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A tritrophic plant-insect-pathogen system used to develop a closely linked *Rag2* and *Rsv1-h* recombinant haplotype in double-resistant soybean germplasm

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Abstract

Background The colocalization of two resistance (*R*) genes on chromosome 13 of soybean (*Glycine max* (L.) Merrill) that confer resistance against the soybean aphid (*Aphis glycines*) and soybean mosaic virus (SMV) gives rise to a very unique *R-avr* tritrophic incompatible interaction system that goes across biological kingdoms. In this tritrophic system, the insect is the only natural vector of the virus and soybean is a host-plant for both pests/pathogen. The almost unavoidable co-evolution of pathogen-vector with that of the *R*-genes in soybean plants through an endless arms race to avoid each other's defense-attack mechanisms raises interesting questions. The objectives of this work were to (*i*) develop double-resistant recombinant inbred lines (RILs) with a *Rag2-Rsv1-h* gene haplotype in coupling phase using resistance alleles from two different genetic sources (PI 243540 *(Rag2)* and Suweon 97 *(Rsv1-h)*), (*ii*) confirm phenotypically the resistant reaction against both pests in double-resistant RILs, and (*iii*) dissect the *Rag2-Rsv1-h* region with molecular markers and investigate the potential for structural variation.

Results We observed a recombination event in identified double-resistant $F_{3:5}$ RILs in a region of chromosome 13 ca. 21 kb long (between positions 30,297,227 and 30,318,949 in Wm82.a2.v1) that lies between the reported locations of the *Rsv1-h* and *Rag2* genes (29,815,463--29,912,369 and 30,412,581--30,466,533 intervals, respectively, based on Wm82.a2.v1), indicating the double-resistant haplotype is in coupling phase. The tight LD estimates obtained between haplotype markers underscored the physical proximity of the two resistance genes. Only 10 recombinant haplotype classes (excluding double heterozygotes) were observed among the 51 that were possible with a four loci haplotype. The 10 recombinant classes represented 15 out of 192 screened individuals. A joint SMV-aphid phenotypic greenhouse screen allowed us to identify the best aphid biotype 1 and SMV-G1, double resistant haplotype class in recombinant progeny. Our molecular marker results agree with previous fine-mapping reports and preclude the presence of resistance genes other than *Rag2* and *Rsv1-h* in double-resistant RILs. A comparative genomic hybridization analysis revealed no obvious structural variants in the region.

Conclusions **To** our knowledge, this is the first report of double-resistant *Rag2-Rsv1-h* soybean RILs that used a plant-insect-pathogen tritrophic system for germplasm enhancement. The co-occurrence of *Rag* and *Rsv* genes in a region that clusters resistance genes on chromosome 13 may be a unique feature of domesticated soybean. The

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recombinant genotypes will be useful in breeding to develop soybean cultivars with resistance to both the vector and the virus. The parental and recombinant genotypes may be helpful in future studies to elucidate interesting evolutionary questions regarding vector, host, and virus tritrophic systems.

Keywords Recombinant haplotype, *R*-gene, Linkage, Comparative genomic hybridization, Resistance, Breeding, Crop improvement, Single nucleotide polymorphism, Candidate genes, Soybean (*Glycine max* (L.) Merrill)

Background

In order to protect US agricultural food production systems we need to be prepared against emerging pests. Pest-specific *R-Avr* gene-dependent incompatible interactions represent excellent systems for the study of tritrophic plant-microbe-insect interactions and host-plant's defense mechanisms in economically important crops. Soybean (*Glycine max* (L.) Merrill) is the leading source of vegetable protein and the largest source of vegetable oil in the world [1, 2]. As the second most planted field crop in the US, soybean represents an important element of food security for North America [3, 4]. Cultivated soybean is hypothesized to have its origins in Asia where its cultivation may have begun 6,000 to 9,000 years ago [5]. As a top commodity in 2022, soybean represented $59.2 billion for the US economy, and the US was the second largest soybean producer in the world with 4.47 billion bushels produced, just behind Brazil's 4.8 [1]. Both soybean aphid and soybean mosaic virus can be a significant threat to soybean production. Annual loss estimates averaging ca. $4.25 billion in North America due to soybean aphid (*Aphis glycines* Matsumura) infestation have been reported [6]. In addition, yield losses caused by the soybean mosaic virus (SMV) can significantly reduce profitability for farmers, particularly if the disease coincides with additional pests [7]. Therefore, it is of vital importance to devote research efforts to the study of agronomically relevant crop-pest interactions.

Soybean aphid herbivory and plant genetic resistance

The soybean aphid, an insect species also native to Asia, is the most detrimental insect pest to soybean growers in North America [8, 9]. Its appearance was first reported in 2000, and since then, the soybean aphid has spread across at least 30 states in the US and several southern provinces in Canada [9, 10], with at least four different biotypes reported: Aphids biotype 1 is avirulent to known *R*-genes in soybean (denominated as *Rag* – resistance to *Aphis glycines*); biotype 2 can overcome *Rag1* type of resistance [11]; biotype 3 can overcome *Rag2* resistance [12]; and biotype 4 can overcome *Rag1* and *Rag2* haplotypes together or individually [13]. Yield losses of up to 40% have been reported in soybean fields of the north central states with high levels of aphid infestation [14] and there is a great deal of concern regarding this pest because of its ability to evolve virulence, its intra-biotype variability, and its capacity to spread relatively quickly in geographical areas [15, 16].

The soybean aphid is known to transmit SMV and other plant viruses, including *Alfalfa mosaic virus*, *Soybean dwarf virus* and *Tobacco ringspot virus,* to soybean plants [17–21]. The relatively recent introduction and spread of the soybean aphid in the north-central region of the US has raised concerns about the risk of increased SMV-disease incidence on production fields [22]. Field experiments have suggested that mitigation of yield loss by application of insecticide to control for aphid populations spreading SMV can be an alternative for pest management [23]. However, effective SMV disease control cannot always be attained by restraining the aphid vector through application of foliar insecticides, which can ultimately result in significant yield losses [22, 23]. Therefore, host plant resistance is the most effective strategy not only to control SMV infection, but also to control aphid insect damage and minimize the use of chemical insecticides [9, 23]. The three mechanisms of plant resistance against soybean aphids are antixenosis, antibiosis and tolerance. Antixenosis has been defined as the non-preference of a plant host given morphological or chemical barriers in the form of toxins, repellents or antinutritional secondary metabolites encountered by the insect [24]. Antibiosis involves the mechanisms that have an effect on aphid biology and reproduction (i.e., aphid mortality and fecundity) [9, 24–26]. Aphid tolerance has been proposed as the ability to withstand and outgrow infestation, as well as the ability to produce new biomass, including seed yield, that is significantly higher than the amounts produced by susceptible individuals [24, 27–30].

There are at least six *Rag* independent genetic loci in the US germplasm. Some of them exhibiting more than one resistant allele and different genic mode of resistance: *Rag1*, *rag1b*, *rag1c*, *Rag2*, *Rag3*, *rag3*, *rag3b*, *Rag3c, rag4*, *Rag4, Rag5*, *Rag6*, and more recently *RagFMD*, a novel gene for aphid resistance also identified in China [31–44]. Soybean accession PI 243540 was reported to carry a single dominant gene providing strong antibiosis resistance against aphid biotypes 1 and 2 [35, 45]. Similarly, an independent study reported that accession PI

200538 carried a single dominant gene conferring strong antibiosis resistance to soybean aphids biotypes 1 and 2 [33]. The aphid resistance genes identified in PI 243540 and PI 200538 mapped to the same genetic region on chromosome 13 of soybean and given the similar resistance reaction to both aphid biotypes, it was hypothesized that an allelic relationship existed between the two genes. Hence, the two genes were referred to as *Rag2* [33, 35, 46]. Multiple soybean accessions have been identified to carry a single *Rag* gene, and more recently, five accessions were found to confer not only different types of resistance to four soybean aphids biotypes, but also to carry as many as three *Rag* resistance genes exhibiting different phenotypic reactions [31–44, 47].

Soybean mosaic virus and plant genetic resistance

Soybean mosaic virus (SMV) is the cause of one of the most common viral diseases in soybean [48]. SMV infection of soybean plants can result in a range of symptoms, some of which can be observed in the leaves and seeds of infected plants and can ultimately lead to severe yield losses and reduced grain quality [48–50]. In North America, SMV was first documented in 1916 and it was given its name in 1921 [51, 52]. The SMV is known to have a relatively narrow host range, which includes primarily soybean and its wild relatives in the *Fabaceae* family, but it can also infect other legumes as well as members of other plant families including *Amaranthaceae*, *Chenopodiaceae*, *Passifloraceae*, *Schropulariaceae* and *Solanaceae* [48, 53]. SMV has been shown to spread through SMV-infected seeds and by over 30 aphid species, including *Aphis glycines*, that can serve as insect vectors for the pathogen and it has been found in all the major soybean-producing countries in the world [48, 54, 55]. SMV belongs to the *Potyvirus* genus, classified as a member of the *Potyviridae*, a family of 228 species known as the largest group of plant-infecting RNA-viruses and responsible for causing severe losses to agricultural crops worldwide [56]. As most potyviruses, SMV is a positive-sense, single-stranded RNA virus whose genome is 9600 nucleotides and contains a polycistronic open reading frame that codes for a large polyprotein, which upon proteolytic cleavage by SMV-encoded proteases yields 10 functional proteins (P1, HC-Pro, P3, 6 K1, CI, 6 K2, NIa-VPg, NIa-Pro, Nib and CP). An additional protein (PIPO) is coded by a frameshift that results in an eleventh functional protein [57–60]. Natural host-plant resistance and the deployment of cultivars enhanced with SMV-*R*-gene backgrounds has been proposed as the best alternative to control for SMV field isolate infection [59, 61]. In the US, a system was established for classification of SMV field isolates using a set of susceptible and resistant cultivars carrying different SMV-*R*-genes that exhibited a differential reaction when challenged with different SMV isolates, thus, giving rise to the widely used *Rsv*-gene G1 to G7 SMV-strain classification system used in the US, Brazil, Ukraine, Iran and South Korea [59, 62–64]. In Japan and China, a different classification of SMV isolates has been used and some of the Chinese identified *R*-genes have been named under that nomenclature [59]. Under the US *Rsv* (resistance to soybean mosaic virus) nomenclature, there are at least four SMV-resistant loci reported to date, *Rsv1, Rsv3, Rsv4* and *Rsv5*, and at least two of them have been reported to have multiple alleles. The gene *Rsv1*, the most complex of all *Rsv* loci, has been reported to harbor 10 different alleles (*Rsv1, Rsv1-t, Rsv1-y, Rsv1-m, Rsv1-k, Rsv1-r, Rsv1-s, Rsv1-n, Rsv1-h,* and *Rsv1-c*) and the locus has been mapped to an *R*-gene cluster on chromosome 13 of soybean [59, 65, 66]. The genes *Rsv3* and *Rsv4* have been recently cloned and characterized, while *Rsv1* has been difficult to positionally clone [59, 60, 67–70].

Incompatible *R-avr* gene-dependent systems have been shown to be associated with host-plant hypersensitive response (HR) mechanisms that lead to necrotic cellular death in the host-plant cells upon pathogen encounter and which limit pathogen infection and systemic spread [71]. As such, necrotic spots observed due to the *Rsv*-mediated defense response are common in soybean resistant plants [59, 60]. Generally, *Rsv*-resistant plants appear symptomless and cannot be distinguished from SMV-uninfected plants. *Rsv1* has been observed as an extreme resistance type where virus detection on inoculated leaves does not occur [59]. In contrast, *Rsv3* and *Rsv4* type of resistance have been shown to allow for limited virus replication at the inoculation site [59, 72–74]. Susceptible symptoms of SMV-infected plants appear to be highly variable and they include leaf mottling, curling, rugosity, chlorotic patterns, broad tissue necrosis and plant stunting. In the seed, SMV-infection can be observed through mottling of the seed coat [59, 60]. Plant-wide necrotic patterns have been reported on *Rsv*-resistant backgrounds and there is ongoing debate about the classification that should be used for the necrotic response [60]. The *Rsv1-h* resistance allele source from cultivar Suweon 97 (PI 483084) confers resistance to all known US SMV strains, G1 to G7. Thus, it has been an attractive source of SMV resistance for plant breeders [75].

The objective of this study was the development of double-resistant *Rag2-Rsv1-h* recombinant inbred lines (RILs) using the aphid resistance source PI 243540 (*Rag2*) and the SMV resistance source Suweon 97 (*Rsv1-h*) as donors in a double-cross type of breeding scheme, and elite experimental lines from the University of Nebraska-Lincoln soybean breeding programs as the susceptible parents for trait introgression (Table 1, Figure 1). The

Table 1 Description of soybean plant genetic materials used in this study. Entries include the plant genetic material used to develop recombinant inbreed lines with resistance to SMV or soybean aphids

Strain[a]	Pedigree[b]	Generation derivation	*Rag2*[c]	*Rsv1-h*[d]	Relative Maturity Group	Purpose
U00-429037	U96-1612 x U96-3601	F4	S	*Rsv1-t*	MGII	Parent
Suweon 97	SS7006 x SS6807	Pureline	S	R	MGIV	Parent
U06-607094	U00-429037 x Suweon 97	F4	S	R	MGII	Parent
PI 243540	Accession collected in Akita, Japan	Pureline	R	S	MGIV	Parent
U03-100612	U99-009019 x P92B12	F4	S	S	MGI	Parent
UX2324	U03-100612 x PI 243540	F2 population	Segregating	Segregating	Segregating	Parent
UX2569	U06-607094 x UX2324	F5	R	R	Segregating	Progeny
PI 200538	Accession collected in Kagawa, Japan	Pureline	R	S	MGVIII	Control
U03-120221	U99-013019 x HS98-3407	F4	S	S	MGII	Control
U06-308474	LNX97164-35 x UX1818	F4	S	R	MGIII	Control

[a] Entries with prefix UX represent populations of individuals

[b] Pedigree information of soybean experimental lines, cultivars, accessions and populations was obtained from the program's records and from GRIN correspondingly

[c] Represents the expected phenotype against soybean aphids biotype 1, e.g., resistant (R) or susceptible (S), based on previous studies and information from soybase [77]

[d] Represents the expected phenotype against SMV-G1, e.g., resistant (R) or susceptible (S), based on previous studies and information from soybase [77]. Line U00-429037 was discovered to possess resistance to SMV-G1 through phenotypic evaluation. We assume the phenotype was the result of *Rsv1-t* allele inheritance via pedigree from soybean cultivar 'Saturn' [78], a grandparent of the line

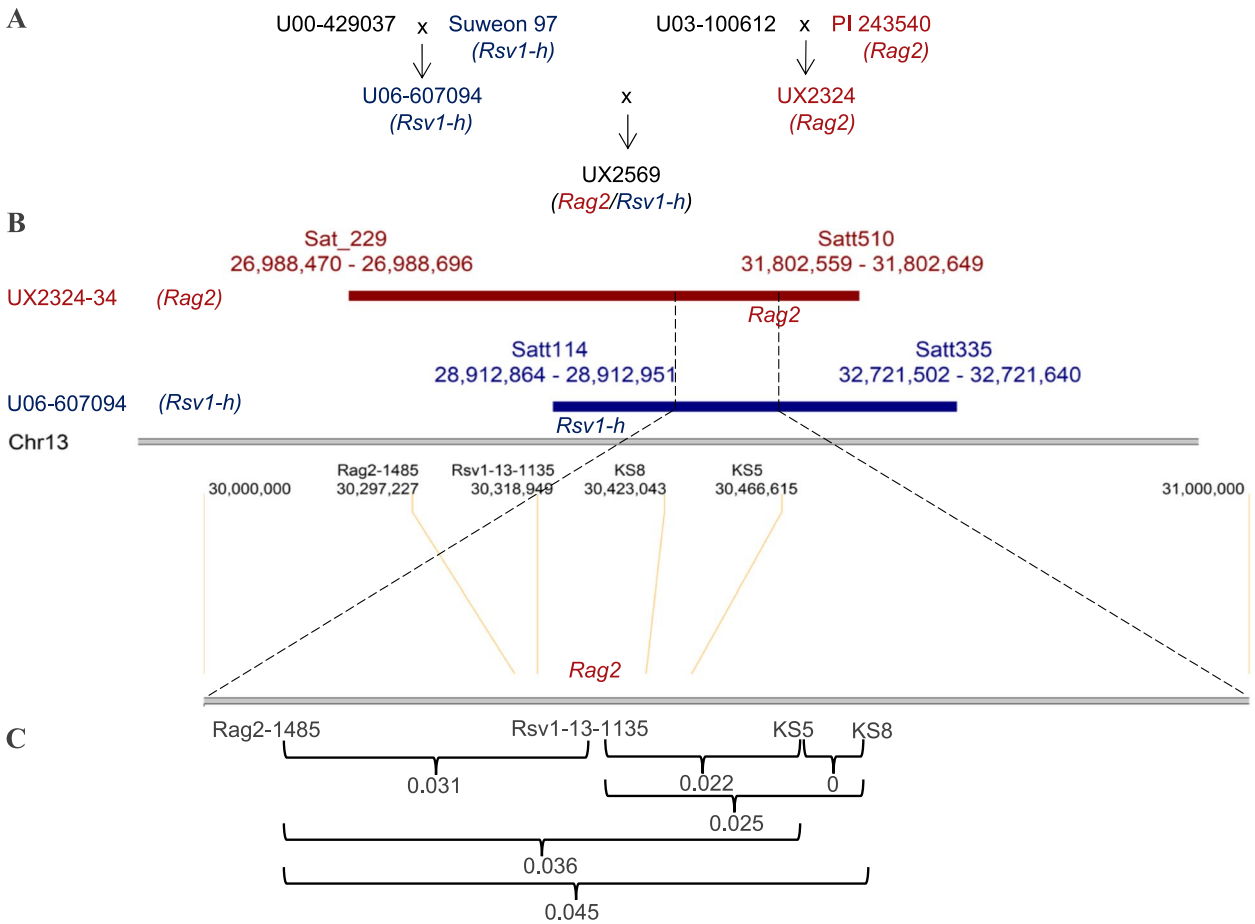

Fig. 1 Breeding scheme and introgression of genomic resistance haplotypes. **A** Breeding scheme used for the development of double-resistance RILs. **B** Allele haplotypes carried in UX2324-34 (from aphid resistant source PI 243540 Rag2) and U06-607094 (from SMV resistant source Suweon 97 Rsv1-h) based on SSR markers flanking the putative positions of Rsv1-h and Rag2 genes. **C** Estimation of LD among marker pairs using genotype data observed from 192 F3 segregating progeny. Genome positions were obtained from assembly version Wm82.a2.v1 soybase [77]

genes *Rag2* and *Rsv1-h* have been shown to colocalize to an NBS-LRR hot-spot on chromosome 13 of soybean [46, 65, 76], thus making the development of a recombinant haplotype in coupling phase challenging. We leveraged information and molecular tools from previous studies together with greenhouse phenotypic screens using both independent and joint pest challenges of soybean aphid biotype 1 and SMV-G1 on segregating progeny. Our results showed that there was a recombination event in identified double-resistant $F_{3:5}$ RILs in a region ca. 21 kb (position between 30,297,227 and 30,318,949 of Wm82.a2.v1) that lies between the reported position of the two genes (*Rag2* positioned between 30,412,581 and 30,466,533 bases, and *Rsv1-h* positioned between 29,815,463 and 29,912,369 bases relative to Wm82.a2.v1) [46, 66].

Methods

Plant genetic materials

The SMV *Rsv1-h* resistance allele source was obtained from soybean cultivar Suweon 97 (PI 483084). The source of the *Rag2* allele conferring resistance to SA biotypes I and II was obtained from soybean accession PI 243540. Several soybean experimental lines with good agronomic traits, but susceptible to both SMV and SA were developed by the University of Nebraska-Lincoln (UNL) Soybean Breeding Program and were used as the elite parents for population development (Table 1, Figure 1). Some of these elite, susceptible experimental lines were also used as controls during the SMV and SA reaction screens carried out in this experiment. In addition, soybean accessions PI 96983, PI 507389, Kwanggyo, Marshall, York, Ogden and Lee 68 were used as differential SMV reaction controls. This set of differentials has been used thoroughly in previous soybean-SMV resistance experiments [60, 62].

Population development and marker assisted selection

SMV and SA double resistant material

Breeding material used for introgression of resistance genes *Rsv1-h* and *Rag2* against SMV and aphids was developed as described in Additional File 1. In the same manner, double-resistant material was developed by crossing RIL U06-607094 (*Rsv1-h*) as female with RIL UX2324 (*Rag2*) as male in an overall double-cross type of breeding scheme (Figure 1, Supplementary Table 1, Supplementary Table 2, Additional File 2). Segregating recombinant $F_{3:5}$ lines were obtained from these breeding efforts.

The initial experimental plan for screening segregating $F_{3:5}$ lines was based on the assumption that the *Rag2* and *Rsv1* genes are 5 or less cM apart, in accordance with previous mapping studies [35, 79]. A gene fine-mapping study presented evidence of the location of *Rag2* from resistance source PI 200538 between positions 30,412,581 and 30,466,533 (SNP markers KS9-3 and KS5, respectively) on chromosome 13 of soybean (Wm82.a2.v1) [46]. Because the aphid resistance locus from PI 243540 had also been named *Rag2* we assumed the location of our gene conferring resistance to aphids would also be positioned between 30,412,581 and 30,466,533 (Wm82.a2.v1) [80]. Furthermore, six clones on chromosome 13 near the *Rsv1* region were reported to have an association with SMV resistance [65]. Three of the clones reported had been completely sequenced (NCBI Gen-Bank accession no. AY518517–AY518519) [81]. It was observed that clone *3 gG2* (AY518517) co-segregated with *Rsv1* with complete linkage in a population derived from PI 96983 (*Rsv1*) [65]. A different study observed that marker Rsv1-f/r co-segregated with the *Rsv1* resistance reaction to SMV in a F_2 population segregating for *Rsv1* and *Rsv3* resistance [79]. Marker Rsv1-f/r had been developed from the putative *Rsv1* allele clone, *3 gG2* gene. Because the *Rsv1-h* allele from Suweon 97 represented a different version of the same *Rsv1* gene and was reported to amplify the characteristic fragment of genotypes containing *Rsv1* alleles, we assumed our *Rsv1* target region to be within 30,425,670 and 30,426,025 (Wm82.a2.v1) on chromosome 13 after a BLAST of the amplicon region of Rsv1-f/r [79, 80]. Thus, it appeared the two resistance genes were located very close to each other. Despite being aware of this complex region being reported with clusters of NB-LRR genes [76], the possibility of the two genes being the same did not elude our discussions. However, the more reasonable scenario was the hypothesis that they are two closely linked genes. With a recombination frequency of 0.05 between two genetic loci, the number of double-resistant (recombinant) plants in the F_2 generation would be six in 10,000 (0.000625), and one in 100 (0.0128) in the F_3 generation. With this in mind, molecular SNP assay Rag2-1485, referred to as #1485 in its original publication [46], was set up in the winter of 2010-2011 in our laboratory and was used for an initial DNA screening of 192 F_3 plants collected during the summer of 2010. Marker polymorphisms for this assay allowed to differentiate the marker allele of *Rag2* donor PI 243540 from the rest of the parental lines used in this study (Table 2), hence the prefix Rag2 in the marker name. This single-marker genotype information of 192 F_3-derived lines was used to identify lines homozygous for the *Rag2* marker alleles. The next step of the experimental plan was to identify lines with any of the following two genotypes (*i*) *Rag2/Rag2:Rsv1/rsv1* (heterozygous at the *Rsv1* locus), and (*ii*) *Rag2/Rag2:Rsv1/Rsv1* (homozygous at the *Rsv1* locus), while also eliminating *Rag2/Rag2:rsv1/rsv1* lines not carrying the *Rsv1-h* alleles. Marker assay Rsv1-f/r [79] did

Table 2 Molecular marker haplotype of parents and 192 F_3 plants of population UX2569

	Marker name				
	Rag2-1485[b]	**Rsv1-13–1135**[c]	**KS8**[b]	**KS5**[b]	**Number of lines**[d]
Type	SNP	SSR	Indel	SNP	--
Start pos.[a]	30,297,227	30,318,939	30,423,043	30,466,615	--
End pos.	--	30,319,184	30,423,810	--	--
Parental	Locus polymorphism-based classification[e]				
strains:	*Rag2*	*Rsv1-h*	*Rag2*	*Rag2*	
U06-607094 (*Rsv1-h*)	B	B	--	B	--
UX2324-34 (*Rag2*)	A	A	A	A	--
Haplotype class of F_3 progeny in UX2569[f]					
	HET	HET	--	HET	48*
	HET	HET	A	A	2*
	HET	B	--	B	1*
	HET	A	A	A	4*
	HET	A	--	B	1
Parental	**A**	**A**	**A**	**A**	**64***
	A	A	--	HET	1*
	A	HET	A	A	1*
	A	M	A	A	1
	B	B	A	A	1*
	B	B	--	HET	1*
Parental	**B**	**B**	--	**B**	**64***
	B	A	A	A	2*
	B	A	--	B	1
Grand Total					**192**

[a] Single nucleotide polymorphism (SNP) physical position where indicated or start position of SSR sequence or forward primer in the case of the indel. Positions based on BLAST results of SSR primer sequences or SNP context sequence based on genome version Wm82.a2.v1 obtained phytozome.org [80]

[b] SNP markers from a previous publication [46]. KS8 is a dominant (presence/absence) marker type that resulted in the null amplification (no band = A) of alleles derived from PI 243540 (*Rag2/Rag2*), and as a discrete band (--) when the homozygote *rag2/rag2* or the heterozygote *rag2/Rag2* marker alleles were amplified

[c] Simple sequence repeat (SSR) marker obtained from personal communication with Dr. Pengyin Chen, University of Arkansas. M represent a missing datapoint

[d] The use an asterisk indicates that a sample of individuals from the haplotype class was selected to undergo phenotypic screening for resistance against SMV and aphids

[e] The locus polymorphism-based classification indicates whether a given marker was useful for discriminating alleles from different resistance sources in order to detect either *Rsv-1 h* alleles derived from Suweon 97 (through U06-607094), or *Rag2* alleles derived from PI 243540 (through UX2324-34). Single letter scores represent homozygous marker genotypes (e.g., B = BB, carries two copies of the marker allele). HET represents the heterozygous state

[f] The parental haplotypes in the F_3 progeny are in bold whereas recombinant and double heterozygotes are not

not yield consistent results and a different polymorphic SSR marker in this region was used instead (Dr. Pengyin Chen at University of Arkansas personal communication). Assay Rsv1-13–1135 yielded consistent results and showed useful polymorphisms that allowed to discriminate the marker allele from *Rsv1-h* donor Suweon 97 and the rest of the parental lines, hence the prefix in the marker name. By the start of summer of 2011, SSR assay KS8 and SNP assay KS5 [46] yielded consistent results and showed useful polymorphisms for the screening of 192 F_3 plants from population UX2569 (Tables 2, and 3). Phenotypic screens were conducted to validate marker information. Reserve $F_{3:5}$ seed was used to perform all subsequent phenotypic screens and molecular work.

Greenhouse aphid antibiosis evaluation, data collection and analysis

Soybean biotype 1 aphid colony development and maintenance were performed as described in Additional File 1. In September 2011, using reserve seed from the $F_{3:5}$ lines and the available marker haplotype information (Table 2), we selected a subset of 40 $F_{3:5}$ lines for further characterization and phenotypic screen for aphid biotype 1 resistance in the entomology greenhouse at UNL. The

Table 3 Plant marker haplotype of the *Rag2-Rsv1* genomic region and results of SMV and aphid resistance phenotypic screens

	Marker haplotype[b]								SMV-G1 scores[e]	
	30.30	30.32	30.42	30.47	Controls[c]	Aphid antibiosis counts[d]			Sep	Jan
	Rag2	Rsv1			aphid/	Sep	Jan	SE	2011	2012
Strain[a]	1485	13–1135	KS8	KS5	SMV-G1	2011	2012	2012	Visual	ELISA
Susceptible					S/na	121	139.4	1.18		
U03-120221	B	A	--	B	S/S	302			MR	
U06-308474	B	B	--	B	S/R	197				
U00-429037	B	A	--	B	S/R	125	59	14.5	MR-R	
Suweon 97	B	B	--	B	S/R	111			MR-R	0.29[fg]
Williams 82	B	A	--	B	S/S	99				
U03-100612	B	A	--	B	S/S	77			MS-S	
U06-607094	B	B	--	B	S/R	77			MR-R	
KS4202					S/S	69	67	17.8		
K03-4686					S/S	37				
IAC-19					R/S	5	26.4***	5.6		
IAC-24					R/S	7	25.8***	5.4		
IAC-17					R/S	na	36.6***	9.8		
PI 243540	A	A	A	A	R/S	22	19.7***	5.8	S	
PI 200538	A	A	A	A	R/S	na	9.5***	3.3		
UX2569-159	B	A	A	A		2	6.7***	1.4	R	0.0[g]
UX2569-176	B	B	--	B		11	30.3***	6.5	R	0.15[g]
UX2569-122	B	A	A	A		6	26.7***	5.9	R	2.5
UX2569-175	B	B	A	A		6	18.6***	3.9	MR-R	0.27[g]
UX2569-128	HET	HET	--	HET		4	13.1***	2.9	MR-R	4.5
UX2569-111	A	A	A	A		2			S	
UX2569-113	A	A	A	A		4			S	
UX2569-003	A	A	A	A		14			na	
UX2569-163	A	A	--	HET		7			S	
UX2569-182	A	HET	A	A		9			S	
UX2569-098	B	B	--	B		7			MS-S	
UX2569-124	HET	A	A	A		2			S	
UX2569-110	HET	HET	--	HET		13			MR	
UX2569-114	HET	HET	--	HET		16			MR-R	
UX2569-058	B	B	--	B		25			MS-S	
UX2569-140	HET	B	--	B		25			MR-R	
UX2569-107	HET	A	A	A		26			S	
UX2569-168	B	B	--	B		29			R	
UX2569-006	HET	HET	--	HET		29			R	
UX2569-147	HET	HET	A	A		29			MR	
UX2569-162	B	B	--	B		35			MR-R	
UX2569-184	B	B	--	HET		45			MR-R	
UX2569-072	B	B	--	B		56			MR-R	
UX2569-009	HET	HET	--	HET		56			MS-S	
UX2569-028	B	B	--	B		59			MR-R	
UX2569-002	B	B	--	B		60			MS-S	
UX2569-102	HET	HET	--	HET		68			MS-S	
UX2569-021	B	B	--	B		73			R	
UX2569-133	HET	HET	--	HET		73			MR-R	
UX2569-053	B	B	--	B		87			MR-R	

Table 3 (continued)

	Marker haplotype[b]								SMV-G1 scores[e]	
	30.30	30.32	30.42	30.47	Controls[c]	Aphid antibiosis counts[d]			Sep	Jan
	Rag2	Rsv1			aphid/	Sep	Jan	SE	2011	2012
Strain[a]	1485	13–1135	KS8	KS5	SMV-G1	2011	2012	2012	Visual	ELISA
UX2569-097	B	B	--	B		94			MR-R	
UX2569-071	B	B	--	B		97			MR-R	
UX2569-186	B	B	--	B		103			MR-R	
UX2569-103	B	B	--	B		108			R	
UX2569-016	B	B	--	B		110			MR	
UX2569-024	B	B	--	B		125			R	
UX2569-047	B	B	--	B		171			R	
UX2569-059	B	B	--	B		198			MR-R	
UX2569-023	B	B	--	B		285			MR-R	
UX2569-067	B	B	--	B		370			MR-R	

[a] List of strains used in the phenotypic screens. The "susceptible" entry represents the susceptible control group with its corresponding mean and standard error estimates

[b] Marker physical position in mega bases (Mb) based on genome version Wm82.a2.v1 [80]. Single letter scores represent homozygous marker genotypes (e.g., B = BB, carries two copies of the marker allele). HET represents the heterozygous state. Genotypes observed correspond to F_3 plant individuals of population UX2569, experimental lines, parents, accessions and controls. Symbol "--" in marker KS8 represents discernable PCR amplification product containing ambiguous *rag2*/_ genotype calls, where heterozygotes cannot be distinguished from homozygotes susceptible marker calls. Call A here represents the absence of amplification PCR product as observed for PI 243540 and PI 200538 (both classified as *Rag2/Rag2,* carrying the resistant marker alleles)

[c] Expected phenotypic reactions of control and parental lines to soybean aphid biotype 1 and SMV-G1 isolates: R, resistant; S, susceptible

[d] Soybean aphid biotype 1 antibiosis assays on 40 $F_{3:5}$ soybean experimental lines, parents, accessions and controls carried out in the entomology greenhouse at UNL: (*i*) on September 2011 and (*ii*) as a joint aphid-SMV trial in January 2012. Arithmetic means are reported for the trial of September 2011. A combined analysis of variance for aphid counts was performed across the two trials (September 2011 and January 2012) for the subset of five $F_{3:5}$ experimental lines and controls re-tested in the trial of January 2012. Mean aphid count estimates and standard errors are reported for the January 2012 trial. Four plants per entry were tested in the September 2011 trial and ca. 20 plants per entry were used in the joint aphid-SMV January 2012 trial

[e] SMV-G1 reaction assays on 40 $F_{3:5}$ soybean experimental lines, parents, accessions and controls carried out in the UNL pathology and entomology greenhouses during September 2011 and January 2012, respectively. Visual classification of the average reaction to SMV-G1 isolate is reported for the September 2011 trial: R, resistant; MR-R, mostly resistant; S, susceptible; MS-S, mostly susceptible; --, no plants available. ELISA assay results reported for the subset of five $F_{3:5}$ experimental lines and controls re-tested in the January 2012 trial. Four plants per entry were tested in the September 2011 trial and ca. 20 plants per entry were used in the joint aphid-SMV January 2012 trial

[f] Entry not part of January 2012 greenhouse trial. Included as SMV-G1 inoculated control for ELISA run. Inoculated tissue from previous trial

[g] Line classified as resistant to SMV-G1 based on the observed average ELISA absorbance values

*** $P<0.001$

selection criteria were based on F_3 progeny marker haplotype classes as described in Table 2. Individuals were chosen at random where applicable among the available haplotype categories as to have a robust representation of all classes observed (Table 2). The final set selected was described in Table 3. Up to four seeds of each of 40 $F_{3:5}$ lines were planted in potting media. Potting media consisted of 34% peat, 31% perlite, 31% vermiculite, and 4% soil mixed in 15-cm-diameter plastic pots. Plants were thinned to one plant per pot after seedling emergence. Greenhouse conditions were 400-W high-intensity lamps, ~23°C temperature, and a photoperiod of 16:8 hours (light:dark). Known resistant and susceptible parental entries and other lines were used in the screen evaluation as controls. Some parental lines were expected to be much later in maturity than the average $F_{3:5}$ line (Table 1). Different planting dates were used for the different maturity groups to ensure that all plants reached the R1 stage at approximately the same time. Seed from PI 200538 did not germinate and this entry was excluded from further analysis. The experimental design was a CRD factorial with 52 genotypes (40 $F_{3:5}$ RILs plus 12 control lines) with four replicates per treatment (Table 3). Enough cages were available for a full greenhouse aphid screen consisting of ca. 208 experimental units total (pots harboring plants). When plants reached the V2 developmental stage, infestation with 10 apterous aphids per plant took place. Aphids were placed on the youngest expanded trifoliate using a soft paintbrush. After infestation, plants were caged in 5-cm wide × 50-cm tall polycarbonate plastic cages (Eplastics, San Diego). We used organdy fabric at the top of each cage to prevent aphid escape.

In the first aphid antibiosis resistance trial, aphid infestation was quantified by counting the number of aphids on experimental units assigned to each of four reproductive stages, R1, R2, R3 and R4; one plant at each

reproductive stage. Stages were assigned at random to experimental units. We collected aphid infestation data across four plant stages to make the experiment more manageable given the size of the experiment and the time-consuming phenotyping method.

Greenhouse SMV resistance evaluation

SMV-inoculum was prepared and maintained in the following way: seeds from SMV-susceptible genotype Lee 68 were grown in August 2011 in the pathology greenhouse at UNL in isolation for inoculum reactivation. Before planting, seeds were inspected to discard any strays with mottling or hilum-bleeding. After emergence, seedlings showing curled or odd leaf shapes were pulled out. At V2, leaf disks were collected from each of 20 plants available to confirm the absence of SMV via double antibody sandwich (DAS)-ELISA with positive controls, reagents and protocol as per manufacturer (Agdia Inc. Elkhart, IN). In September of the same year a stock of infected leaf tissue with SMV-G1 was obtained for inoculum reactivation and subsequent experimentation (Dr. Sue Tolin at Virginia Tech personal communication). In order to prepare a virus culture of uniform days-after-inoculation for reproducibility purposes, SMV-G1 virus isolate was reactivated by inoculating 20 Lee 68 plants at ~V3 stage. SMV inoculum for reactivation was prepared by grinding infected leaf tissue in a mortar a pestle and adding volumes of 0.025 M potassium sulfate buffer in a 1:10 g/ml ratio at pH 7.2. Manual homogenization continued until obtaining a uniform mixture. Virus extract was filtered using cheese cloth material to remove insoluble plant material. Plant inoculation proceeded immediately after inoculum preparation. Moments before inoculation of Lee 68 plants for virus reactivation, carborundum (Fisher Scientific, 320 grit) was sprinkled in a small quantity to mortar containing inoculum and mixed briefly with pestle. We inoculated Lee 68 plants using the method of "rubbing leaves with a pestle" that is common for routine transfers of virus cultures (Dr. Sue Tolin at Virginia Tech personal communication). The underside of one unifoliolate leaf of each plant at V3 was rubbed with one swipe of the pestle carrying inoculum. Twenty minutes after inoculation, leaves were rinsed off with tap water using a squeeze bottle to help observe the reaction. Fourteen days after inoculation of Lee 68 plants, virus symptoms were recorded visually and leaf disks were collected to confirm SMV presence via DAS-ELISA test. At the same time, leaf tissue from SMV-infected Lee 68 plants was harvested for subsequent virus-resistance screenings and long-term storage. Harvested leaves showing symptoms were immediately sliced into small pieces on a sterile plastic surface. Sliced pieces were placed in cryovials and transported in liquid nitrogen for long-term storage in an ultra-low freezer. Inoculation success rate of susceptible Lee 68 individuals was 100%.

In early September of 2011, in a parallel experiment to the aphid screen above, seed from the same set of $F_{3:5}$ lines and controls was planted in the pathology greenhouse at UNL (Table 3). Up to four seeds of each of 40 $F_{3:5}$ lines were planted in potting media. In addition, four seeds from each of nine SMV-differential designated controls (Dr. Sue Tolin at Virginia Tech personal communication) were planted in each of two pots for confirmation of the SMV-G1 isolate reaction (data not shown). Potting media consisted of 34% peat, 31% perlite, 31% vermiculite, and 4% soil mixed in 30-cm-diameter plastic pots. Greenhouse conditions were 400-W high-intensity lamps, ~23°C temperature, and a photoperiod of 16:8 hours (light:dark). Germplasm inoculation took place when plants had reached the V1 stage. Inoculum of SMV-G1 was prepared on the same day of plant inoculation using frozen infected leaves that had been harvested after isolate reactivation as described above. Fourteen days after plant inoculation virus symptoms were recorded visually (Table 3). Visually scored material was classified as: R for resistant, MR-R for mostly resistant, S for susceptible, MS-S for mostly susceptible; -- when no plants were available.

SMV resistance and aphid antibiosis joint greenhouse assay

In January of 2012, based on the results of the first soybean aphid and SMV phenotypic screens carried out in separate greenhouses, (i.e., the entomology and pathology greenhouses, respectively), a subset of five $F_{3:5}$ lines with the lowest aphid count means and with resistant phenotypic reactions (R) to SMV-G1 inoculation, was chosen for further evaluation and characterization. The seed source for this follow-up screen was also the reserve seed from $F_{3:5}$ line harvest. Entries and control lines were planted in the entomology greenhouse at UNL in January of 2012. One seed from each entry was planted in each of twenty-five 15-cm diameter plastic pots. The number of pots was reduced to 20 experimental units per genotype treatment after emergence for uniformity (i.e., 20 replicates per genotype treatment). Potting media consisted of 34% peat, 31% perlite, 31% vermiculite, and 4% soil mixed. Greenhouse conditions were 400-W high-intensity lamps, ~23 C temperature, and a photoperiod of 16:8 hours (light:dark). Known resistant and susceptible parental entries and other lines were used in the screen evaluation as controls. As before, different planting dates were used for the different maturity groups to ensure that all plants reached the R1 stage at approximately the same time.

Plant virus-inoculation took place at the V1 stage. SMV-G1 inoculum was prepared a few hours in advance using frozen infected leaves that had been harvested after isolate

reactivation as described above. The phenotypic response to viral challenge was quantified fourteen days after inoculation with a DAS-ELISA test as described in Additional File 1 (Table 3). When this same set of plants reached the V2 developmental stage, infestation with 10 apterous aphids per plant took place. Aphids were placed on the youngest expanded trifoliate using a soft paintbrush. After infestation, plants were caged in 5-cm wide × 50-cm tall polycarbonate plastic cages (Eplastics, San Diego). We used organdy fabric at the top of each cage to prevent aphid escape. Aphid infestation was quantified by counting the number of aphids on experimental plants at the R1 reproductive stage. After collecting aphid data, plants were transplanted to 30 cm pots for seed production after a soil drenched treatment with a solution of systemic insecticide imidacloprid {1-[(6-chloro-3-pyridinyl) methyl]-N-nitro-2-imidazolidinimine}, which rendered the surviving aphids dead. Plants were relocated and tissue sampled for genotype confirmation and grown to seed. At maturity, each plant was harvested individually as $F_{5:6}$ seed.

Comparative genomic hybridization analysis

The microarray used for the CGH exploration of the *Rag2-Rsv1* region in chromosome 13 of soybean has been described previously [82]. Briefly, the microarray consisted of 696,139 unique oligonucleotide probes ranging from 50 to 75 bp in length. The probes tile the assembled soybean genome sequence at a median interval length of 1,120 bp between adjacent probes. CGH protocols, including DNA labeling, microarray hybridization, and scanning, were performed as described [82]. Genotype PI 243540 was used as the Cy5 reference in all hybridizations. Briefly, the segMNT algorithm in the Nimblescan software (version 2.5) was used to extract the raw data and make segmentation calls. The parameters of the algorithm were as follows: minimum segment difference = 0.1, minimum segment length (number of probes) = 2, acceptance percentile = 0.99, number of permutations = 10; nonunique probes were included, and spatial correction and qspline normalization were applied. The list of resulting segments was processed to identify significant segments. Segments were significant if the log2 ratio mean of the probes within the segment was beyond the threshold level for that genotype. The upper threshold was the log2 ratio value of the 95 th percentile of all data points for each individual genotype. The comparative genomic hybridization data for this study can be found as accession number GSE285240 in the National Center for Biotechnology Information Gene Expression Omnibus.

Statistical analysis

Analysis of variance was conducted using the SAS 9.4 Generalized Mixed Linear Model procedure [83]. A combined analysis of variance across the two aphid greenhouse experiments (i.e., trials conducted in September of 2011 and January of 2012) was performed fitting a negative binomial distribution for aphid counts with the default log link function. Entries, reproductive stages and trials were treated as fixed effects. Since different reproductive stages for data collection were only used in the first trial (September of 2011) but not in the second, stages were nested within trial. The following model was used:

$$\eta_{ijkl} = \mu + a_i + b(a)_{ji} + e_k$$

where η_{ijkl} is the linear predictor of the k^{th} entry evaluated at the j^{th} reproductive stage within the i^{th} trail, μ is the overall mean, a_i, is the effect of the *ith* trial, $b(a)_{ji}$ is the effect of the *jth* reproductive stage within the *ith* trail and e_k is the effect of entry *k*. Least squares means for entries and contrasts estimates between each entry and the mean of the susceptible genotypes were requested from the procedure to answer pre-planned questions. The entry lsmean estimates were back transformed to the count scale by exponentiating the estimates. Occurrences of significant differences between each entry and the mean of the susceptible genotypes are reported in Table 3. For single plant classification into resistant or susceptible categories, a susceptible bound of 45 aphids (alpha level of 0.05) was established for the reaction against soybean aphid biotype 1, consistent with the generalized model used for the aphid count data in the combined analysis and their corresponding standard errors.

For marker linkage disequilibrium (LD) the expected genotype frequencies in the F_3 generation were calculated assuming two generations of selfing from a digenic F_1 dihybrid scenario. All combinations of different loci pairs as functions of varying LD levels were used to find the best fit for the observed data. The minimum chi-square estimation was approximated by finding the LD value that yielded the best fit (i.e., the largest *P*-value) (Figure 1). A single factor ANOVA analysis was performed to calculate allele dosage effect on aphid biotype 1 count estimates for each of the four markers in the *Rag2-Rsv1* haplotype. An additional single factor ANOVA analysis was performed to calculate haplotype estimates using the aphid biotype 1 count data from the January 2012 trial (Table 4).

Results

We identified double-resistant recombinant progeny in F_3-derived, F_5 experimental lines from a cross between U06-607094 (*Rsv1-h*) x UX2324 (*Rag2*) (Table 1) carrying resistance alleles from sources Suweon 97 and PI 243540, respectively (Figure 1). Using information from a four-marker haplotype in the *Rag2-Rsv1* region for 192 F_3 plants derived from this cross, we were able to identify at

Table 4 Allele dosage effect on aphid biotype 1 counts and their corresponding trend coefficients for each of the four haplotype markers

Marker	Effect[a]	Estimate[b]	SE	Sample size	*P*-value
Rag2-1485					
	A	9	1.6	40	***
	HET	35	1.4		***
	B	90	1.2		***
	Quadratic	0.04	2		***
	3-class X^2	1.81		192	0.405
Rsv1-13–1135					
	A	9	1.5	40	***
	HET	41	1.4		***
	B	96	1.2		***
	Quadratic	0.04	2		***
	3-class X^2	0.55		192	0.761
KS8					
	A	13	1.4	40	***
	--	84	1.2		***
	Linear	0.15	1.5		***
	2-class X^2	0.2		192	0.655
KS5					
	A	13	1.4	40	***
	HET	43	1.4		***
	B	112	1.2		***
	Quadratic	0.4	1.9		***
	3-class X^2	0.56		192	0.757
Four-Marker haplotype					
	BAAA	16.7a	4.5	91	***
	BB--B	53.2b	6		***
	BBAA	33.3c	5.9		***
	HETHET--HET	24ac	6.2		***

[a] Symbol "--"represents discernable PCR amplification product containing ambiguous *rag2*/_ genotype calls, where heterozygotes cannot be distinguished from homozygotes calls. Call A here represents the absence of amplification PCR product as observed for PI 243540 and PI 200538 (both classified as *Rag2/Rag2*). Single letter scores for the marker haplotype represent homozygous marker genotypes (e.g., B = BB, carries two copies of the marker allele)

[b] Allele dosage effect and trend coefficient estimates obtained from the adjusted means of the combined analysis of variance of 40 $F_{3:5}$ soybean experimental lines. Chi-square estimates and *P*-values of single marker genotype class expectations of individuals in the F_3 generation. Haplotype aphid count estimates that share the same letter following the mean statistic are not significantly different at the 0.05 probability level

*** $P<0.001$

least 13 different haplotype categories out of the expected 54 possible (Table 2). As expected, the largest proportion of F_3 genotypes represented the parental and double heterozygote haplotype classes. However, we were surprised to observe different recombinant haplotypes in this initial screen as the LD between these markers was assumed to be very high. The minimum chi-square estimators that we calculated using the expected recombination frequencies with varying degrees of LD in the F_3 generation revealed very tight linkage among the four marker loci, whether assuming coupling (e.g., from F_2 double heterozygote marker genotypes of a marker pair as AB/ab) or repulsion (e.g., F_2 double heterozygote as Ab/aB) phases for different marker pairs (Figure 1). According to soybean genome assembly Wm82.a2.v1, these markers reside within a 200 kb window on chromosome 13 [80]. Different LD estimates were observed among different marker pairs with nearly complete LD (coupling phase) between KS8 and KS5 and as large as 0.045 (4.5% recombination frequency) between Rag2-1485 and KS8 (coupling phase), the most distant markers. Our marker analysis revealed a slightly different genetic arrangement for markers KS8 and KS5 relative to the other loci as per physical coordinates (Figure 1). The *Rag2-Rsv1* region had been placed within the euchromatic region of chromosome 13 according to a previous study, thus, some degree of recombination was expected [84]. The individual marker genotype counts for all four markers fit the expected segregation ratio of plants in F_3 (Table 4).

The *Rag2-Rsv1* region marker haplotype information we obtained from 192 F_3 plants allowed us to select a subset of 40 $F_{3:5}$ lines for phenotypic evaluation of resistance against aphid biotype 1 and SMV-G1. As described above, individuals were chosen from different haplotype classes as depicted in Table 2 to maximize representation of parental and recombinant haplotypes. The phenotypic trials were conducted in separate greenhouses, each with their corresponding controls (September 2011 trials in Table 3). These independent phenotypic screens allowed us to select a subset of five recombinant lines that appeared to possess double phenotypic resistance. The selected lines represented four different F_3 plant marker haplotypes (BAAA, BBAA, BB—B and HETHET—HET) for the molecular markers used in this study (Table 3). We then carried out a joint aphid-SMV greenhouse trial with the five selected RILs and controls (January 2012 trial in Table 3) for confirmation of double resistance. Combined analysis of variance of the two aphid biotype 1 antibiosis trials showed that all five selected $F_{3:5}$ RILs had significantly lower ($p<0.001$) mean aphid counts relative to the mean of susceptible controls used in this experiment (Table 3). Not all five RILs were confirmed to be resistant to SMV-G1, however, with only three RILs being classified as resistant given the ELISA absorbance cutoff threshold of 0.3. Among the three double-resistant RILs, line UX2569-159 showed the smallest aphid count estimate ($p<0.001$), the smallest average ELISA absorbance value (Table 3), and largest number of plants classified as resistant to both, aphids and to SMV-G1 (Table 5).

Table 5 Resistant and susceptible soybean plant phenotype counts observed from the reaction to SMV-G1 and aphid biotype 1 challenge

						Single dominant gene		Two dominant genes	
						Exact test	X^2	Exact Test	X^2
Strain[a]	Test[b]	Resistant[c]		Susceptible[c]		*P*-Value[d]	*P*-Value[e]	*P*-Value[d]	*P*-Value[e]
UX2569-122		--- *n* = 17 ---							
	SMV	(5)	6	(2)	7	0.258	0.224	**	***
	Aphids		13		4	0.316	0.234	0.285	0.262
UX2569-128		--- *n* = 18 ---							
	SMV	(3)	4	(0)	10	*	**	***	***
	Aphids		16		2	*	*	0.766	0.719
UX2569-159		--- *n* = 20 ---							
	SMV	(16)	17	(0)	1	**	**	0.355	0.299
	Aphids		19		1	**	**	0.346	0.244
UX2569-175		--- *n* = 20 ---							
	SMV	(14)	16	(0)	1	**	**	0.497	0.332
	Aphids		17		3	0.061	*	1	0.904
UX2569-176		--- *n* = 19 ---							
	SMV	(11)	13	(2)	3	0.195	0.121	0.716	0.59
	Aphids		12		7	1	0.953	*	**

[a] Each of the five F_3-derived, F_5 recombinant lines evaluated in the January 2012 trial

[b] Biological pest challenge

[c] Numbers in parenthesis within each phenotypic class represent double-resistant or double-susceptible counts, in each column respectively. For example: In UX2569-122 line, out of the six plants resistant to SMV, five plants were also found to be resistant to aphids. In the same manner, out of the 13 plants found to be resistant to aphids, five were also classified as resistant to SMV, while the rest were found either susceptible or died. The largest number of double-resistant individuals was observed in line UX2569-159

[d] Expected frequencies were calculated under the assumption of heterozygous F_3 plants at the resistance gene loci with F_5 progeny segregating for the reactions

[e] Chi-square and exact test *P*-values for two types of gene segregation scenarios in F_3-derived, F_5 recombinant lines, with two-degrees of freedom

* $P<0.05$; ** $P<0.01$; *** $P<0.001$

The three double-resistant RILs identified, UX2569-159, UX2569-175 and UX2569-176, revealed three different F_3 plant marker-haplotype versions in the *Rag2-Rsv1* region: BAAA, BBAA, and BB--B, respectively (Table 3). We then asked if mean aphid counts were significantly different among the three double-resistant RILs. The mean aphid count estimate of UX2569-159 was significantly smaller ($p<0.001$) than the mean of either UX2569-175 or UX2569-176, suggesting that BAAA is the preferred haplotype for aphid biotype 1 resistance. To further validate the four-marker haplotype used, single marker allele dosage effect on aphid counts showed an average reduction of 42 aphids per copy of allele *A* present ($p<0.001$) across the four markers (Table 4), with a significant although small quadratic effect, suggesting an incomplete dominance, intra-genic type of interaction for codominant markers. Furthermore, single factor ANOVA analysis of the maker haplotype on aphid adjusted mean counts obtained from the combined analysis of variance confirmed that BAAA is the preferred haplotype for resistance to aphid biotype 1 ($p<0.001$) (Table 4). We then set out to confirm the molecular haplotype of some of the $F_{3:5}$ plants that displayed the double-resistant phenotype. In addition, we asked if the *Rag2-Rsv1* region colocalized with regions harboring structural variation (i.e., duplications, copy number variation, translocations, etc.) [85]. A CGH analysis revealed no clear structural variants observed in this region. The analysis revealed, however, that there was a recombination event within a 100 kb window between positions ca. 30.24 and 30.34 of chromosome 13 (Wm82.a2.v1) in double-resistant plants UX2569-159-2, UX2569-176-5 and UX2569-122-3 (Figure 2), confirming the results of the four-marker haplotype in two out of these three instances (UX2569-159 and UX2569-122 with the BAAA haplotype). Based on the F_3 plant genotypes, this recombination event appeared to have occurred between markers Rag2-1485 (30,297,227 bases) and Rsv1-13–1135 (30,318,949 bases), narrowing down the recombination region to ~22 kb. These recombinant plants showed very small aphid counts (3, 8, and 0, respectively) and low SMV-ELISA absorbance levels (ODs of 0.2, 0.2 and 0.05, respectively) in the joint aphid-SMV trial. Plant UX2569-176-5, however, was not expected to be a recombinant in this region, but instead

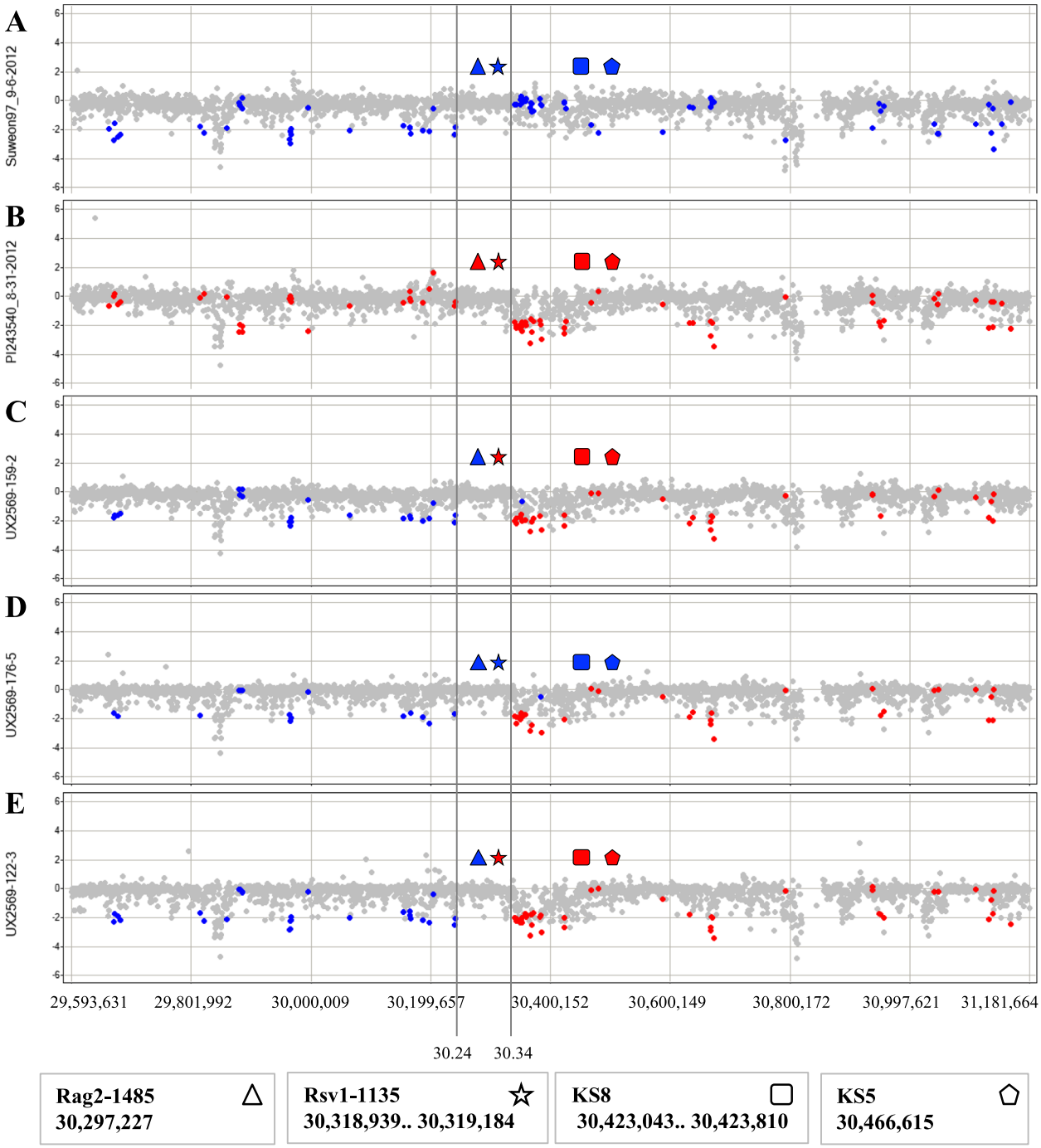

Fig. 2 Genomic hybridization profiles of the *Rag2-Rsv1* region on chromosome 13 of soybean. **A** Suweon 97 Rsv1-h. **B** PI 243540 Rag2. **C**, **D**, **E** Double-resistant F5 plants with a recombination event of ca. 100 kb between positions 30.24 Mb and 30.34 Mb. Blue and red polymorphisms represent Rsv1-h and Rag2 alleles, respectively. Marker genomic positions were obtained from assembly version Wm82.a2.v1 soybase [77]

to carry the full Suweon 97 haplotype (*Rsv-1-h*) as suggested by the four-marker haplotype identified in the F_3 generation (BB-B) (Table 3).

The molecular evidence presented allowed us to confirm the off-type individuals identified in the lines evaluated and helped us correctly characterize UX2569-159 as the best double-resistant RIL in this experiment across two independent greenhouse trials and with sufficient plant replicates. The data presented here, namely: (*i*) the genomic hybridization data confirming the recombination event within the *Rag2-Rsv1* region in the $F_{3:5}$ plants analyzed (Figure 3), (*ii*) which in turn is supporting

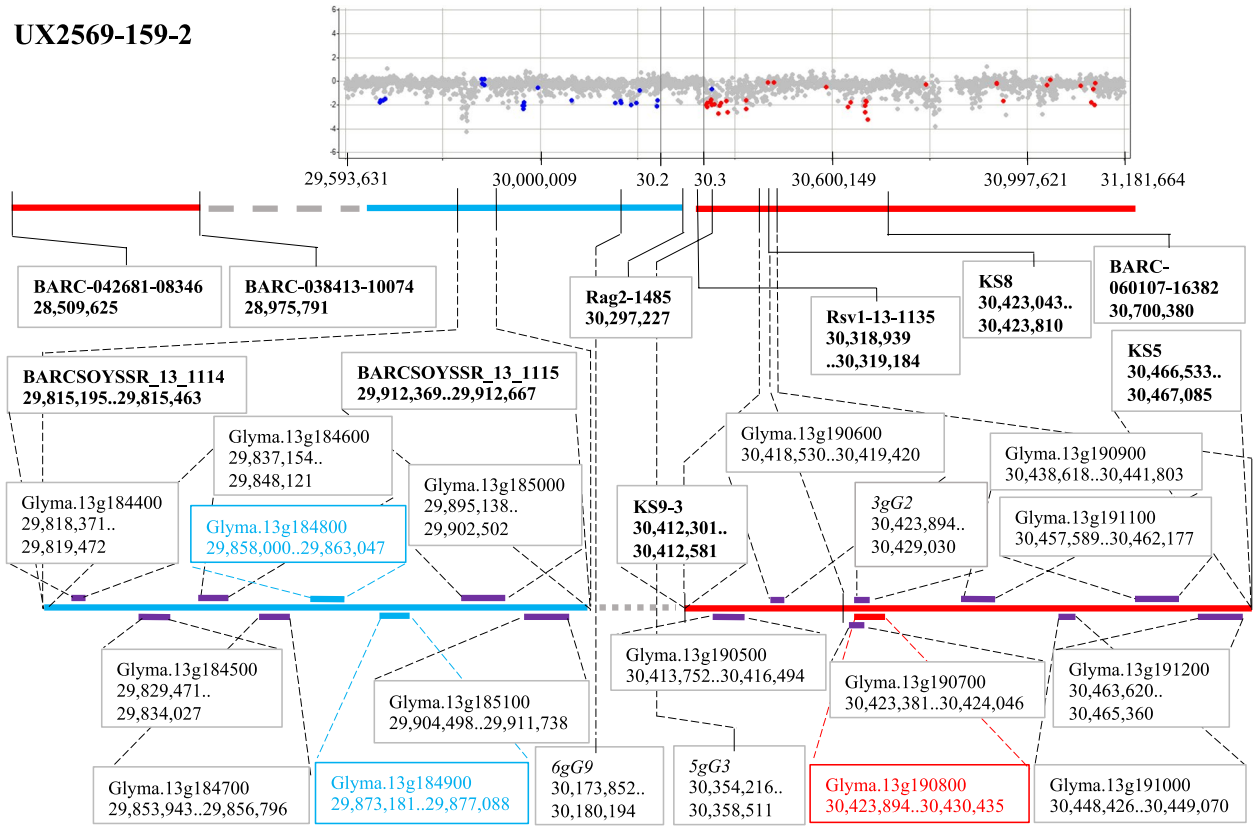

Fig. 3 Genomic hybridization profile of double-resistant RIL UX2569-159-2 in the region containing the *Rag2* and *Rsv1* genes on chromosome 13 of soybean. Polymorphisms are shown in blue and red to differentiate Rsv1-h and Rag2 alleles, respectively. Zoomed-in genomic regions where candidate genes have been proposed. Molecular markers are shown in bold. Physical positions and gene information were obtained from assembly version Wm82.a2.v1 in soybase [77]. All three candidate genes (Glyma.13g184800, Glyma.13g184900 and Glyma.13g190800) are leucine-rich repeat-containing proteins

evidence of the recombination point first captured by the four-marker haplotype obtained for F_3 plant genotypes, (*iii*) the confirmation of the *Rsv1-h/Rsv1-h*, *rsv3/rsv3* and the *rsv4/rsv4* marker haplotypes observed in the female parent of this RIL (U06-607094) (Supplementary Table 3, Additional File 2) and its susceptibility to aphids (Table 3), (*iv*) the susceptibility of the male grandparents of this RIL to SMV-G1 (U03-100612 x PI 243540) (Table 3), (*v*) the susceptibility of the female grandparent of this RIL (U03-100612) to aphids (Table 3), and finally, (*vi*) the significantly lowest mean aphid counts along with the lowest average SMV-ELISA absorbance values observed in this RIL, are strong supporting evidence of UX2569-159 as a *Rag2-Rsv-1-h* double-resistant RIL against aphid biotype 1 and SMV-G1 (Table 3).

At the onset of this experiment, we did not know the allelic status of line U00-429037 at the *Rsv1* locus, but we knew that the soybean cultivar 'Saturn' [78] was a grandparent of this line. We also knew that Saturn carried the *Rsv1-t* allele at the *Rsv1* locus and it amplified the characteristic fragment of genotypes containing *Rsv1* alleles [79]. We discovered through the phenotypic screens performed in this study that line U00-429037 showed resistance to SMV-G1 based on ELISA values (mean OD of 0.107, %CV= 0.7%) and visually symptomless plants (n= 12). To characterize allelic status at other known *Rsv* loci, SSR markers spanning the *Rsv4* region in chromosome 2 fine-mapped in a previous study were used to confirm the allelic status of female parent U00-429037 as *rsv4/rsv4*, based on the polymorphisms observed compared to a known source of the *Rsv4* resistance gene in our program (Essex *Rsv4*) (Supplementary Table 4, Additional File 2) [86]. The GoldenGate assay platform was also used to characterize the allelic status of U06-607094, U00-429037 and Suweon 97 in chromosomes 2 and 14 spanning the *Rsv4* and *Rsv3* regions, respectively, which suggested U06-607094 carried the Suweon 97 alleles at said loci (Supplementary Table 3, Additional File 2). Hence, the resistance observed in U00-429037 is likely mediated by *Rsv1-t* and U06-607094 appears to have inherited haplotypes from Suweon 97 at the *Rsv1*, *Rsv3* and *Rsv4* loci, thus, only carrying the *Rsv1-h* resistant alleles.

Discussion

Soybean-SMV and the *Rsv* Genes

The challenges and complexity of the SMV-soybean pathosystem including the current knowledge about the *Rsv1* SMV-resistance locus have been previously described [59, 60]. The *Rsv1* locus is likely the most complex among the four independent *Rsv* resistance loci identified thus far in the United States (*Rsv1, Rsv3, Rsv4* and more recently, *Rsv5*). The *Rsv1* locus exhibits at least 10 different alleles (*Rsv1, Rsv1-t, Rsv1-y, Rsv1-m, Rsv1-k, Rsv1-r, Rsv1-s, Rsv1-n, Rsv1-h,* and *Rsv1-c*) present in different plant genetic sources and expressing different phenotypic reactions to different US-SMV strains [87, 87–91]. The *Rsv1* genomic region is also rich in NBS-LRR resistance type of genes (*R*) [65, 76, 92, 93], and some of them have been reported to be involved in the resistance observed against other pathogens, including *Rpv1* (resistance to peanut mottle virus), *Rps3* (resistance to Phytophthora *sojae*), *Rpg1* (resistance to bacterial blight) *Rcs* (resistance to Cercospora leaf spot) [92, 94–97], among others. In addition, at least six SMV-resistance genes on chromosome 13 have been identified in Chinese soybean germplasm and which have been named using a different gene nomenclature (*Rsc14Q, Rsc11, Rsc12, Rsc3Q, SC18*-resistance gene, *Rsc-pm* and *Rsc-ps*) [98–105]. Some of these genes may be different or the same allelomorphic series of the *Rsv1* alleles. Here, we add another layer of complexity by introducing the *Rag2* resistance gene against soybean aphids, which happens to reside in the same NBS-LRR rich region and was mapped very close to *Rsv1* [46]. By introducing the *Rag2-Rsv1-h* digenic system in this study, we are also introducing a very interesting biological research model in the form of a plant tritrophic interaction with a viral pathogen (SMV) and one of its natural vectors, the soybean aphid, and the soybean host plant with *R*-genes against both pests. Given the complexity of the *Rag2 Rsv1* locus, perhaps it should not come as a surprise these genes have not been cloned to date eluding scientists for decades.

Soybean-Aphid and the *Rag* Genes

Of the at least six independent *Rag* genetic loci (*Rag1*, *rag1b*, *rag1c*, *Rag2*, *Rag3*, *rag3*, *rag3b*, *Rag3c, rag4*, *Rag4, Rag5*, *Rag6*, and *RagFMD*), it is worth noting that *Rag2*, *Rag5* and *RagFMD* map to a nearby region on chromosome 13, although they have been shown to be independent genes [31–44]. Phenotype expression of plant resistance to aphids is diverse and highly dependent on aphid genotype and plant host species [26]. The main mechanisms of aphid resistance have been classified into antixenosis or non-preference via morphological or chemical barriers in the form of toxins, repellents or antinutritional secondary metabolites; antibiosis affecting aphid's biology and reproduction and which can range from mild to lethal; and tolerance as the host-plant's ability to outgrow and recover from damage caused by arthropod infestation levels similar to those observed in susceptible plants [9, 24–30]. An example of morphological barriers that can deter pest damage was shown via lower aphid-delivered SMV-infection incidence in susceptible soybean plants in backgrounds with high pubescence density compared to glabrous cultivars [106]. The role of chemical defenses in soybean plant resistance has been shown to involve high-levels of lignin in the leaves of host plants, while (more aphid-attractive) high-levels of leaf nitrogen content were found in susceptible individuals [107, 108]. The phenolic precursors of lignin and the free-radical molecules produced during lignin polymerization are known to have an effect on pathogen membrane composition, but they could also act as inactivators of pathogen elicitors, toxins and enzymes, thus hindering biotrophic parasitism on host plants [71, 109]. Therefore, evidence suggests that the same chemical defense mechanism can act as a deterrent of both, arthropods and microbes. Recently, Chapman et al. [30] showed that in a soybean aphid-tolerant background there is constitutive expression of jasmonic and abscisic acid, compared to susceptible soybean genotypes. They also pointed out that aphid-induced transcriptional responses in tolerant genetic backgrounds appear to occur at a slower rate compared to changes in *R*-gene resistant genotypes. A rapid induction of defense-related genes and transcription factors involved in phytohormone synthesis was observed in a *Rag1* genetic background upon aphid priming [110]. Likely, susceptible genotypes exhibit an even slower transcriptional response rate to aphid infestation than tolerant backgrounds. Thus, *Rag2 R*-mediated defense mechanisms against soybean aphid may involve a more rapid response to aphid priming in addition to effector-induced (not constitutive) activation of phytohormone mechanisms involving the well-known salicylic acid and jasmonic acid hormones common in herbivory-induced interactions with obligate parasites [30, 110, 111]. One has to wonder about the energetic cost of the constitutive expression phytohormone signaling pathways from an agronomic point of view. All this complexity in a ditrophic, two-organism type of interaction without considering the presence of a third partner, the viral parasite that seems to have its own evolutionary agenda in this complex tripartite system.

Soybean-Aphid-SMV tritrophic interactions

Soybean represents a valuable resource as a model plant system. Research on plant defense response, pest elicitors and effectors has increased our understanding of

each system separately, but limited research has been focused on the study of tritrophic interactions [112]. The resources developed as part of this study can be used to further our knowledge not only on plant defense response to effectors coming from perhaps both organisms (i.e., the aphid and the virus as it has been hypothesized) that may give rise to effector-triggered immunity, but also on the intricate mechanisms of this unique, cross-kingdom, tritrophic system where the interaction between the pathogen and the insect appears to be almost inevitable since the insect vector is the only biological means of plant-host transmission for the virus, thus, suggesting a pathogen-insect system that has co-evolved together [112]. If this SMV-aphid association has co-evolved together with plant defense mechanisms in the evolutionary arms race where they seek to avoid each other's defenses, is the presence of the *Rag2* and *Rsv1* genes on the same NBS-LRR hot-spot on chromosome 13 a coincidence? The co-occurrence of *Rag* and *Rsv* genes in a region that clusters resistance genes on chromosome 13 may be a unique feature of domesticated soybean. In addition to the *Rsv1* allelomorphic series described above, the cluster includes the *Rsv5*, *Rag2*, *Rag5* and *RagFMD* genes present in different domesticated soybean accessions, but none of these have been identified in its wild relative *G. soja* (*Glycine soja* Siebold & Zucc.), although other *Rag* and *Rsv* genes (*Rag3c* and *Rag6* on chromosomes 16 and 8, respectively, and an *Rsv4*-signature deletion on chromosome 2) have been identified in *G. soja* accessions [43, 68, 113]. In *G. max,* dual-pest *R*-gene colocalization models similar to the *Rag2-Rsv1* association identified in this study, seem to occur in at least two other instances where resistance genes against a virus and its insect vector colocalize to the same genomic region [114–118].

Additional questions remain regarding the mechanisms of host-plant resistance. How do *Rag2-Rsv1-h* double-resistant plants respond to SMV-aphid effectors in the *R*-mediated incompatible tritrophic interaction, versus the molecular mechanisms that take place when insect-mediated sap-feeding injury delivers viral invasion followed by disease in susceptible *rag-rsv* individuals? Are the aphid elicitors derived from salivary proteins [119] or from the chitin polymer that is part of the insect exoskeleton? An additional interesting layer suggests that in some systems it could be possible the *R*-gene-recognized aphid-elicitors may be derived from endosymbiotic bacteria living in the hemocoel of the aphid [120]. Is the interaction between SMV and soybean aphids on double-resistant soybean plants antagonistic as it has been shown to be the case for susceptible backgrounds [121]? Previous research using a tritrophic system composed of soybean, SMV and soybean aphids, suggested that there is a negative effect of SMV-infected plants on aphid performance. The study measured the antagonistic effects of common soybean viruses, SMV (Potyviridae), alfalfa mosaic virus (Bromoviddidae), and bean pod mottle virus (Comoviridae), on aphid population growth using different susceptible soybean varieties [121]. On average, aphid abundance and population growth rates were lower on artificially inoculated virus-infected plants in field and laboratory settings, suggesting that viral infection decreased the attractiveness of host plants or hindered aphid's ability to survive and reproduce. In our experiment, we did observe an overall reduction in aphid counts in the second antibiosis trial. In the first trial, September 2011, aphid antiobiosis and SMV resistance were evaluated in separate greenhouses. In the second trial, January 2012, the joint SMV-aphid trial was carried out in the same greenhouse. First, plants were mechanically inoculated with SMV and a few days later, when plants had reached the V2 stage, they were inoculated with aphids. Overall, mean aphid counts of susceptible controls in the January 2012 trial exhibited less than half the aphid counts of susceptible controls in the September 2011 trial, suggesting an antagonistic effect of virus infection on aphid fitness. Further work is needed in order to confirm the observed trend.

Our understanding of plant-insect-virus systems is limited, but we have gained some knowledge in tritrophic models of this nature so far. A review of virus-dependent modulation of plant host interactions with aphids pointed out early evidence of tripartite studies from the 1930's where it was suggested the interaction between viruses and vectors is complex and that vector and plant-host responses, in many cases, are contingent upon the virus-specific interaction with its vector [122, 123]. The current knowledge suggests that non-persistent viruses (as is the case for SMV), thought to be acquired by their insect vector through fast-probing of plant epidermal cells, followed by quick vector dispersal onto a different healthy plant that is then infected -- as opposed to persistent viruses that are thought to persistently feed on the phloem of infected plants for prolonged periods of time in order to acquire the virus for transmission -- have a negative effect on aphid performance in infected host plants, in a way forcing the aphid to seek further dispersal to continue their infectious cycle onto uninfected plants [121, 124, 125]. More recently, a study found that there are important biochemical changes in host-infected plants that modulate aphid behavior, and thus, induce virus propagation and transmission [126]. The authors observed that cultivated squash (*Cucurbita pepo*) plants infected by the cucumber mosaic virus (also a non-persistent virus, CMV) had a detrimental effect on aphid phagostimulation as the levels of sugars were decreased and those of free amino acids were increased

in non-vascular leaf cells of infected plants compared to healthy hosts. Infected squash plants also exhibited higher baseline levels of salicylic acid, a phytohormone known to be negatively correlated with jasmonic acid, the latter known as a hormone that is upregulated in herbivory-induced defense mechanisms in plants. In *Arabidopsis thaliana*, evidence suggests that upregulation of salicylic acid production in response to phloem-feeding herbivory may be the result of a manipulative strategy used by the aphid and other insects to suppress the upregulation of the jasmonic acid pathway [126, 127]. Elevated levels of ethylene and other signaling volatile compounds have also been found in CMV-infected plants, suggesting a number of physiological alterations that occur in the host plant with the ultimate goal of virus-vector dispersal [126]. Interestingly, this study also found that both, CMV-infected and non-infected plants emit similar volatile blends that attract aphids to the plant host [124, 126]. It is possible that a similar biochemical manipulation is taking place in *rag-rsv* plant host backgrounds of the SMV-soybean-aphid system, but which is halted in *Rag2-Rsv1* individuals. A hypothesis that remains to be elucidated.

Rag2 and *Rsv1* Genes

The recombination event that we observed in double-resistant RILs between 30,297,227 and 30,318,949 bases (as per markers Rag2-1485 and Rsv1-13–1135 in Wm82.a2.v1) showed that our best haplotype, BAAA, is in agreement with the findings of a previous *Rag2* fine-mapping study, using resistance source PI 200538, placing the gene between positions 30,412,581 and 30,466,533 (SNP markers KS9-3 and KS5, respectively), and who proposed NBS-LRR gene *Glyma.13 g190800* (Wm82.a2.v1, also known as *Glyma13 g26000* in Wm82.a1.v1.1) as the strongest *Rag2* candidate based on their findings [46]. Our findings further support the claim that the *Rag2* gene present in PI 243540 is the same as in PI 200538, although further investigation is needed to confirm the exact identity of the gene. Our findings are also in agreement with those of a previous fine-mapping study of the *Rsv1-h* gene from the Suweon 97 source to an interval on chromosome 13 between 29,815,463 and 29,912,369 bases (Figure 3) [66]. In the study, NBS-LRR genes *Glyma.13 g184800* and *Glyma.13 g184900* (Wm82.a2.v1) were proposed as potential candidates of *Rsv1-h* (Figure 3) [66]. A different study has suggested that Suweon 97 also carries the *Rsv3* gene [128]. However, our local Suweon 97 cultivar could be different from that reported elsewhere [128]. The presence of genetic heterogeneity among subcultivar versions of Williams 82, for example, has been demonstrated previously, in which differences among local versions of the cultivar were reported [82]. Thus, it is possible that the study in which *Rsv3* was identified in the Suweon 97 background involved the use of a different "local" version of this cultivar, possibly giving rise to issues of resistance heterogeneity [66, 82, 128]. Furthermore, the NBS-LRR *3 gG2* gene reported as one of three candidate genes for *Rsv1* derived from PI 96983 co-localizes with gene *Glyma.13 g190800* (Wm82.a2.v1) of the reference genome Williams 82, interestingly the same candidate gene proposed for the *Rag2* gene by another study [46, 65]. It has been reported that resistance from PI 96983 involved additional genes (*5 gG3* and *6 gG9*) at the *Rsv1* locus, however our findings suggest that *3 gG2* is not involved in resistance to SMV-G1 from Suweon 97, which is in agreement with other studies (Figure 3) [65, 66]. The *Rsv1-h* fine-mapped interval reported previously does not include the physical positions of *5 gG3* and *6 gG9* genes [66]. Our data also exclude gene *5 gG3*, as the region of the haplotype allele at the position of this gene was inherited from PI 243540 according to maker Rsv1-13–1135 in our double-resistant RIL UX2569-159-2 (Figure 3). In our study, the haplotype inherited from Suweon 97 overlaps the position of the *6 gG9* gene, however, it remains to be demonstrated whether the *6 gG9* gene is involved in *Rsv1-h*-mediated SMV resistance. Overall, these results suggest that the *Rsv1* resistance mechanisms in PI 96983 may be different than those in Suweon 97, despite the vast amount of evidence supporting *Rsv1* and *Rsv1-h* as allelomorphic series. Our results indicate that resistance to soybean aphids and SMV is mediated by two independent genes. Suweon 97, the source of *Rsv1-h,* was found to be susceptible to aphid biotype 1, and PI 243540, the source of *Rag2*, was found to be susceptible to SMV-G1. In addition, the susceptible parents used to develop the double-resistant RILs were confirmed to be susceptible to each pest (Table 3), with one notable exception in U00-429037 which was described in more detail above. Lastly, the best double-resistant RILs were shown to have a recombination event in the *Rag2-Rsv1* region where the double-resistant recombinant haplotype was shown in coupling phase (Table 3, Figure 3).

Conclusions

Subsequent research work is needed in order to answer some of the questions posed here. We are currently working to carry out additional double-resistance experiments to confirm the *R*-mediated phenotypic reactions of RIL UX2569-159 using our tritrophic system. There is ongoing work to obtain more dense SNP marker information to improve the molecular characterization of the different haplotypes of the RILs and parent germplasm evaluated in this study. This work will help confirm the allelic status at different haplotype regions where the resistant loci are thought to reside. This germplasm could facilitate

future gene mapping efforts, and we are in the process of developing a follow-up experiment that will evaluate the agronomic performance of the double-recombinant haplotype in elite, high-yielding backgrounds. This work will allow us to document any challenges that may arise from the inheritance of the double-recombinant haplotype through a backcrossing process. To our knowledge, this is the first report of double-resistant *Rag2-Rsv1-h* soybean RILs using a plant-insect-pathogen tritrophic system during their development in which the insect is the natural vector of the pathogen, thus, raising important evolutionary questions. The recombinant genotypes will be useful in breeding to develop soybean cultivars with resistance to both the vector and the virus. The parental and recombinant genotypes may be helpful in future studies to elucidate interesting evolutionary questions regarding vector, host, and virus tritrophic systems.

Abbreviations

ANOVA	analysis of variance
Avr-gene	avirulence gene
CGH	comparative genomic hybridization
cM	centimorgan
ELISA	enzyme-linked immunosorbent assay
NBS-LRR	nucleotide-binding domain leucine-rich repeat genes
R	resistance
Rag	resistance to *Aphids glycines*
RIL	recombinant inbred line
Rsv	resistance to soybean mosaic virus
SSR	simple sequence repeat
SMV	soybean mosaic virus
SSD	single seed descent

Supplementary Information

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Additional file 1

Additional file 2

Additional file 3

Additional file 4

Additional file 5

Additional file 6

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Authors' contributions
GLG conceived the study. LGP and GLG contributed to population development. LGP, ELLB, LM and SS and JYZ contributed to phenotypic evaluation and data collection. LGP and RMS contributed to genotyping. LGP, KME, TMH and GLG contributed to experimental design and analysis. LGP contributed to the writing of this paper. All authors contributed edits and updates and approved the final manuscript version.

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Data availability
Greenhouse phenotype and genotype data generated in this study are included in this article as supplementary files. The comparative genomic hybridization data for this study can be found as accession number GSE285240 in the National Center for Biotechnology Information Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo). Genetic tools generated in this study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
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Competing interests
The authors declare no competing interests.

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