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ORIGINAL ARTICLE

Fine mapping and candidate gene analysis of two loci conferring resistance to *Phytophthora sojae* in soybean

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Abstract

***Key message RpsUN1* and *RpsUN2* were fine mapped to two genomic regions harboring disease resistance-like genes. The haplotypes and instability of the regions and candidate genes for the two resistance loci were characterized.**

Abstract Phytophthora root and stem rot caused by *Phytophthora sojae*, is one of the most destructive diseases of soybean. Deploying soybean cultivars carrying race-specific resistance conferred by *Rps* genes is the most practical approach to managing this disease. Previously, two *Rps* genes, *RpsUN1* and *RpsUN2* were identified in a landrace PI 567139B and mapped to a 6.5 cM region on chromosome 3 and a 3.0 cM region on chromosome 16, corresponding to 1387 and 423 kb of the soybean reference genome sequences. By analyzing recombinants defined by genotypic and phenotypic screening of the 826 $F_{2:3}$ families derived from two reciprocal crosses between the two parental lines, *RpsUN1* and *RpsUN2,* were further narrowed to a 151 kb region that harbors five genes including three disease resistance (R)-like genes, and a 36 kb region that contains four genes including five R-like genes, respectively, according to the reference genome. Expressional changes of these nine genes before and after inoculation with the pathogen, as revealed by RNA-seq, suggest that *Glyma.03g034600* in the *RpsUN1* region and *Glyma.16g215200* and *Glyma.16g214900* in the *RpsUN2* region of PI 567139B may be associated with the resistance to *P. sojae*. It is also suggested that unequal recombination between/among R-like genes may have occurred, resulting in the formation of two recombinants with inconsistent genotypic and phenotypic observations. The haplotype variation of genomic regions where *RpsUN1* and *RpsUN2* reside in the entire soybean germplasm deposited in the US soybean germplasm collection suggests that *RpsUN1* and *RpsUN2* are most likely novel genes.

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Introduction

Phytophthora root and stem rot (PRSR) caused by the oomycete pathogen *Phytophthora sojae*, is a prevalent

soybean disease in most soybean growing regions throughout the world. It was estimated that PRSR caused an annual yield loss of approximately 44.7 million bu from 1996 to 2009 in the United States (Wrather and Koenning 2006, 2009). In certain year with heavy rainfall and poor drainage, the yield losses could reach 100 % in the affected fields. In the past decades, soybean yield losses to *P. sojae* have been limited by incorporating *Rps* genes conferring resistance to prevalent races of the pathogen into elite cultivars. Today, deploying resistant cultivars remains the most effective, economical, and environmental friendly approach to managing this disease.

So far a total of 20 *Rps* loci including 26 alleles have been identified, which are distributed on seven chromosomes (Hanson et al. 1988; Anderson and Buzzell 1992; Polzin et al. 1994; Demirbas et al. 2001; Gardner et al. 2001; Weng et al. 2001; Gao et al. 2005; Sandhu et al. 2004, 2005; Gordon et al. 2006; Sugimoto et al. 2008; Fan et al. 2009; Yao et al. 2010; Wu et al. 2011; Sun et al. 2011; Lin et al. 2013; Zhang et al. 2013; Ping et al. 2016). The *Rps1* locus including five alleles (*Rps1-a*, *Rps1-b*, *Rps1-c*, *Rps1-d*, and *Rps1-k*), *Rps7*, *Rps9*, *RpsYu25*, *RpsYD29*, and *RpsUN1* are located on the short arm of chromosome 3. *Rps2* and *RpsUN2* are located on the long arm of chromosome 16. The *Rps3* locus including three "so-called" alleles (*Rps3-a*, *Rps3-b,* and *Rps3-c*), which is either linked to or allelic with *Rps8,* is located on chromosome 13. *Rps4*, *Rps5,* and *Rps6* are linked and located on chromosome 18. *Rps11* is located on the short arm of chromosome 7. Because many of these *Rps* loci were genetically anchored to linkage groups (LGs) or chromosomes using different types/sets of molecular markers and mapping populations of relatively small sizes, the order of most *Rps* loci on a same chromosome and their relative distances remain blurry. In addition, the multiple alleles at the *Rps1* or *Rps3* locus were roughly defined by such markers and populations without further fine-scale mapping using same sets of molecular markers, as such, whether they are really allelic with each other or they actually belong to different loci is also unclear. Fine mapping of these genes/alleles would enable more accurate definition of these genes/alleles and marker-assisted selection for breeding new cultivars resistant to *P. sojae*.

Among the race-specific *Rps* genes/alleles that have been identified, *Rps1-k*, *Rps1-c,* and *Rps3-a* are the primary ones deployed for soybean protection in the past few decades. In particular, *Rps1-k* has been isolated by a map-based cloning approach, and widely used for developing resistant cultivars (Kasuga et al. 1997; Gao et al. 2005). Intriguingly, the two *Rps1-k* candidate genes, both of which encode nucleotide-binding site-leucine-rich repeat (NBS-LRR) proteins, identified by sequencing three overlapping Bacterial Artificial Chromosome (BAC) clones from Williams 82, could not be found in the Williams 82 soybean reference genome sequence. As a result, functional markers that would most effectively target the causative mutations/genetic variations underlying resistance to *P. sojae* have not been developed in any of the previously identified *Rps* genes.

In general, the resistance contributed by individual *Rps* genes is non-durable. Such genes each, if used alone, would be effective for only 8-15 years due to rapid variation of the pathogen under selection pressure (Schmitthenner 1985). It is thus not surprising that *Rps1-k*, the most widely deployed *Rps* genes for breeding commercial soybean cultivars has lost its effectiveness to many emerging isolates of the pathogen (Sugimoto et al. 2012). A promising strategy for breeding more durable resistance is to pyramid multiple, broad-spectrum *Rps* genes into a single cultivar. However, several *Rps* genes/alleles are distributed in the same or adjacent genomic regions, and may not be simultaneously tagged unless they are fine mapped or cloned. When multiple *Rps* gene donors are involved in breeding, defining haplotypes of genomic regions surrounding the target *Rps* loci would also be essential for design of effective markers to distinguish individual target regions harboring different *Rps* genes.

Recently, we have identified two *Rps* genes, *RpsUN1* and *RpsUN2* from a soybean landrace, which together confer complete resistance to all 16 *P. sojae* races/isolates, including the prevalent ones identified in Indiana State used in resistance evaluation (Lin et al. 2013). *RpsUN1* was defined to a 6.6 cM region between SSR markers Satt159 and BARCSOYSSR_03_0250 that spans the *Rps1* locus on chromosome 3, corresponding to 1387 kb of genomic region in the soybean reference genome. *RpsUN2* was defined to a 3.0 cM region between BARCSOYSSR_16_1275 and Sat_144 that is closely linked to the *Rps2* locus on chromosome 16, corresponding to 423 kb of genomic region in the reference genome. These genomic regions represent two major NBS-LRR (R) gene clusters, which account for approximately a quarter of all R gene models predicted in the reference genome (Schmutz et al. 2010). It is documented that the R gene clusters increase the probability of structural and copy number variation of R genes by equal or unequal chromosomal recombination events, resulting in acquisition or loss of resistance, suggesting that these regions may be hotspots for novel *Rps* genes. Indeed, marker-assisted resistance spectrum analysis suggested that both *RpsUN1* and *RpsUN2* are potentially novel *Rps* genes/alleles, representing a new source of resistance for enhancing the durability and level of resistance to *P. sojae*. However, because different *Rps* genes in the two R gene clusters are closely linked, selection and pyramiding of individual genes from different resources into a single cultivar would be ineffective with markers distant from the target genes. To implement effective and precise selection

for this new source of *Rps* genes for deployment in soybean breeding programs, we have conducted fine mapping of *RpsUN1* and *RpsUN2*, identification of candidates for these two genes, and haplotype analysis of genomic regions surrounding the two genes.

Materials and methods

Plant materials

The mapping populations generated by reciprocal crosses between Williams and PI 567139B, which include $F_{2:3}$ families from 44 survived F_2 seedlings derived from the "Williams × PI 567139B" cross (dubbed the 'WPT' families), which were transferred to larger pots after inoculation to produce seeds. The additional 403 $F_{2:3}$ families (dubbed the "WP" families) derived from the "Williams × PI 567139B" cross as described previously (Lin et al. 2013), and 379 $F_{2:3}$ families derived from the cross of "PI 567139B × Williams" (dubbed the 'PW' families), making a total of 826 $F_{2:3}$ families for fine mapping of the two loci.

Inoculation treatment and disease evaluation

Phytophthora sojae, *pmg*(*17*)*-1,* and *pmg*(*25*)*-1*, with pathotypes corresponding to races 17 and 25, respectively, were used to evaluate the resistance of the $F_{2:3}$ families using a protocol previously described (Dorrance et al. 2008; Lin et al. 2013).

Sample collection and DNA extraction of mapping population

Approximately 15-20 progeny $F_{2:3}$ seedling leaf samples from each of the F_2 recombinants determined by molecular markers surrounding *RpsUN1* and *RpsUN2*, respectively, were equally mixed for DNA isolation. Genotyping of detected F_2 recombinants with SSR (Song et al. 2010), CAPS, and SNP markers were conducted following methods described previously (Lin et al. 2013; Ping et al. 2016) using primers and enzymes listed in Online Resource 1.

Evaluation of gene expression by RNA-seq

Approximately 20 seedlings of PI 567139B were inoculated with *P. sojae* race 1 (*pmg*(*1*)*-3*) and 20 seedlings from the same line were wounded without inoculation. After 24 h, stems of 2–3 cm in length across the wounded sites from the inoculated and wounded seedling were sectioned to form the inoculated and wounded groups, respectively. RNA isolation, RNA-seq, and data analysis were performed following a protocol described earlier. RNA-seq data from Williams and 10 *Rps* gene isogenic lines (*Rps1-a*, *Rps1-b*, *Rps1-c*, *Rps1-k*, *Rps3-a*, *Rps3-b*, *Rps3-c*, *Rps4*, *Rps5,* and *Rps6*) under the same treatment as performed for PI 567139B (Lin et al. 2014) were analyzed using the same protocol as used for PI 567139B. The expression values (FPKM, fragments per kb of exon per million fragments mapped) of the genes in the mapped *RpsUN1* and *RpsUN2* regions were measured and compared by Cufflinks (Trapnell et al. 2012). The RNA-seq data obtained from PI 567139B in this study have been deposited at the National Center for Biotechnology Information Gene Expression Omnibus under accession number GSE82240.

Phylogenetic analysis

The phylogenetic neighbor-joining trees were constructed using SNPs extracted from the two defined genomic regions among all the USDA soybean germplasm accessions genotyped with the SoySNP50K iSelect BeadChip containing 52,041 SNPs (Song et al. 2013, 2015) following the methods previously described (Tian et al. 2010).

Results

Fine mapping of *RpsUN1* on chromosome 3

To further narrow the region harboring *RpsUN1*, SSR markers near or at the boundaries of previously mapped *RpsUN1* region, BARCSOYSSR_03_180 and BARCSOYSSR_03_250, were used to genotype the 826 F_2 plants derived from the reciprocal crosses between Williams and PI 567139B, and nine recombinants between the two SSR markers were identified. Subsequently, the $F_{2:3}$ families derived from these recombinants were inoculated with the *P. sojae* race 17 (Online Resource 2), which is avirulent to *RpsUN1* but virulent to *RpsUN2*. The corresponding F_2 plants or pools of F_3 seedlings from individual F_2 plants were genotyped using additional seven SSR markers exhibiting polymorphisms between the two parental lines. As showN in Fig. 1, the recombinants WP133 and PW202 defined the *RpsUN1* locus to the downstream of the marker BARCSOYSSR_03_0233, while the recombinants W243 and A318 defined the *RpsUN1* locus to the upstream of the marker BARCSOYSSR_03_0246. The genotypic and phenotypic data from the remaining five recombinants were as expected and consistent to the data from the four recombinants WP133, PW202, WP243, and PW318. Thus, the candidate gene for *RpsUN1* was defined to the region between BARCSOYSSR_03_0233 and BARCSOYSSR_03_0246. According to the Williams 82 soybean reference genome, these two markers span an ~151 kb region, which contains five annotated genes, including three predicted R-like genes (Online Resource 3).

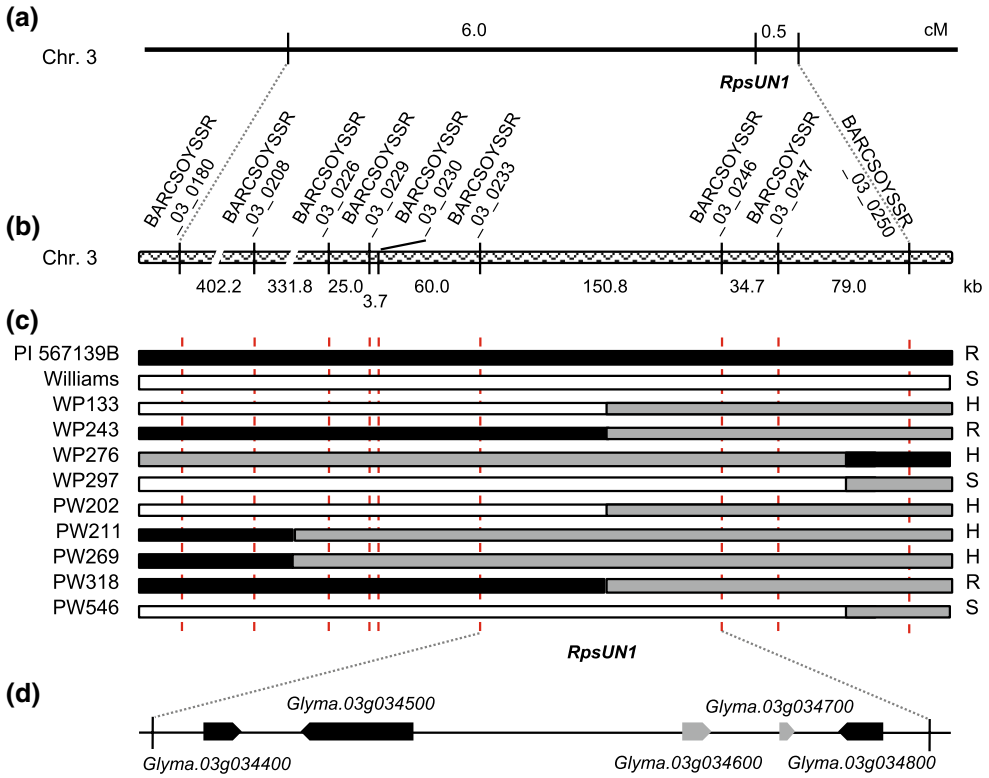

Fig. 1 Fine mapping of the *RpsUN1* locus. **a** Linkage map of the *RpsUN1* locus. **b** Physical positions of molecular markers (according to the Williams 82 reference genome) used for fine mapping of the *RpsUN1* locus. **c** Recombinants carrying crossovers as determined by molecular markers and genotypes of the F_2 plants deduced by phenotyping the $F_{2:3}$ families. *Black*, *white*, and *gray bars* represent homozygous segments from PI 567139B, Williams 82, and heterozygous segments from both parental lines as determined by molecular markers, respectively. **d** Annotated genes in the fine mapped region of the Williams 82 reference genome. *Black boxes* indicate disease resistance-related genes, whereas *gray boxes* indicate other types of genes. *Arrows* indicate the transcriptional orientation of these genes

Fine mapping of *RpsUN2* on chromosome 16

To further narrow the region harboring *RpsUN2*, SSR markers near or at the boundaries of previously mapped *RpsUN2* region, BARCSOYSSR_16_1275 and Sat_114, were used to genotype the 826 F_2 plants, and 23 recombinants between these two SSR markers were identified. Subsequently, the $F_{2:3}$ families derived from these recombinants were phenotyped by inoculation with the *P. sojae* race 25 (Online Resource 2), which is avirulent to *RpsUN2* but virulent to *RpsUN1*. The corresponding F_2 plants or pools of F_3 seedlings from individual F_2 plants were genotyped using additional two SSR markers showing polymorphisms between the two parental lines and four cleaved amplified polymorphic sequence (CAPS) markers designed based on genic sequences in the mapped region from the two parental lines. As shown in Fig. 2b, the recombinants WPT074, WP401133, and PW555 defined the *RpsUN2* locus to the downstream of the marker CAPS3, while the recombinants WP015, WP062, WP364, PW568, and PW547 defined the *RpsUN2* to the upstream of the marker CAPS4. The genotypic and phenotypic data from the remaining 15 recombinants were as expected and supportive of the data from these eight recombinants. Additional four CAPS markers and two SNP markers detected by direct sequencing PCR fragments were designed based on sequences between CAPS3 and CAPS4 from the two parental lines and used to genotype the eight recombinants defined by CAPS3 and CPAS 4. Based on the genotypic and phenotypic data from the six recombinants WP062, WP364, WP404, PW568, PW547, and PW555, the candidate for the *RpsUN2* locus was defined to the genomic region between CAPS6 and SNP2. According to the soybean Williams 82 reference genome (Schmutz et al. 2010), this region contains four genes, all of which are of the typical R type (Online Resource 3). Unexpectedly, the genotypic data and phenotypic data from two recombinants AP015 and WPT074 were inconsistent. The former suggests that the *RpsUN2* locus is located in the upstream of CAPS6, while the latter suggests that the *RpsUN2* locus is located in the downstream of CAPS8. Additional replicates were performed and the phenotypic and genotypic data remain the same.

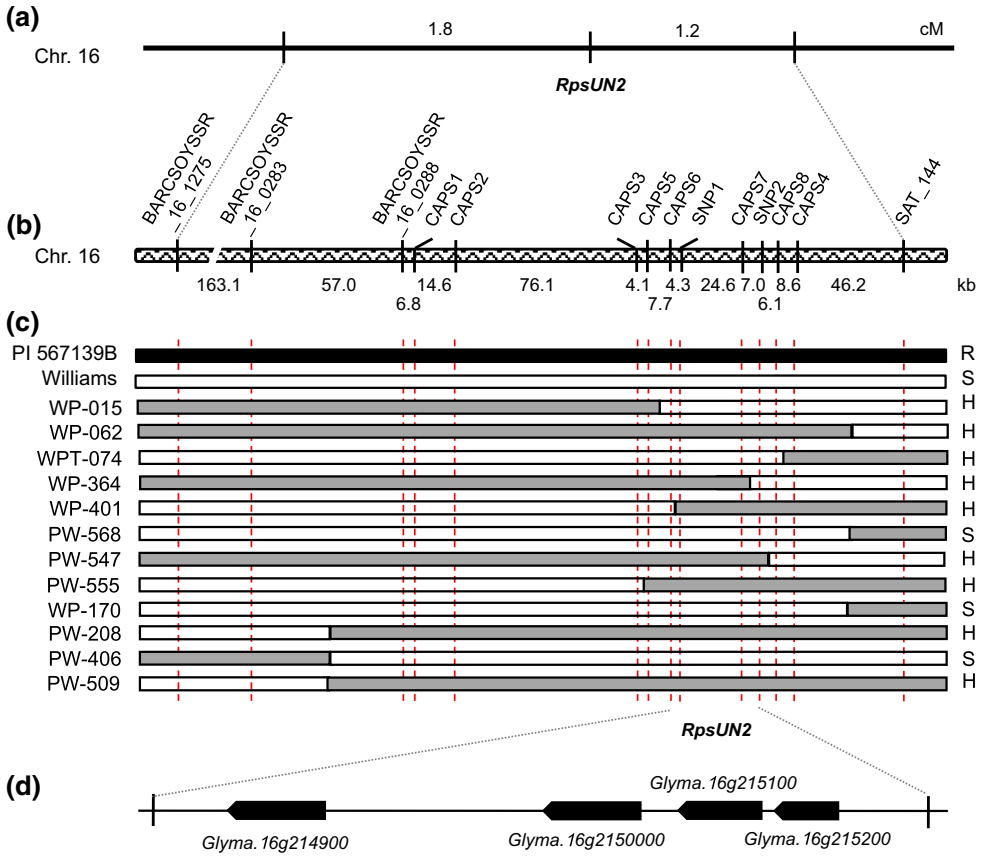

Fig. 2 Fine mapping of the *RpsUN2* locus. **a** Linkage map of the *RpsUN2* locus. **b** Physical positions of molecular markers (according to the Williams 82 reference genome) used for fine mapping of the *RpsUN2* locus. **c** Recombinants carrying crossovers as determined by molecular markers and genotypes of the F_2 plants deduced by phenotyping the $F_{2:3}$ families. *Black*, *white*, and *gray bars* represent homozygous segments from PI 567139B, Williams 82, and heterozygous segments from both parental lines as determined by molecular markers, respectively. **d** Annotated genes in the fine mapped region of the Williams 82 reference genome. *Black boxes* indicate disease resistance-related genes, whereas *gray boxes* indicate other types of genes. *Arrows* indicate the transcriptional orientations of these genes

Expressional changes of genes in the fine mapped *RpsUN1* and *RpsUN2* regions in responsive to *P. sojae*

Because the majority of the annotated genes in the fine mapped *RpsUN1* and *RpsUN2* regions in the Williams 82 reference genome are of the R type such as NBS-LRRs, which are highly similar and often hotspots for unequal recombination that lead to chimeric structure and/or copy number variation (Michelmore and Meyers 1998; Hulbert 1998; Ellis et al. 2000; Nagy and Bennetzen 2008), we thought that further fine mapping of the two loci may not be effective towards pinpointing the candidate genes for *RpsUN1* and *RpsUN2*. To understand the genomic difference in the *RpsUN1* and *RpsUN2* regions between the two parental lines and potential causative variation that defers the resistant parental line from the susceptible parental line, primers were designed based on the three NB-ARC genes in the *RpsUN1* region and the four NBS-LRR genes in the *RpsUN2* region of Williams 82 and used to amplify their genomic and transcriptomic counterparts in PI 567139B. However, in many attempts, these genes in PI 567139B are either too similar to be distinguishable or difficult to be amplified possibly due to sequence diversity between the parental lines. As a result, the genomic and transcriptomic counterparts of these NBS-LRR genes in PI 567139B have not been accurately determined.

In an attempt to pinpoint the candidate genes for the *RpsUN1* and *RpsUN2* loci, we evaluated and compared the expression levels of the five genes in the *RpsUN1* region and the four genes in the *RpsUN2* regions, before and after inoculation with *P. sojae*, in the two parental lines PI 567139B,Williams, and 10 *Rps* gene isogenic lines in the Williams background, by RNA-seq. To increase the accuracy of the evaluation, only the RNA-seq reads uniquely mapped to the reference genome were used to calculate the relative abundance of transcripts from each gene. The changes in relative abundance of transcripts from the nine genes upon inoculation with the pathogen were shown in

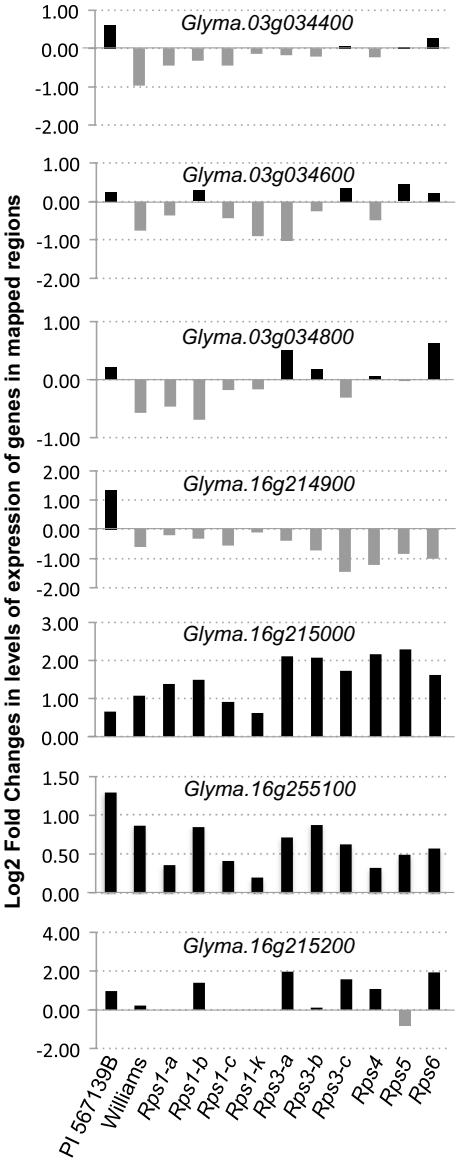

Fig. 3 Expressional changes of genes in the *RpsUN1* and *RpsUN2* regions revealed by RNA-seq. The abundance of each gene relative to the abundance of *Cos4* was used to evaluate the relative expression level of the gene. No any RNA-seq reads uniquely mapped to *Glyma.03g034500* or *Glyma.03g034700* were identified in any of the 12 lines

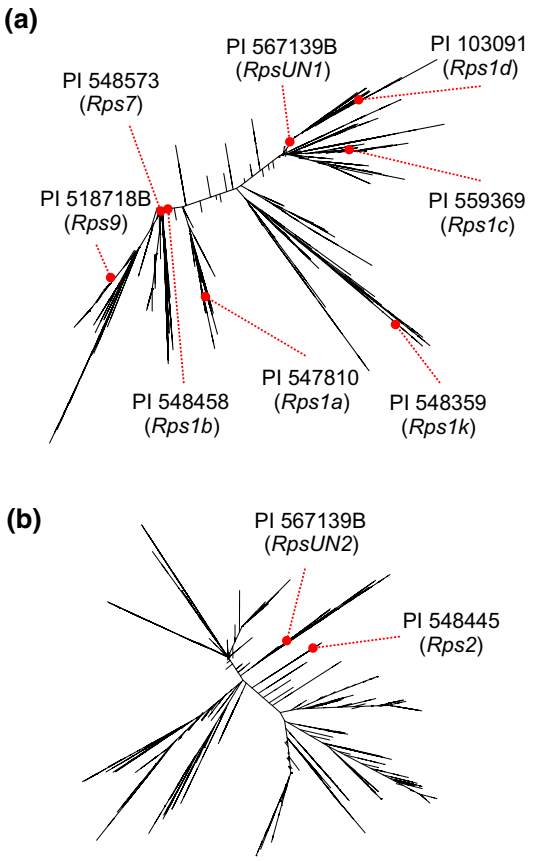

Fig. 4 Haplotype variation of the *RpsUN1* and *RpsUN2* regions among the entire USDA Soybean Germplasm Collection as revealed by SNPs distributed in the two regions. **a** Phylogenetic tree constructed with SNPs in the *RpsUN1* region. **b** Phylogenetic tree constructed with SNPs in the *RpsUN2* region

Fig. 3 and Online Resource 4. Among the five genes in the *RpsUN1* region, *Glyma.03g034400*, *Glyma.03g034600*, and *Glyma.03g034800* were all up-regulated in PI 567139B, but down-regulated in Williams, upon inoculation with the pathogen. Up-regulation of these three genes were also detected in a few other *Rps* gene isogenic lines, but only *Glyma.03g034400* showed highest levels of up-regulation among the 12 lines examined. Thus, *Glyma.03g034400* may be the candidate for the *RpsUN1* locus. Among the four genes in the *RpsUN2* region, *Glyma.16g214900* showed up-regulation in PI 567139B but down-regulation in Williams and all the 10 *Rps* gene isogenic lines. *Glyma.16g215000*, *Glyma.16g215100*, and *Glyma.16g215200* were also up-regulated upon inoculation with the pathogen, but such up-regulation was also detected in the majority of the *Rps* gene isogenic lines. Therefore, *Glyma.16g214900* would be the best candidate for the *RpsUN2* locus.

Haplotype variation of the *RpsUN1* and *RpsUN2* regions in the entire USDA soybean germplasm collection

Although *RpsUN1* and *RpsUN2* were fine mapped to two considerably small genomic regions, whether these two loci different from any other *Rps* loci in the overlapping/adjacent regions was unclear. This is mainly due to the fact that different *Rps* genes were mapped at different scales and with different sets/types of molecular markers, making the genetic maps of these *Rps* genes incomparable. To

shed light on this question, we extracted the SNP genotypic data from a 560 kb genomic region surrounding the *RpsUN1* locus and a 110 kb genomic region surrounding the *RpsUN2* locus from the USDA Soybean Germplasm Collection that is comprised of 19,652 *Glycine max* and *Glycine soja* accessions. The SNP data were generated using the SoySNP50K iSelect BeadChip, and each region contains 10 SNPs. Using these SNP data, we analyzed the phylogenetic population structure of the two regions. As shown in Fig. 4, the branch for the *RpsUN1* region of PI 567139B is considerably distinct from the branches for the corresponding regions from the donor or ancestral lines of *Rps1-a*, *Rps1-b*, *Rps1-c*, *Rps1-d*, and Rps1-k, and the branch containing the *RpsUN2* region of PI 567139B is considerably distinct from the branch containing the corresponding region from an ancestral line carrying *Rps2*.

Discussion

In this study, we fine mapped the *RpsUN1* and *RpsUN2* loci to two small genomic regions, which only harbor five and four genes, respectively, according to the soybean reference genome. In these regions, only two and three recombinants between the closest markers and the *RpsUN1* and *RpsUN2* loci were found in the mapping populations comprising 826 F_2 plants and/or $F_{2:3}$ families, thus recombination frequencies between these markers and the two resistance loci are extremely low (0.0024 and 0.0036, respectively). If these markers are used for marker-assisted selection of these two genes in breeding programs, the accuracy of selection for the two resistance loci would be higher than 0.4 %. The accuracy can be further increased if effective markers within the mapped regions are identified and used in marker-assisted selection.

Nevertheless, we would like to note again that two recombinants (WP-015 and WPT-074) which showed inconsistent phenotypic and genotypic data with some markers in the mapped genomic regions were found. These two recombinants controversially positioned the *RpsUN2* locus to the upstream and downstream of the fine mapped *RpsUN2* region. As the *RpsUN2* region in PI 567139B and these two recombinants have not been fully sequenced, it remains unknown how such an inconsistency was formed. However, given the fact that the fine mapped *RpsUN2* region in Williams 82 contains only four NBS-LRR genes, which are flanked by additional NBS-LRR genes as part of a genomic region enriched with NBS-LRR genes (Schmutz et al. 2010), it is expected that the *RpsUN2* region in PI 567139B is also enriched with NBS-LRR genes. It has been documented that NBS-LRR genes or gene clusters are generally inherently unstable and fast-evolving via recombination and rearrangements, such as duplication and deletions by unequal recombination (Michelmore and Meyers 1998; Hulbert. 1998; Ellis et al. 2000; Leister 2004; Nagy and Bennetzen 2008), resulting in new forms of NBS-LRR genes as well as their copy number variation, it thus is possible that the two recombinants showing inconsistent phenotypic and genotypic data are the outcome of unequal recombination mediated by NBS-LRR genes.

Although a single trait is generally controlled by a single gene, a genetic locus underlying a specific trait such as disease resistance could consist of multiple genes of the same or different types. Such genes could act independently from each other or function at variable levels according to their copy number variation. For example, the *Rps1-k* locus is composed of four tandemly arranged NBS-LRR genes, among which two individual copies were each fully responsible for complete resistance to *P. sojae* (Gao et al. 2005; Gao and Bhattacharyya 2008). By contrast, a recent study demonstrates that copy number variation of multiple genes at the *Rhg1* locus mediates nematode resistance in soybean (Cook et al. 2012). Thus, additional genes in the mapped regions of PI 567139B, if any, which are absent in the corresponding regions of the reference genome, could also be involved in the control of resistance to *P. sojae*. If this is the case, the candidate genes for the *RpsUN1* and *RpsUN2* loci may have not been fully deduced based on the observed changes in relative abundance of the RNA-seq reads uniquely mapped to the two genomic regions of the reference genome.

Allelic test is routinely used to determine whether a trait is controlled by two different alleles of a single gene or by two different genes. However, due to the complexity of many resistance loci and the potential instability of such loci and underlying traits, it would be difficult or ineffective to determine exclusively whether *RpsUN1* and *RpsUN2* are different from other *Rps* genes mapped to similar regions by allelic test. Nevertheless, the haplotypic variation of the *RpsUN1* and *RpsUN2* regions between PI 567139B and the donor/ancestral lines carrying other *Rps* genes are obvious, which may reflect the distinct origins of the *RpsUN1* and *RpsUN2* loci and other known *Rps* genes/alleles mapped in their overlapping or adjacent regions.

Author contribution statement JM & CC designed the research; LL, FL, WW, JP, JCF, MZ, SL performed the research; LL, FL, LS, CC, and JM analyzed the data; JM wrote the manuscript.

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Compliance with ethical standards

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

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