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ORIGINAL PAPER

Identification and molecular mapping of two soybean aphid resistance genes in soybean PI 587732

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Abstract Soybean [*Glycine max* (L.) Merr.] continues to be plagued by the soybean aphid (*Aphis glycines* Matsumura: SA) in North America. New soybean resistance sources are needed to combat the four identified SA biotypes. The objectives of this study were to determine the inheritance of SA resistance in PI 587732 and to map resistance gene(s). For this study, 323 F_2 and 214 F_3 plants developed from crossing PI 587732 to two susceptible genotypes were challenged with three SA biotypes and evaluated with genetic markers. Choice tests showed that resistance to SA Biotype 1 in the first F_2 population was controlled by a gene in the *Rag1* region on chromosome 7, while resistance to SA Biotype 2 in the second population was controlled by a gene in the *Rag2* region on chromosome 13. When 134 F_3 plants segregating in both the *Rag1* and *Rag2* regions were tested with a 1:1 mixture of SA Biotypes 1 and 2, the *Rag2* region and an interaction between the *Rag1* and *Rag2* regions were significantly associated with the resistance. Based on the results of the non-choice tests, the resistance gene in the *Rag1* region in PI 587732 may be a different allele or gene from *Rag1* from Dowling because the PI 587732 gene showed antibiosis type resistance to SA Biotype 2 while *Rag1* from Dowling did not. The two SA resistance loci and genetic marker information from this study will be useful in increasing diversity of SA resistance sources and marker-assisted selection for soybean breeding programs.

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Introduction

Soybean aphid (*Aphis glycines* Matsumura: SA) was first reported in North America in 2000 (Hartman et al. 2001) on its summer host, soybean [*Glycine max* (L.) Merr.]. Although SA has a short history in North America, it has quickly spread across 22 states in the USA and three provinces of Canada (Venette and Ragsdale 2004) and has caused significant yield losses in most of the upper Midwest states including Wisconsin, Michigan, Minnesota, Illinois, and Iowa (Ostlie 2001; Steffey 2003; Rice et al. 2005). Severe yield loss by SA was also reported in China (Wang et al. 1996). Soybean aphid was able to quickly spread in North America because common buckthorn (*Rhamnus cathartica* L.), its primary overwintering host, was already established in North America (Ragsdale et al. 2004). Dense SA colonies can inhibit growth and development of soybean plants and reduce seed yield by causing severe plant damage including leaf distortion, desiccation, plant stunting, and reduced pod and seed numbers (Li et al. 2004; Sun et al. 1990; Beckendorf et al. 2008). Indirect damage from SAs is caused by the transmission of certain plant viruses including alfalfa mosaic virus, soybean dwarf virus, soybean mosaic virus, and tobacco ringspot virus (Hartman et al. 2001; Iwaki et al. 1980; Sama et al. 1974). In addition, the black sooty mold fungus growing on

honeydew produced by SA feeding can lead to the inhibition of photosynthesis (Hartman et al. 2001).

Host plant resistance is often the most important component of an integrated pest management system for insect control (Auclair 1989: Harrewijn and Minks 1989; Luginbill 1969). Accessions from the USDA Soybean Germplasm Collection have been screened to identify sources of both antibiosis and antixenosis type of host plant resistance to SA. Several SA resistance genes have been identified and genetically mapped onto soybean chromosomes. The SA resistance gene *Rag1* from Dowling, *Rag* from Jackson (Li et al. 2007), and *rag1c* from plant introduction (PI) 567541B (Zhang et al. 2009) were genetically mapped to the same region on soybean chromosome 7. The SA resistance gene *Rag2* from PI 243540 (Mian et al. 2008), PI 200538 (Hill et al. 2009), and *rag4* from PI 567541B (Zhang et al. 2009) were mapped on soybean chromosome 13. On soybean chromosome 16, *Rag3* from PI 567543C and *Rag3b* from PI 567537 were genetically mapped in the same region (Zhang et al. 2010, 2013). A soybean aphid resistance gene in PI 567301B was recently mapped to the same region where *Rag2* from PI 200538 and PI 243540 mapped, but PI 567301B conferred antixenosis type of resistance to SA, which differed from the antibiosis type resistance reported in either PI 200538 or PI 243540 (Jun et al. 2012). Among the mapped SA resistance genes, *Rag1* from Dowling was fine mapped to a 115 kb region (Kim et al. 2010a) and *Rag2* from PI 200538 to a 54 kb region (Kim et al. 2010b) in the reference soybean genome of Williams 82.

The use of host plant resistance to control SA has been complicated by the discovery of multiple SA biotypes in North America. Kim et al. (2008) first reported SA biotype variation in North America and identified that a SA isolate from Illinois (Biotype 1) was different from an isolate from Ohio (Biotype 2) based on their ability to reproduce on resistance sources. Dowling was found to confer resistance to SA Biotype 1 but not to Biotype 2 while the resistance in PI 200538, PI 567541B, and PI 567597C could control both Biotypes 1 and 2 (Kim et al. 2008). Hill et al. (2010) discovered SA Biotype 3 in North America and this biotype overcame the resistance of *rag1c, Rag2, rag3*, and *rag4* while it did not overcome the resistance of *Rag1* from Dowling especially in non-choice tests. Recently, SA Biotype 4 that can overcome the resistance of *Rag1* and *Rag2* was identified in North America (Alt and Ryan-Mahmutagic 2013). The existence of at least four SA biotypes in North America suggests that there is high virulence variability present in North American soybean aphid populations, which would increase the vulnerability of host plant resistance controlled by single genes in soybean (Hill et al. 2012).

Continued screening of soybean germplasm is needed to identify SA resistance alleles at new loci or new alleles at known SA resistance loci. The introduction of new resistance genes or alleles into soybean breeding programs will increase diversity of SA resistance and may delay the ability of SA populations to overcome resistance in soybean. PI 587732 was selected as the SA resistance source for this study because it presented resistance to both SA Biotypes 1 and 2 in our preliminary tests. Therefore, the objectives of this study were to determine the inheritance of resistance and map gene(s) conferring resistance to SA biotypes from PI 587732.

Materials and methods

Plant material

A total of 323 F_2 plants from two crosses and 214 F_3 plants from four different F_2 plants ($F_{2:3}$ lines) were used in this study. The two F_2 populations were first used to determine the inheritance of SA resistance and to map the genetic position of the resistance genes in PI 587732 in choice tests. During the summer of 2007, crosses were made using PI 587732 as a male parent and soybean aphid-susceptible cultivars as female parents at the Illinois Agricultural Experimental Station. The subsequent F_1 plant from each cross was selfed in a greenhouse in 2008 to produce F_2 populations for this study. The 4485-population consisted of 163 random F_2 plants derived from a single F_1 plant from a cross between LD02-4485 and PI 587732. PI 587732 is a maturity group (MG) VI germplasm accession originating from Hubei, China (USDA–ARS Germplasm Resources Information Network, http//:www.ars–grin.gov/npgs/; accessed 18 May, 2012) and LD02-4485 is a MG II cultivar. The 3309-population consisted of 160 random F_2 plants that were derived from a single F_1 plant from a cross between the cultivar LD00-3309 (Diers et al. 2006) and PI 587732. Both LD02-4485 and LD00-3309 are high yielding cultivars developed by the University of Illinois that are susceptible to soybean rust (Kim et al. 2012) and SA (Kim et al. 2010b), but have resistance to soybean cyst nematode (SCN) from PI 88788, which provides resistance to some HG (*Heterodera glycines* Ichinohe) types (Cary and Diers 2011).

Two $F_{2:3}$ lines were used to test the reaction of SA resistance genes to a mixture of SA Biotypes 1 and 2 in choice tests. The lines 116 and 135 were selected from the 4485-population because they were derived from F_2 plants that were heterozygous for both the *Rag1* and *Rag2* regions based on genetic markers. Sixty-two F_3 plants from the line 116 and 72 F_3 plants from the line 135 were used for the choice tests.

Two $F_{2:3}$ lines were used to test resistance genes in the *Rag1* and *Rag2* intervals separately for antibiosis type of resistance in PI 587732 to SA Biotypes 1 and 2 in non-choice tests. Line 89 was selected from the 4485-population because it was derived from an F_2 plant that was

heterozygous for the *Rag1* region but homozygous for the region from the susceptible parent at *Rag2*. Line 147 was selected because it was derived from a plant that was heterozygous at the *Rag2* region but homozygous for the region from the susceptible parent at *Rag1*. Twenty plants from each line, parents, Dowling, PI 200538, an $F_{2:3}$ line (line RR) that is homozygous resistant for both the *Rag1* and *Rag2* regions, and a $F_{2:3}$ line (line SS) that is homozygous susceptible for both the *Rag1* and *Rag2* regions were used as checks in tests with each SA biotype. The RR and SS lines were also selected from the 4485-population based on their genotypes and reactions to SAs.

Soybean aphid culture

Three SA biotypes were used for this study. The SA Biotype 1 was collected at Urbana, IL in 2000 (Hill et al. 2004) and Biotype 2 was collected at the Ohio Agricultural Research and Development Center (OARDC), Wooster, OH in 2005 (Kim et al. 2008). Biotype 3 was collected from the glossy buckthorn (*Frangula alnus*) in Springfield Fen, IN during spring 2007 (Hill et al. 2010). All SA biotypes were maintained in a plant growth chamber at 22 °C and under daily photoperiod of 16 h. Soybean aphid Biotype 1 was maintained on the cultivar Williams 82, Biotype 2 on the soybean breeding line LD05-16611, which has *Rag1* from Dowling, and Biotype 3 on the *Rag2* resistance source PI 200538 (Hill et al. 2009). The SA biotypes were maintained in growth chambers located in different buildings in the Department of Crop Sciences, University of Illinois, to avoid admixture.

Choice tests with soybean aphid Biotypes 1, 2, and mixture of 1 and 2

Four choice tests were conducted in a greenhouse or a growth chamber under environmental conditions described by Hill et al. (2004). All plants in this study were genotyped with genetic markers flanking the *Rag1* and *Rag2* regions to test for associations between resistance reactions and these regions. In all choice tests, the photoperiod was 14 h and the temperature was maintained at 22–25 °C.

The first and second choice tests with the 4485-population were simultaneously conducted in a greenhouse and growth chamber. In the first test, 83 F_2 plants from the 4485-population were tested with Biotype 1 in the greenhouse along with six replications of the check cultivars Williams 82 and Dowling, and the parents of the population. In the second test, 80 F_2 plants from the 4485-population, four replications of the parents, and the checks were tested with Biotype 1 in the growth chamber. After completion of the test in the growth chamber, the 80 plants were transplanted and grown in the greenhouse to produce $F_{2:3}$ seed for progeny testing following treatment with the systemic insecticide imidacloprid, Marathon II (Olympic Horticultural Products, Mainland, PA). The third and fourth choice tests were conducted in the growth chamber. In the third choice test, 160 F_2 plants from the 3309-population, eight replications of the parents of the population, and the checks PI 200538 and Dowling were tested for resistance to SA Biotype 2. In the fourth choice test, 16 replications of Dowling, PI 200538, the parents, and 134 F_3 plants from lines 116 and 135 from the 4485-population were tested for resistance to a mixture of SA Biotypes 1 and 2.

Individual F_2 and F_3 plants were grown in 60 by 60 by 60 mm plastic 48-pot inserts (Hummert Intl., Earth City, MO) contained inside plastic trays without holes as described by Kim et al. (2008). Each 48-pot insert included 40 F_2 or F_3 plants from the populations and two replications of the parents and checks. The 48 plants in an insert were arranged by a completely randomized design (CRD). At the VE growth stage (Fehr et al. 1971), leaves of Williams 82 infested with Biotype 1 or LD05-16611 infested with Biotype 2 were placed on top of soil between the plants. For the choice tests with the mixture of Biotypes 1 and 2, approximately equal amount of the each biotype was inoculated as described above. Resistance to SA in the choice test was visually rated for the level of SA colonization at 15 and 21 days after SA infestation using a scale of 1–5 where 1 = 0–10 SAs; 2 = few solitary live SAs on young leaves; 3 = several transient SAs without dense colonies on young leaves and stems; 4 = dense SA colonies on all leaves and stems; and 5 = dense SA colonies on the whole plant with plant damage including leaf distortion and stunting (Kim et al. 2008). In qualitative analyses, plants with phenotypic ratings of 1 or 2 were considered resistant and plants with ratings of 3, 4 or 5 were considered susceptible.

Non-choice tests with soybean aphid Biotypes 1 and 2

To test antibiosis type of resistance of the two SA resistance genes in PI 587732 to SA Biotypes 1 and 2, two non-choice tests were simultaneously conducted on lines 147 and 89 (Table 1). In the first test, 64 plants including 20 plants from each $F_{2:3}$ line and four replications of the parents (PI 587732 and LD02-4485) and the checks (PI 200538, Dowling, line RR, and line SS) were inoculated with SA Biotype 1. In the second test, plants from the same lines, parents and checks were tested using SA Biotype 2 as inoculum. Each plant was grown in an 11 cm diameter pot and arranged in a CRD in the greenhouse with the same conditions as the choice test. At the V1 growth stage (Fehr et al. 1971), three-third instar nymphs were placed on the upper side of a unifoliolate leaf of each plant with a moist camel's hair brush (Kim et al. 2008). After infestation, the plants were isolated with screened cylindrical cages

Table 1 Association between soybean aphid (SA) resistance (antibiosis) and segregating markers at *Rag1* or *Rag2* regions in non-choice tests

Line	Genotypes		Marker used in *F* test	Aphid number[a] and standard deviation			$P > F$[b]	R^2[c]
	Rag1 region on chromosome 7	*Rag2* region on chromosome 13		R	H	S		
SA Biotype 1 test								
Line 89	Segregating	Homozygous susceptible	SNP28455[d]	25 ± 9	59 ± 14	169 ± 83	0.0002	0.63
Line 147	Homozygous susceptible	Segregating	SNP #20[e]	1 ± 1	1 ± 1	180 ± 86	<0.0001	0.82
SA Biotype 2 test								
Line 89	Segregating	Homozygous susceptible	SNP28455	213 ± 35	396 ± 60	726 ± 114	<0.0001	0.85
Line 147	Homozygous susceptible	Segregating	SNP #20	0 ± 1	1 ± 2	607 ± 245	<0.0001	0.84

[a] Mean number of aphids on each plant and standard deviations for plants predicted to be homozygous resistant (R), heterozygous (H), and homozygous susceptible (S) for *Rag1* or *Rag2* regions based on the segregation of the listed marker

[b] Significance level of the marker association

[c] R^2 value of the marker association

[d] SNP28455 is the SNP marker flanking the *Rag1* region (Kim et al. 2010a)

[e] SNP #20 is the SNP marker flanking the *Rag2* region (Kim et al. 2010b)

to restrict aphid movement among pots. The cages were 100 by 300 mm clear plastic cylinders with 80 by 180 mm side windows and tops covered with a plastic mesh with 100 μm opening (Hill et al. 2004). Fifteen days after infestation, the number of SAs on each plant was counted to test the antibiosis type of resistance to each SA biotype.

Non-choice test with soybean aphid Biotype 3

To test for resistance to SA Biotype 3 in PI 587732, a non-choice test was conducted in a growth chamber with environmental conditions as described above. In this test, Dowling (*Rag1*), PI 200538 (*Rag2*), PI 567541B (*rag1c* and *rag4*), PI 587732, and PI 437696, which has strong resistance to the biotype 3 (Hill et al. 2010), were evaluated. Five plants of each soybean genotype were grown in an 11 cm diameter pot and the pots were arranged in a CRD. Three nymphs of the Biotype 3 were placed on the top of a unifoliolate leaf of each plant at the V1 growth stage (Fehr et al. 1971). After infestation, each pot was isolated as described above. Fifteen days after infestation, the resistance was evaluated by counting the number of SAs on each plant.

DNA extraction and genetic marker analysis

Small trifoliolate leaves from each plant were used to extract DNA. Genomic DNA was extracted by the CTAB (hexadecylatri methylammonium bromide) method described by Saghai Maroof et al. (1984) with slight modifications in speed and time of centrifugation. ND-1000 Spectrophotometer (NanoDrop Technologies, USA) was used to quantify DNA concentration and all genomic DNA samples were diluted to 30 ng ul^{-1} for simple sequence repeat (SSR) and single nucleotide polymorphism (SNP) marker genotyping.

To identify genetic region(s) associated with SA resistance in PI 587732, genetic markers flanking the known SA resistance gene were first screened to find polymorphisms between the parents. From the screening, SNP markers SNP28455 for *Rag1* (Kim et al. 2010a), #20 for *Rag2* (Kim et al. 2010b), SNP16-10 for *Rag3* (personal communication with Dr. Dechun Wang in Michigan State University), and Satt269 for *rag4* (Zhang et al. 2009) were selected. These markers were used to genotype all plants from the segregating populations in the four choice tests. Plants in non-choice tests were genotyped with genetic markers flanking the *Rag1* and *Rag2* regions to test for an association between resistance phenotypes and genetic marker genotypes.

To increase the map resolution of the resistance gene in the 4485-population, 20 SSR and four SNP markers located near the *Rag1* locus on chromosome 7 were screened for parental polymorphism, and five markers were polymorphic. All F_2 plants in the population were then tested with these five markers and resistance was mapped (Table 2; Fig. 1). To improve the map resolution of the resistance gene in the 3309-population, 30 additional SSR and two SNP markers located near the *Rag2* locus on chromosome 13 were screened for polymorphism between the parents. Seven markers were polymorphic and used to test the entire population (Table 3; Fig. 1).

Polymerase chain reaction (PCR) for SSR markers and electrophoresis of PCR products were conducted as described by Wang et al. (2003). PCR consisted of 36 cycles of denaturation at 94 °C for 25 s, annealing at 47 − 58 °C for 25 s, and extension at 68 °C for 25 s with a PTC 100 Programmable Thermal Controller (MJ Research Inc.,

Table 2 Chi-square analysis of the segregation of soybean aphid (SA) resistance and the linked genetic markers in 163 F_2 plants from the 4485-population tested for resistance to soybean aphid Biotype 1

Locus	Number of F_2 plants for each genotype			$\chi^2_{3:1}$	*P*
	R[a]	H	S		
SA resistance[b]	121	–	42	0.05	0.82
Satt540	42	80	41	0.01	0.96
Satt435	46	75	42	0.05	0.82
SNP65906.2	46	75	42	0.05	0.82
SNP28455	44	75	44	0.13	0.72
BARC0320	45	76	42	0.05	0.82
BARC0342	41	81	41	0.002	0.96

[a] Co-dominant SSR or SNP markers were scored as 'R' = homozygous for the allele of the resistant parent (PI 587732), 'H' = heterozygous, and 'S' = homozygous for the allele of the susceptible parent (LD02-4485)

[b] Segregation of SA resistance in the F_2 population when it was tested with SA Biotype 1. Plants were scored as resistant (phenotype rating 1 and 2) or susceptible (phenotype rating 3, 4, and 5)

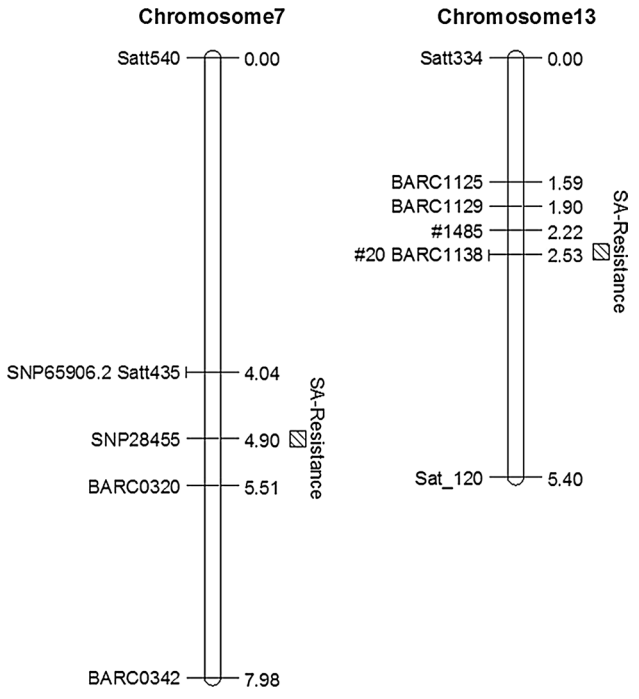

Fig. 1 Genetic maps showing the location of soybean aphid (SA) resistance gene on chromosome 7 in the PI 587732 × LD02-4485 population and on chromosome 13 in the PI 587732 × LD00-3309 population. *Shaded boxes* represent the estimated position of the genes based on the results of interval mapping

Watertown, MA, USA). The PCR products were analyzed by electrophoresis in 3 % agarose gels (BMA, Rockland, ME, USA) or 6 % nondenaturing polyacrylamide gels based on the polymorphism sizes. TaqMan assay was used for SNP marker genotyping and genotyping with the Roche LightCycler® 480 System (Roche Diagnostics, Indianapolis, IN, USA) was conducted as described by Kim et al. (2010b).

Table 3 Chi-square analysis of the segregation of soybean aphid (SA) resistance and the linked genetic markers in 160 F_2 plants from the 3309-population tested with soybean aphid Biotype 2

Locus	Number of F_2 plants for each genotype			$\chi^2_{3:1}$	*P*
	R[a]	H	S		
SA resistance[b]	124	–	36	0.53	0.47
Satt334	48	69	43	0.3	0.58
BARC1125	48	71	41	0.03	0.86
BARC1129	47	72	41	0.03	0.86
SNP #1485	47	71	42	0.13	0.72
BARC1138	48	70	42	0.13	0.72
SNP #20	48	70	42	0.13	0.72
Sat_120	46	69	45	0.83	0.36

[a] Co-dominant SSR or SNP markers were scored as 'R' = homozygous for the allele of the resistant parent (PI 587732), 'H' = heterozygous, and 'S' = homozygous for the allele of the susceptible parent (LD00-3309)

[b] Segregation of SA resistance in the F_2 population when it was tested with SA Biotype 2. Plants were scored as resistant (phenotype rating 1 and 2) or susceptible (phenotype rating 3, 4, and 5)

Statistical analysis and genetic mapping

The Chi-square tests were performed to test the goodness-of-fit for the observed segregation of qualitative resistance ratings and molecular marker genotypes in each F_2 population. Phenotypic and genotypic data were analyzed using the PROC GLM function in SAS 9.3 to identify associations between SA resistance and the genetic makers.

Linkage mapping was completed using JoinMap 3.0 with the Kosambi map function and LOD score of 5.0 (Van Ooijen and Voorrips 2001). Genomic region(s) associated with SA resistance were then mapped as quantitative trait loci (QTL) using the interval mapping (IM) functions in MapQTL® 4.0 (Van Ooijen et al. 2002) with the 1–5 SA resistance scores. The LOD score threshold for declaring a putative locus significant was determined by 1,000 permutations and the gene position was defined as the point of maximum LOD score.

Results

Inheritance of soybean aphid resistance in PI 587732

In the choice tests with the two F_2 populations, PI 587732 displayed resistance to both SA Biotypes 1 and 2 with

Table 4 Chi-square analysis of the segregation of soybean aphid (SA) resistance and association between SA resistance and segregating markers at the *Rag1* or *Rag2* regions among 134 F_3 plants tested with mixture of soybean aphid Biotypes 1 and 2

Locus	Number of F_3 plants with each genotype[a]			$\chi^2_{3:1}$	P	Phenotypic rating[b]			$P > F$[c]	R^{2d}
	R	H	S			R	H	S		
SA resistance[e]	98	–	36	0.25	0.62	–	–	–	–	–
SNP28455[f]	9	89	36	0.25	0.62	2.22	2.03	2.36	0.41	0.01
BARC0320[g]	28	73	33	0.01	0.92	2.07	2.05	2.36	0.48	0.02
SNP #20[h]	31	73	30	0.49	0.49	1.13	1.75	4.10	<0.0001	0.75

[a] Co-dominant SSR or SNP markers were scored as 'R' = homozygous for the allele of the resistant parent (PI 587732), 'H' = heterozygous, and 'S' = homozygous for the allele of the susceptible parent (LD02-4485)

[b] Mean phenotypic rating of plants genotyped as 'R' = homozygous for the allele of the resistant parent (PI 587732), 'H' = heterozygous, and 'S' = homozygous for the allele of the susceptible parent (LD02-4485) based on the segregation of the marker listed in the first column

[c] Significance level of the marker association

[d] R^2 value of the marker association

[e] Segregation of the SA resistance in the F_3 population when it was tested with mixture of SA Biotypes 1 and 2. Plants were scored as resistant (phenotype rating 1 and 2) or susceptible (phenotype rating 3, 4, and 5)

[f] SNP28455 is a SNP marker in the *Rag1* region (Kim et al. 2010a)

[g] BARC0320 is a SSR marker in the *Rag1* region based on results from this study (Fig. 1)

[h] SNP #20 a SNP marker in the *Rag2* region (Kim et al. 2010b)

rating of 1 or 2 while the susceptible parents (LD02-4485 or LD00-3309) and the susceptible checks (Williams 82 for the Biotype 1 test or Dowling for the Biotype 2 test) had ratings of 4 or 5.

The number of resistant and susceptible plants in the 4485-population tested with Biotype 1 was 121 and 42, respectively, and this fits the 3:1 segregation ratio for a single dominant gene ($\chi^2 = 0.05$, $P = 0.82$) (Table 2). In the 3309-population tested with Biotype 2, the number of resistant and susceptible plants was 124 and 36, respectively, which also fits a 3:1 ratio ($\chi^2 = 0.53$, $P = 0.47$) (Table 3). These results indicated that a single dominant gene controlled SA resistance to both SA Biotypes 1 and 2 in PI 587732. In the fourth choice test, 134 F_3 plants segregating for *Rag1* and *Rag2* from the 4485-population were treated with a mixture of SA Biotypes 1 and 2, and the number of resistant and susceptible plants was 98 and 36, respectively (Table 4). This segregation pattern also fits a 3:1 ratio ($\chi^2 = 0.25$, $P = 0.62$), supporting the results of the F_2 analysis that indicated the resistance in PI 587732 was controlled by a single gene.

Genetic mapping of the soybean aphid resistance genes in PI 587732

The F_2 populations were initially screened with a single marker near each of the four resistance genes. The SNP marker 28455 (SNP28455), which is closely linked to *Rag1* on chromosome 7, was found to be significantly associated with the resistance to Biotype 1 ($P < 0.0001$, $R^2 = 0.79$) in tests 1 and 2 with the 4485-population while markers linked to *Rag2* ($P = 0.20$, $R^2 = 0.02$), *Rag3* ($P = 0.69$, $R^2 = 0.004$), and *rag4* ($P = 0.72$, $R^2 = 0.004$) were not. In the 3309-population, which was tested with SA Biotype 2, only the SNP marker #20, which is linked to *Rag2* on chromosome 13, was significantly associated with the resistance ($P < 0.0001$, $R^2 = 0.82$). Therefore, the *Rag1* region on chromosome 7 and *Rag2* region on chromosome 13 were selected for future genetic mapping of SA resistance in PI 587732.

The 4485-population was tested with additional markers on chromosome 7 resulting in the mapping of a single dominant SA resistance gene into a 1.5 cM interval between Satt435 and BARCSOYSSR_07_0320 (BARC0320) with SNP28455 having the highest LOD score (57.6) and R^2 value (80.4). The genetic positions of Satt435, SNP28455, and BARC0320 were 4.0, 4.9, and 5.5 cM from the Satt540 anchor, respectively (Fig. 1). The testing of the 3309-population with additional markers placed the resistance gene on chromosome 13 between the SNP markers #1485 and #20 with the SSR marker BARCSOYSSR_13_1138 (BARC1138) having the greatest LOD score (74.1) and R^2 value (88.2).

When the 134 plants from the two $F_{2:3}$ lines (selected from the 4485-population), which segregated at both the *Rag1* and *Rag2* regions, were evaluated with a mixture of SA biotypes 1 and 2, the *Rag2* region ($P < 0.0001$) and an interaction between *Rag1* and *Rag2* region ($P = 0.02$) were significantly associated with the resistance while the *Rag1* region did not have an individual effect ($P = 0.50$). The

Table 5 Colonization of soybean aphid (SA) Biotypes 1, 2 and 3 in a non-choice test

Soybean genotype	SA resistance locus	Number of SAs		
		Biotype 1	Biotype 2	Biotype 3
Dowling	*Rag1*	0.5a[a]	624a	7a
PI 437696	No information	–[b]	–	4a
PI 200538	*Rag2*	0a	1b	175b
PI 567541B	*rag1c* and *rag4*	–	–	170b
PI 587732	*Rag1* and *Rag2*	0a	2b	146b
Line RR[c]	*Rag1* and *Rag2*	0a	1b	–
Line SS[d]	Susceptible check	303b	675a	–
LD02-4485	Susceptible check	311b	650a	–

[a] Means followed by the same letters in each column are not significantly different by the least significant difference test ($P = 0.05$)

[b] PI was not tested with the biotype

[c] Line RR is a $F_{2:3}$ line selected from the PI 587732 × LD02-4485 population that is homozygous resistant for alleles from PI 587732 at both the *Rag1* and *Rag2* regions

[d] Line SS is a $F_{2:3}$ line selected from the PI 587732 × LD02-4485 population that is homozygous susceptible for alleles from the susceptible parent, LD02-4485, at both the *Rag1* and *Rag2* regions

Rag2 locus explained the majority (70.6 %) of the phenotypic variation while the interaction explained only a small amount of the variation (3.4 %).

Non-choice tests with soybean aphid Biotypes 1 and 2

$F_{2:3}$ lines segregating for one resistance locus but fixed for the susceptible allele for the other locus were evaluated in non-choice tests to determine whether the two SA resistance genes in PI 587732 have antibiosis type of resistance. In a test with Biotype 1 of the $F_{2:3}$ line 89, which was segregating at the *Rag1* region, a significant association between resistance and segregation of SNP28455 was observed (Table 1). Plants homozygous for the PI 587732 allele at SNP28455 averaged 25 SAs while plants homozygous for the susceptible allele averaged 169 SAs per plant. The *Rag2* region was significantly associated with resistance to Biotype 1 in the line 147, which only segregates for the *Rag2* interval. Plants homozygous for the PI 587732 allele in the *Rag2* interval averaged 0.4 aphids per plant while plants homozygous for the susceptible allele averaged 180 aphids.

Unlike *Rag1* from Dowling, the PI 587732 allele in the *Rag1* region presented antibiosis resistance to SA Biotype 2 in tests of line 89 (Table 1). This is shown by the highly significant association ($P < 0.0001$) between SA resistance and the segregation of the SNP28455 in the line. The segregation of the marker in the *Rag1* region described 85 % of the phenotypic variation in the line (Table 1). The *Rag2* interval showed stronger resistance to Biotype 2 than *Rag1* with plants homozygous for the PI 587732 allele for this region having no SA while homozygous susceptible plants averaged 607 SAs per plant.

Non-choice test with soybean aphid Biotype 3

Fifteen days after infestation, the number of SAs on each plant was counted to test whether PI 587732 had resistance to SA Biotype 3. PI 437696 and Dowling (*Rag1*) showed a strong antibiosis to the Biotype 3 while the Biotype 3 overcame the resistance in PI 567541B (*rag1c* and *rag4*), PI 200538 (*Rag2*), and PI 587732 (Table 5). This result from the checks was consistent with the previous report on the ability of SA Biotype 3 to colonize soybeans with *rag1c*, *Rag2*, and *rag4* (Hill et al. 2010).

Discussion

PI 587732 was selected for the present study because it conferred resistance to both SA Biotypes 1 and 2 in our germplasm screen for SA resistance. Phenotypic segregation ratios of resistance in the two F_2 populations fit single dominant gene inheritance and only the *Rag1* locus was significantly associated with resistance to SA Biotype 1 in the 4485-population test. To evaluate whether the *Rag1* region in PI 587732 was also responsible for resistance to Biotype 2, the second F_2 population (3309-population) was tested with Biotype 2 and genetic markers. Unlike the result from the 4485-population test, only the *Rag2* region was responsible for providing resistance to Biotype 2.

The relative genetic positions of the markers used to map the SA resistance genes in PI 587732 were consistent with their genetic and physical locations on the Soybean-Consensus map 4.0 (Hyten et al. 2010) (Fig. 1). The resistance gene on chromosome 7 mapped to an interval consistent with the position that Kim et al. (2010a) fine mapped *Rag1* from Dowling. In addition, the resistance gene on chromosome 13 was mapped to the same interval that *Rag2* from PI 200538 was fine mapped by Kim et al. (2010b).

Although the resistance gene on chromosome 7 mapped to the *Rag1* region, this study provided evidence that compared to *Rag1* from Dowling, the gene from PI 587732 is either a different allele at *Rag1* or a closely linked, but different locus. There are two lines of evidence to support this. The first is that the gene controlled different reactions to SA Biotype 2 compared to the *Rag1* from Dowling. In non-choice tests with SA Biotype 2, the gene in the *Rag1* interval from PI 587732 conferred antibiosis to SA Biotype 2 while Dowling was completely susceptible to this biotype (Tables 1 and 5). The susceptibility of Dowling to Biotype 2 also is consistent with Kim et al. (2008)

and Hill et al. (2010). The second line of evidence is that in the Biotype 3 test, PI 587732 was highly susceptible while Dowling exhibited strong resistance (Table 5). It is likely that these differences in reaction are the results of these resistance sources having different alleles or genes in the *Rag1* region. However, it is possible that Dowling has another unmapped gene(s) that gives the resistance to Biotype 3 because genetic mapping of *Rag1* from Dowling was conducted using only Biotype 1 (Li et al. 2007). Additional proof of the presence of a new gene or allele would come from allelism tests in populations developed from crossing PI 587732 and germplasm carrying *Rag1* from Dowling.

Although some results of our test with *Rag2* were unexpected, there is less convincing data to support a new gene or allele at *Rag2* from PI 587732 than the evidence for a new gene or allele at *Rag1*. *Rag2* from PI 200538 confers resistance to both Biotypes 1 and 2 (Li et al. 2004; Kim et al. 2008), so we were surprised to find that the gene from PI 587732 linked to *Rag2* was not detected in the 4485-population when plants were inoculated with SA Biotype 1. Both *Rag1* and *Rag2* were segregating in the 4485-population and it was expected that both genes would be detected after infestation with Biotype 1 resulting in a ratio of resistant to susceptible plants of 15:1, however, only the *Rag1* interval was detected. In contrast, *Rag2* from PI 587732 was detected after infestation with Biotype 1 in the F_3 line 147, which segregated only for the gene in the *Rag2* interval from PI 587732. This shows that the *Rag2* interval in PI 587732 confers resistance to Biotype 1 when *Rag1* is not present. Unfortunately, we do not have results to compare whether known alleles at *Rag1* and *Rag2* are each expected to be detected when they are both segregating in populations. Future work is needed to determine whether *Rag2* from PI 200538 and the resistance gene in *Rag2* region in PI 587732 are the same or different gene or allele.

It is unclear why in populations segregating for both *Rag1* and *Rag2* intervals only the *Rag1* interval was significant when plants were infested with Biotype 1 and when plants were infested with Biotype 2, only the *Rag2* interval was significant. This is in contrast to each gene being significant for each biotype when the genes are tested separately. This suggests that in the two gene combinations, an interaction between genes occurs resulting in the expression of resistance from only one gene and the repression of the second gene.

Another unexpected result was from the test of the two $F_{2:3}$ lines segregating for *Rag1* and *Rag2* with a mixture of SA Biotypes 1 and 2. Only the *Rag2* region and an interaction between the *Rag1* and *Rag2* regions were significantly associated with resistance to this mixture, although the resistance was mainly controlled by *Rag2* region. It was expected that both the *Rag1* and *Rag2* intervals would be detected after infestation with the mixture because when populations segregating for both intervals were inoculated with Biotype 1, the *Rag1* interval was detected and after infestation with Biotype 2, *Rag2* interval was detected. One explanation for this result is that Biotype 2 out competed Biotype 1 in the mixture, and because the *Rag2* interval provides stronger resistance to Biotype 2 than to *Rag1*, this resulted in mostly *Rag2* being detected. Although we attempted to inoculate plants with an equal number of Biotype 1 and 2 aphids, there was no way to monitor reproduction of each biotype in the mixture. Another explanation could be that Biotype 2 confers stronger virulence than Biotype 1, resulting in a greater effect of *Rag2* after inoculation with the two biotypes.

Artificial infestation tests using SAs with known virulence patterns are needed in soybean germplasm screening to identify sources of SA resistance and to map resistance gene(s). However, to maintain different SA biotypes is laborious and difficult because of the need for a continuous supply of susceptible soybean plants and the difficulty in keeping biotypes separate while they are being maintained. In the present study, if we used either SA Biotype 1 or 2 during the germplasm selection and genetic mapping, we would have not identified whether PI 587732 had the two resistance genes and different expression patterns based on the SA biotypes. The results of this study suggest that at least two different SA biotypes may be necessary to map the full range of resistance genes from aphid resistance sources. The information on the SA resistance gene at the *Rag1* region on chromosome 7 and the flanking genetic makers in this study will be useful in improving genetic diversity of SA resistance by MAS in soybean breeding program.

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