_1859 and BARCOSYSSR_18_1860, and RpsJS co-segregates with SSRG60685K and BARCOSYSSR_18_1861. The genetic distance between BARCOSYSSR_18_1859 and BARCOSYSSR_18_1861 was reported to be 0.9 cM\(^3\). In our study, the genetic distance between these two SSR markers is 0.4 cM. The physical distance between BARCOSYSSR_18_1860 and BARCOSYSSR_18_1861 is 71 kb based on the soybean Williams 82 genome sequence (Fig. 5). The candidate annotated disease resistance gene-like sequence among the 10 predicted genes of the 92.7 kb Rps13 region between SSBP56.32 and BARCOSYSSR_18_1861 markers in the Williams 82 genome is an NB-ARC domain-containing gene, Glyma.18g51930, Glyma18g51950, and Glyma18g51960, identified from the RpsJS\(^4\) region between markers BARCOSYSSR_18_1859 and BARCOSYSSR_18_1861. The four NB-LRR genes with high similarity, are presumably paralogous sequences (Supplementary Fig. S1). They were identified from the Williams 82 haplotype that does not contain any known functional Rps genes. Based on the genetic and physical distances between BARCOSYSSR_18_1860 and BARCOSYSSR_18_1861 markers and differences in candidate NB-LRR-like resistance gene sequences, Rps13 and RpsJS are unlikely allelic or the same gene.

We propose that the five Rps genes, Rps4, 6, 12, 13 and JS, might have evolved from a single progenitor Rps gene. Identification of these Rps genes will shed light on how Rps genes evolved in soybean to confer effector triggered immunity against a serious oomycete pathogen, P. sojae.

In this study we have shown that the broad-spectrum Phytophthora resistance is encoded by two Rps genes, Rps12 and Rps13, with distinct race-specificity. The genetic distance between the two Rps genes is 4 cM. Therefore, to maintain the broad-spectrum Phytophthora resistance encoded by Rps12 and Rps13, we must select both genes using molecular markers. We report here several SSR markers that should be ideal for introgressing Rps12 and Rps13 into new soybean cultivars.

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Author contributions
M.K.B. conceived the project and received the grant to conduct the research. D.K.S. conducted all biological experiments. A.D. and X.H. conducted soybean genome analyses for identifying the polymorphic nucleotides of the Rps12/Rps13 genomic region. D.K.S. prepared all figures and tables and wrote the first draft of the manuscript. M.K.B. supervised D.K.S. and prepared the final draft. All authors reviewed the manuscript.

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The authors declare no competing interests.

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