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Characterization and genetics of multiple soybean aphid biotype resistance in five soybean plant introductions

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Abstract

Key message **Five soybean plant introductions expressed antibiosis resistance to multiple soybean aphid biotypes. Two introductions had resistance genes located in the *Rag1, Rag2,* and *Rag3* regions; one introduction had resistance genes located in the *Rag1, Rag2,* and *rag4* regions; one introduction had resistance genes located in the *Rag1* and *Rag2* regions; and one introduction had a resistance gene located in the *Rag2* region.**

Abstract Soybean aphid (*Aphis glycines* Matsumura) is the most important soybean [*Glycine max* (L.) Merr.] insect pest in the USA. The objectives of this study were to characterize the resistance expressed in five plant introductions (PIs) to four soybean aphid biotypes, determine the mode of resistance inheritance, and identify markers associated with genes controlling resistance in these accessions. Five soybean PIs, from an initial set of 3000 PIs, were tested for resistance against soybean aphid biotypes 1, 2, 3, and 4 in choice and no-choice tests. Of these five PIs, PI 587663, PI 587677, and PI 587685 expressed antibiosis against all four biotypes, while PI 587972 and PI 594592 expressed

antibiosis against biotypes 1, 2, and 3. F_2 populations derived from PI 587663 and PI 587972 were evaluated for resistance against soybean aphid biotype 1, and populations derived from PIs 587677, 587685, and 594592 were tested against biotype 3. In addition, $F_{2:3}$ plants were tested against biotypes 2 and 3. Genomic DNA from F_2 plants was screened with markers linked to *Rag1, Rag2, Rag3,* and *rag4* soybean aphid-resistance genes. Results showed that PI 587663 and PI 594592 each had three genes with variable gene action located in the *Rag1, Rag2,* and *Rag3* regions. PI 587677 had three genes with variable gene action located in the *Rag1, Rag2* and *rag4* regions. PI 587685 had one dominant gene located in the *Rag1* region and an additive gene in the *Rag2* region. PI 587972 had one dominant gene located in the *Rag2* region controlling antixenosis- or antibiosis-type resistance to soybean aphid biotypes 1, 2, or 3. PIs 587663, 587677, and 587685 also showed antibiosis-type resistance against biotype 4. Information on multi-biotype aphid resistance and resistance gene markers will be useful for improving soybean aphid resistance in commercial soybean cultivars.

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Abbreviations

PCR   Polymerase chain reaction
SNP   Single nucleotide polymorphisms
SSR   Simple sequence repeat

Introduction

Soybean is the second most important field crop cultivated in the USA, with 36 million ha harvested in 2014, producing 1.09 MMT of grain valued at over $40.3 billion (NASS 2015). The soybean aphid, *Aphis glycines* Matsumura, now established since its introduction from Asia in 2000

(Hartman et al. 2001), has become one of the most economically important insect pests threatening USA soybean production (Kim et al. 2008a; Tilmon et al. 2011), prompting major changes in soybean pest management practices (Fernandez-Cornejo et al. 2014; Hill et al. 2012; Ragsdale et al. 2011). Heavy aphid colonization of soybean plants decreases plant productivity by reducing pod and seed set (Beckendorf et al. 2008), seed oil content (Riedell and Catangui 2006), and photosynthetic efficiency (Macedo et al. 2003). The soybean aphid is capable of transmitting soybean viruses (Hartman et al. 2001; Hill et al. 2001), although no major soybean virus epidemics have been reported to date.

Initially, the application of insecticides was the only commercially effective tool to control the soybean aphid (Ragsdale et al. 2011), but soybean cultivars with resistance have now been deployed in some USA soybean production areas (Hesler et al. 2013; Hill et al. 2012; McCarville et al. 2014). Even before soybean aphid-resistant cultivars became commercially available, at least four soybean aphid biotypes were discovered, three of which could overcome major resistance genes in the USA (Alt and Ryan-Mahmutagic 2013; Hill et al. 2010, 2012; Kim et al. 2008b; Pawlowski et al. 2014). Biotype 1 is avirulent on plants with *Rag1* or *Rag2*. Biotype 2 is virulent on *Rag1* and avirulent on *Rag2*. Biotype 3 is avirulent on *Rag1* and virulent on *Rag2*. Biotype 4 is virulent on both *Rag1* and *Rag2*, including pyramided lines. Biotypes were also subsequently reported from China (Liang et al. 2013; Zhong et al. 2014). The genetics of virulence of soybean aphid biotypes is unknown.

Evaluation of soybean germplasm resources for genetic resistance to the soybean aphid began soon after the pest arrived and continues today in the USA (Bansal et al. 2013; Bhusal et al. 2013, 2014; Fox et al. 2014; Hesler et al. 2012; Hill et al. 2012; Kim et al. 2014) and in China (Liu et al. 2014). Identification of sources of resistance effective against multiple soybean aphid biotypes has been discussed (Bansal et al. 2013; Hill et al. 2012; McCarville et al. 2014), and multi-biotype resistant germplasm accessions expressing resistance against three soybean aphid biotypes have been reported (Bansal et al. 2013).

An evaluation of over 3000 plant introductions (PIs) from the USDA Germplasm Collection, conducted at the University of Illinois, identified 50 PIs with resistance to both soybean aphid biotypes 1 and 2 (Kim et al. 2008b) and the genetics of resistance in 21 of the accessions was determined (Fox et al. 2014; Kim et al. 2014). We subsequently identified five additional PIs that expressed resistance to soybean aphid biotype 3 (Hill et al. 2010). With the discovery of soybean aphid biotype 4 in 2011 (Alt and Ryan-Mahmutagic 2013), the five PIs, 587663, 587677, 587685, 597972, and 594592 with resistance to soybean aphid

biotypes 1, 2, and 3 were evaluated for resistance to biotype 4. The objectives of this study were to (1) characterize the resistance expressed in these five PIs to four soybean aphid biotypes through choice and no-choice testing to determines the type of resistance expression (antibiosis or antixenosis), (2) determine the mode of resistance inheritance, and (3) identify markers associated with resistance regions in these accessions.

Materials and methods

Aphid isolates and culturing

Soybean aphid isolates included soybean aphid biotypes 1, 2, 3 and 4. Biotype 1 was first collected on soybean in Illinois in 2000 (Hill et al. 2004), biotype 2 was found on soybean plants with the *Rag1* resistance gene in Ohio in 2005 (Kim et al. 2008b), biotype 3 was collected from *Frangula alnus* in Indiana in 2007 (Hill et al. 2010), and biotype 4 was identified from soybean in Wisconsin in 2011 (Alt and Ryan-Mahmutagic 2013). Isolates of biotypes 1, 2, and 3 were maintained in our laboratory since their discovery. Researchers from the University of Wisconsin provided biotype 4 (Crossley and Hogg 2015). Methods to rear and maintain the aphids were previously described (Hill et al. 2004, 2010; Kim et al. 2008b). The soybean aphid biotypes 1, 2, 3, and 4 were maintained, respectively, on cultivar Williams 82, soybean breeding lines with *Rag1*, with *Rag2*, and on lines with both *Rag1* and *Rag2*. Aphid isolates were cloned periodically from isolated nymphs and maintained in separate growth chambers for several viviparous, parthenogenetic generations prior to use.

Plant material

Known soybean aphid-resistant and -susceptible soybean genotypes were selected to use as checks and to compare resistance sources in resistance characterization tests (Table 1). Populations were generated from crosses made between the five aphid-resistant soybean PIs and aphid-susceptible soybean breeding lines. The crosses were LD02-5320 × PI 587663, LD03-6566 × PI 587677, LD03-10504 × PI 587685, LD03-10504 × PI 587972, and LD02-5320 × PI 594592, with soybean aphid-resistant PIs as male parents in all crosses. All five PIs originated from China. PIs 587663, 587677, 587685, and 594592 are classified as maturity group (MG) VII and PI 587972 as MG VI. LD02-5320, LD03-6566, and LD03-10504, (soybean breeding lines developed at the University of Illinois) were susceptible to biotypes 1, 2, and 3 in

Table 1 Soybean genotypes used in experiments to characterize the expression and determine the inheritance of resistance in five multi-biotype resistant plant introductions (PI)

Soybean genotype	Description	Aphid resistance	Recent references
PI 587663	Investigated accession	Resistant	This study
PI 587677	Investigated accession	Resistant	This study
PI 587685	Investigated accession	Resistant	This study
PI 587972	Investigated accession	Resistant	This study
PI 594592	Investigated accession	Resistant	This study
LD05-16611	Resistance check	*Rag1*	Chirumamilla et al. (2014)
LD11-4576a	Resistance check	*Rag1*	Pawlowski et al. (2014)
LD08-12422a	Resistance check	*Rag2*	This study
Williams 82	Susceptible check	Susceptible	Chirumamilla et al. (2014)
LD02-5320	Parent	Susceptible	Fox et al. (2014)
LD03-10504	Parent	Susceptible	Fox et al. (2014)
LD03-6566	Parent	Susceptible	Fox et al. (2014)
PI 567597C	Resistance check	Antixenosis	Mensah et al. (2005)
PI 71506	Resistance check	Antixenosis	Fox et al. (2014)
LD11-5431a	Resistance check	*Rag2*	Pawlowski et al. (2014)
PI 567543C	Resistance check	*Rag3*	Zhang et al. (2010)
PI 567598B	Resistance check	*rag1b, rag3*	Bales et al. (2013)
PI 567541B	Resistance check	*rag1c, rag4*	Zhang et al. (2009)
PI 437696	Resistance check	Two resistant genes	Fox et al. (2014)

preliminary tests. They were not tested against biotype 4, which was not known when the crosses were made. The F_1, F_2, and $F_{2:3}$ generations were developed as previously described (Fox et al. 2014; Hill et al. 2009).

Plant culture

Plants used in greenhouse experiments were planted in soil-less potting medium (Sunshine Mix, LC1, Sun Gro Horticulture Inc., Bellevue, WA, USA). For choice tests, plastic multi-pot inserts (Hummert Intl., Earth City, MO, USA) with each pot insert size $30 \times 40 \times 60$ mm were placed inside plastic trays with holes (Hummert Intl.). The size of multi-pot inserts sometimes differed depending on the experimental design and number of test entries. The inserts were filled with the soil-less potting medium and then moistened to field capacity. Two seeds of each entry were placed in a shallow depression (approximately 0.5 cm deep) in the potting medium. Seedlings were thinned to one plant per pot after emergence. For no-choice tests, three seeds of each soybean genotype were planted into 13-cm-diameter plastic pots, filled with the soil-less medium and seedlings were thinned to one plant per pot after emergence. Fifteen ml of slow-release fertilizer pellets (Osmocote 19-6-12, NPK) were spread evenly on top of the soil-less growth medium to an approximate density of 2 to 3 pellets per cm² following planting. Plants were manually irrigated as required to avoid drought stress.

Choice tests of resistance sources

The resistance sources were evaluated in choice tests conducted with each of the four soybean aphid biotypes over time to compare the overall soybean aphid resistance, which includes antixenosis and antibiosis-type resistance. The five PIs were tested along with eight soybean genotypes previously reported to have aphid resistance and the susceptible genotype Williams 82 (Table 1). The 14 genotypes were arranged in a randomized complete block design with four blocks. The soybean breeding lines introgressed with *Rag1* (LD05-16611) and *Rag2* (LD08-12422a) used in the biotype 1, 2, and 3 tests were different from the ones used in the biotype 4 test (LD11-4576a *Rag1* and LD11-5431a *Rag2*) because seed was not available for the older (year after LD in the name) introgressed breeding lines. The experimental unit was one plant of each soybean genotype. There were two trials (repeat) of each biotype choice test and each trial had a different randomization of genotypes. Each test was infested with a single aphid biotype using methods previously described (Hill et al. 2010, 2009; Kim et al. 2008b; Pawlowski et al. 2014). Briefly, the tests were conducted inside an air-conditioned greenhouse set to maintain a constant 22 °C with a mixture of 1000 w high-pressure sodium vapor and metal halide lamps providing supplemental light 16 h day:8 h night. Soybean seedlings were infested with aphids during the V_C to V_1 growth stages (Fehr et al. 1971) by evenly distributing aphid-infested Williams 82 leaves and stems, containing aphids of all viviparous life stages, on top of the seedlings.

Aphid colonization on each plant was evaluated when there was maximum aphid colonization on the susceptible check genotypes about 3 weeks after inoculation. A visual assessment of aphid colonization using a non-parametric, ordinal 1–4 scale of the degree of colonization and plant damage caused by aphid feeding was used (Fox et al. 2014; Hill et al. 2009, 2006a, b; Pawlowski et al. 2014). A rating of 1 = few solitary live aphids, often with dead aphids; 2 = several transient aphids present along with some viviparous aptera surrounded by a few nymphs, but without established colonies; 3 = dense aphid colonies; and 4 = dense colonies accompanied by plant damage such as leaf distortion and stunting. This scale describes four qualitative classes of phenotypes observed on the plants in response to infestation by soybean aphids, and has been consistently found to be appropriate for use in phenotyping qualitatively expressed soybean aphid resistance in our past research. The numbers 1–4 were used to rank the level of aphid colonization and not to quantify the number of aphids or be used for parametric analyses.

No-choice tests of resistance sources

No-choice tests were conducted over time to compare antibiosis-type resistance performance between multiple soybean genotypes. A randomized complete block design with three blocks was used with the five aphid-resistant PIs, eight genotypes reported to be aphid-resistant, and susceptible Williams 82 randomized within each block. The soybean breeding lines with *Rag1* (LD05-16611) and *Rag2* (LD08-12422a) used in the biotype 1, 2, and 3 tests were different from the ones used in the biotype 4 test (LD11-4576a *Rag1* and LD11-5431a *Rag2*) due to insufficient amount of viable seed of the older breeding lines. The experimental unit was a pot with a single soybean genotype inoculated with a single soybean aphid biotype. The plants were inoculated at the V_c to V_1 growth stage with aphids of a single biotype using 10 second or third instar nymphs as previously described (Chirumamilla et al. 2014; Hill et al. 2010; Pawlowski et al. 2014). Immediately following inoculation, each plant was isolated by covering it with a custom-made plastic (Cleartec® Packaging, Park Hills, MO) cylinder, 40 cm tall × 10.5 cm in diameter, closed on the top with a clear plastic cover and with the open bottom end pushed into the soil-less medium. Two rectangular windows (25 cm long × 6 cm wide) were cut out of each cylinder and covered with silk to enable ventilation and prevent aphid escape. The total number of aphids on each plant was counted 14 days after inoculation. The tests were conducted in a plant growth chamber (Conviron PGR15, Manitoba, Canada) illuminated with 500 μmol m^2s^{-1} PAR fluorescent and incandescent lamps programmed for

a 16- and 8-h light–dark cycle and a constant 22 °C. Each no-choice test for each biotype was repeated once and the two trials were conducted at different times.

Choice test evaluation of resistance in F_2 populations and progeny testing

F_2 populations derived from PI 587663 and PI 587972 were tested for resistance to soybean aphid biotype 1, and populations derived from PI 587677, PI 587685, and PI 594592 were tested against biotype 3. The parental breeding lines, 99–279 F_2 plants from each population derived from the five aphid-resistant PI accessions (Table 2), and aphid-resistant and susceptible checks, were tested in the greenhouse using choice testing methods previously reported (Fox et al. 2014; Hill et al. 2006a, b, 2009). Each population was tested separately over time in a greenhouse with supplemental lighting set to provide a 13-h photoperiod and induce early blooming for subsequent F_3 seed production. The greenhouse was air-conditioned and the temperature was set to 24 °C day and

Table 2 Modes of an ordinal 1–4 aphid colonization rating scale used in a choice test consisting of 14 soybean genotypes infested with soybean aphid biotypes 1, 2, 3, and 4

Soybean genotype	Aphid resistance	Biotype			
		1	2	3	4
Rag1 line[a]	*Rag1*	2[c]	3	3	3
Rag2 line[b]	*Rag2*	2	2	3	3
PI 437696	Antibiosis	2	2	2	2
PI 567541B	*rag1c, rag4*	2	2	2	3
PI 567543C	*Rag3*	2	2	2	3
PI 567597C	Antixenosis	2	3	4	3
PI 567598B	*rag1b, rag3*	2	2	3	3
PI 587663	Antibiosis	2	2	2	3
PI 587677	Antibiosis	2	2	2	3
PI 587685	Antibiosis	2	2	2	3
PI 587972	Antibiosis	2	2	2	3
PI 594592	Antibiosis	2	2	3	3
PI 71506	Antixenosis	2	2	2	3
Williams 82	Susceptible	3	3	3	4

[a]*Rag1*-introgressed line: LD05-16611 in biotype 1, 2, and 3 tests, and LD11-4576a in biotype 4 test

[b]*Rag2*-introgressed line: LD08-12422a in biotype 1, 2, and 3 tests, and LD11-5431a in biotype 4 test

[c]Visual assessment of aphid colonization using a non-parametric, ordinal 1–4 scale of the degree of colonization and plant damage caused by aphid feeding, with 1 = few solitary live aphids, often with dead aphids; 2 = several transient aphids present along with some viviparous aptera surrounded by a few nymphs, but without established colonies; 3 = dense aphid colonies; and 4 = dense colonies accompanied by plant damage such as leaf distortion and stunting

18 °C night. The experimental design used to infest and phenotype the aphid resistance in the F_2 populations was previously reported (Fox et al. 2014; Hill et al. 2006a, b, 2009). Four-pot rows of each F_2 population, parental breeding lines, and resistant and susceptible checks were randomly planted in plastic multi-pot inserts containing 48 pots (Hummert Intl., Earth City, MO, USA). Each test included 25% aphid-susceptible genotypes to create aphid refuges and maintain high aphid pressure after the plants were infested. Infestation and evaluation of aphid colonization were conducted as described above for resistance characterization. The experimental unit was an individual plant of each soybean genotype or F_2 plant.

F_2 progeny testing was conducted using choice tests to determine the soybean aphid resistance genotypes of each F_2 plant. After completion of F_2 tests, plants were grown to maturity to produce $F_{2:3}$ seed for progeny testing. $F_{2:3}$ lines that had at least 12 seeds, regardless of F_2 aphid resistance phenotype, were used in progeny tests. The tests of $F_{2:3}$ progenies were conducted separately for each population at different times. Twelve to 16 F_3 seeds were planted in multi-pot inserts in flats with drainage holes as described above. The progeny tests were set up as choice tests as previously described (Hill et al. 2006a, b, 2009). Biotype 3 was initially used in all F_2 progeny tests. $F_{2:3}$ plants, parents, and resistant and susceptible checks were planted and randomized in four-pot rows as described above, with the experimental unit each individual plant. Following the biotype 3 test, four seeds from each $F_{2:3}$ family (of those families that still had four seeds remaining) were screened for resistance against soybean aphid biotype 2 using the same experimental design. Infestation and evaluation of aphid colonization using the 1–4 nonparametric ordinal scale, were as previously described.

Analysis of F_2 plant and $F_{2:3}$ family segregation

A rating of 1 or 2 was considered as a resistant response and a rating of 3 or 4 was considered susceptible (Fox et al. 2014; Hill et al. 2006a, b, 2009). For analysis of segregation in F_2 populations, phenotypic ratios of resistant:susceptible plants were used to test Mendelian genetic models to determine the number of major genes controlling resistance in each PI resistance source. For analysis of the segregation of $F_{2:3}$ families for resistance against biotypes 2 and 3, only families with at least 11 plants were used. Prior to Chi-square analyses, families were classified as all resistant if no susceptible plants were found, segregating if there were both resistant and susceptible plants, and all susceptible if no resistant plants were observed.

Genotyping and marker associations

Methods used to genotype F_2 plants in the populations were previously described (Fox et al. 2014). Briefly, genomic DNA was extracted from young trifoliolate leaves of all F_2 plants and the parents in each population using the CTAB extraction procedure (Keim and Shoemaker 1988). Single nucleotide polymorphisms (SNPs) and simple sequence repeat (SSR) genetic markers that map near the *Rag1, Rag2, Rag3*, and *rag4* regions were used to identify markers that were polymorphic between the susceptible and resistant parents of the populations. One or two polymorphic markers mapping within each *Rag* region were selected (Fox et al. 2014) to genotype each F_2 plant in each of the five populations. Polymerase chain reactions (PCR) for the SSR markers were performed following a previously reported protocol (Cregan and Quigley 1997) and the PCR products were analyzed using non-denatured polyacrylamide gel electrophoresis, using a previously reported protocol (Wang et al. 2003). SNP marker analyses were performed using TaqMan assays conducted with a Roche LightCycler 480 System (Roche Diagnostics, Indianapolis, IN) (Kaczorowski et al. 2008). Additionally, bulked segregant analysis (BSA) (Michelmore et al. 1991) was performed on DNA samples collected from the PI 587663, PI 587685, and PI 587972 parents and their F_2-derived populations using the Illumina GoldenGate 1,536 Universal Soy Linkage Panel 1.0 (Hyten et al. 2010, 2008) after the initial marker screening. For each population, DNA from 10 F_2 plants with resistant phenotypes and 10 plants with susceptible phenotypes were selected and bulked into two separate samples (Fox et al. 2014). The SNP genotypes of the resistant and susceptible bulks were compared with the SNP genotypes of the population parents to determine probable genetic regions associated with soybean aphid resistance. Marker genotypes present in $F_{2:3}$ families were inferred from the parental F_2 plant genotypes.

Not all F_2 plants that were sampled for genotyping produced seed for subsequent testing. Also, the numbers of $F_{2:3}$ plants derived from F_2 plants that were genotyped were sometimes limited by the amount of seed F_2 plants produced, which resulted in lower numbers of $F_{2:3}$ families available for testing against different soybean aphid biotypes. Therefore, F_2 plant seed productivity determined the population sizes that were evaluated for associations between marker genotypes and aphid colonization phenotypes.

Statistical analyses

Statistical analyses were performed with the aid of JMP 12 (SAS Institute, Cary, NC) software. Modes of aphid colonization ratings recorded from the resistance characterization

choice tests were calculated using the JMP distribution procedure. Because there was an exponential range among no-choice aphid population numbers, the count data were transformed by adding 1 to the count and then taking the $\log_{10}$ of the total to correct for the heterogeneity of variance of aphid populations among the soybean genotypes. Using the JMP fit Y × X procedure and the unequal variance tests, with Y = transformed counts and X = trials, homogeneity of variance between trials of each no-choice test was analyzed to determine if the data from both trials could be combined for analysis of variance. The JMP Fit Model procedure was used to perform analysis of variance on the transformed no-choice count data using the model Y ($\log_{10}$ aphid count) = block + biotype + genotype + biotype × genotype + error. Soybean aphid biotype, soybean genotype, and block were fixed effects. Means were separated by least significant difference (LSD) at a significance level of $\alpha = 0.05$, upon confirmation of significant differences among the sources of variation in the analysis of variance, and then were de-transformed by taking the antilog and subtracting 1 before presentation. χ^2 analyses were calculated using Microsoft Excel (v. 14) to analyze the segregation of resistant and susceptible plants in the F_2 populations, and among the all resistant, segregating, and all susceptible $F_{2:3}$ family classifications infested with biotype 3, based upon classes determined by the non-parametric, ordinal scale, as described above. Yates correction for continuity (0.5) was used in the calculations for the F_2 analyses (Little and Hills 1978).

For statistical analyses of associations between F_2 and $F_{2:3}$ aphid colonization phenotypes and parent marker genotypes, F_2 genotypes were inferred and assigned the value 0, 1, or 2, based on homozygosity for the marker alleles from the susceptible parent, or heterozygous or homozygous for the resistant parent marker alleles, respectively. $F_{2:3}$ families were classified based on segregation of resistant plants, using the assigned value 0 for families with all susceptible plants (F_2 homozygous susceptible), 1 for families segregating for resistance and susceptibility (F_2 heterozygous), and 2 for families with all resistant plants (F_2 homozygous resistant), and their F_2 resistant PI parental genotypes were inferred based on the segregation classifications.

Because the 1–4 aphid colonization rating scale is a non-parametric ordinal scale, no parametric tests such as ANOVA were used to test for marker associations. Therefore, associations between F_2 marker genotypes and F_2 aphid colonization ratings (1, 2, 3, 4) against biotypes 1 or 3, and between $F_{2:3}$ family resistance segregation classifications (0, 1, 2) against biotypes 2 or 3 were analyzed using the JMP multivariate procedure and Spearman's correlation test. Spearman's ρ is a correlation coefficient computed on the ranks of the data values instead of on the values themselves (Lehman et al. 2013). In addition, the

non-parametric Wilcoxon/Kruskal–Wallis rank sums test was performed using the JMP Fit Y by X Non-Parametric procedure to test whether medians of the rank sums were located similarly across the three genotype groups (0, 1, 2). Paired comparisons between all three genotype groupings (0 and 1, 0 and 2, or 1 and 2) were then performed using the JMP Wilcoxon each pair test procedure. The Wilcoxon test for paired samples, the non-parametric equivalent of the paired samples t test, was used when the sample data were not normally distributed and log transformation did not normalize the data. The null hypothesis was that the median difference between pairs of observations is zero, or in other words, the group medians were in the same location in both groups. The level of significance for all statistical tests was $P < 0.05$.

Results

Choice tests of resistance sources

Table 2 shows the modes of the 1–4 non-parametric aphid colonization ratings for all soybean genotypes tested against the four soybean aphid biotypes. Four of the accessions under investigation in this study, PI 587663, PI 587677, PI 587685 and PI 587972, had rating modes of 2 for biotypes 1, 2, and 3, but each of them had a rating mode of 3 against biotype 4, showing that they expressed resistance to biotypes 1, 2, and 3, but not to biotype 4. The fifth accession, PI 594592, had relatively low colonization (rating mode = 2) by biotypes 1 and 2 and higher colonization by biotypes 3 and 4 (rating mode = 3). The soybean breeding line LD05-16611, containing the introgressed *Rag1* gene, was tested against biotypes 1, 2, and 3, and had low aphid colonization levels (rating mode = 2) against biotype 1, indicating that it was resistant to biotype 1, however, it was susceptible to biotypes 2 and 3 (rating mode = 3). LD11-4576a, with the introgressed *Rag1* gene, was tested against biotype 4 and had a rating mode = 3, indicating it was susceptible to that biotype. For soybean genotypes containing the introgressed *Rag2* gene, LD08-12422a had lower colonization ratings (rating mode = 2) against biotypes 1 and 2 than against biotype 3 (rating mode = 3), and LD11-5431a, tested against biotype 4, had a relatively high level of biotype 4 colonization (rating mode = 3). Resistant checks PI 71506 (antixenosis resistance), PI 567541B, with *rag1c* and *rag4*, and PI 567543C, with *Rag3*, had low colonization by biotypes 1, 2, and 3 (rating mode = 2), but not biotype 4 (rating mode = 3). The mode for resistant check line PI 437696 was 2 across the four biotypes, indicating that accession had low levels of aphid colonization and expressed resistance against all of the biotypes. PI 567597C (antixenosis resistance) only had low levels of colonization

against biotype 1 (rating mode = 2). Williams 82 had high levels of colonization by all four biotypes.

No-choice tests of resistance sources

Results of four tests for homogeneity of variance between the two trials of each no-choice biotype test, including O'Brien's, Brown–Forsythe, Levene's, and Bartlett's tests, were non-significant ($P > 0.05$), indicating homogeneous variances. Therefore, the data from both trials, where the second trial was a replication of the first with different genotype randomizations, were combined for analysis of variance. A highly significant ($P < 0.0001$) soybean aphid biotype x soybean genotype interaction (Fig. 1) was found for aphid population counts, indicating that aphid colonization was dependent on both biotype and soybean genotype. There were also highly significant differences found among the biotypes and among the genotypes. Differences among blocks with trials were non-significant ($P > 0.05$). Overall, resistant check genotypes PI 437696, PI 567543 C (*Rag3*), and PI 567597 C (antixenosis) had significantly lower populations of all four biotypes compared with Williams 82 (Fig. 1). Of the five PI accessions under investigation in this study, PI 587663, PI 587677, and PI 587685 also had significantly lower populations of all four biotypes compared with Williams 82. PI 587972 and PI 594592 had significantly lower populations of biotypes 1, 2, and 3, but not biotype 4, compared to Williams 82. The *Rag1* introgressed breeding lines had significantly lower biotype 1 and biotype 3 populations, but non-significantly different populations of biotypes 2 and 4 compared to Williams 82. The *Rag2* introgressed breeding lines had significantly lower biotype

1 and 2 populations, but non-significantly different biotype 3 and 4 populations than Williams 82. Resistant check genotypes PI 567541B (*rag1c, rag4*), PI 567598B (*rag1b, rag3*), and PI 71506 had significantly lower populations of biotypes 1, 2, and 3, but not biotype 4 compared to Williams 82. The biotype 4 isolate consistently had the highest levels of colonization on all of the soybean genotypes in this panel, compared to the other three soybean aphid isolates, with an overall mean of 461 aphids per plant, which was much higher than biotype 3 (117 aphids per plant), and an order of magnitude greater than colonization for biotype 1 (20 aphids per plant) and biotype 2 (14 aphids per plant).

Evaluation of resistance in F_2 populations and progeny tests

Table 3 shows the number of F_2 plants that were used in F_2 resistance phenotyping, F_2 marker genotyping, $F_{2:3}$ progeny testing against biotype 3, and $F_{2:3}$ progeny testing against biotype 2. The numbers of $F_{2:3}$ families used were restricted by low seed production of some genotyped F_2 plants.

No deviation from of the expected 3 resistant to 1 susceptible F_2 plant segregation ratio was found in the χ^2 analyses for resistance to soybean aphid biotype 1 in PI 587663 and PI 587972, and resistance to biotype 3 in PI 587677 and PI 587685 (Table 4). This indicated that resistance to the biotypes tested was controlled by single dominant genes. The χ^2 test of a 3R:1S segregation for the PI 594592 F_2 population was rejected, but a test of a 9R:7S ratio was not, which provided evidence two dominant genes with complementary epistatic interaction control resistance to biotype 3 exist in that accession. The χ^2 analyses

Fig. 1 Interactions between 14 soybean genotypes and four soybean aphid biotypes for aphid populations per plant 14 days after inoculation with 10 viviparous second or third instar aphids in a no-choice test. *Letters* above the bars indicate groupings of means not significantly different by least significant difference (LSD) at a significance level of $\alpha = 0.05$. The *Rag1* and *Rag2* lines were *Rag*-region introgressed lines
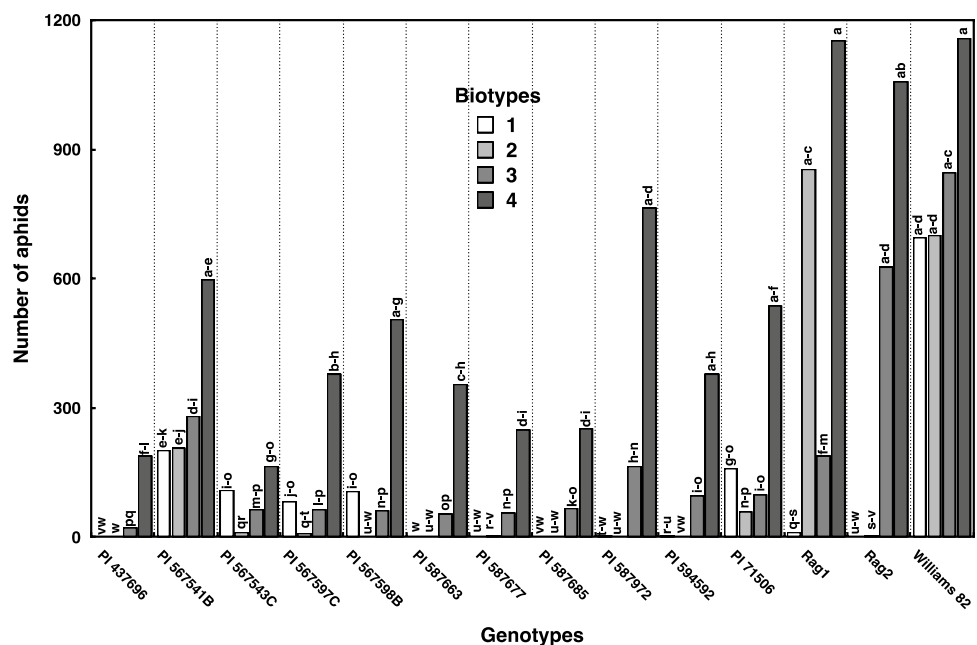
Table 3 Number of F_2 plants derived from crossing soybean aphid-resistant soybean plant introduction (PI) accessions with susceptible soybean breeding lines used in F_2 resistance phenotyping, F_2 marker genotyping, $F_{2:3}$ biotype 3 progeny testing, and $F_{2:3}$ biotype 2 progeny testing

PI accession	F_2 resistance phenotyping	F_2 plants with marker data that produced seed	$F_{2:3}$ progeny tested with biotype 3	$F_{2:3}$ progeny with marker data tested with biotype 3	$F_{2:3}$ progeny with marker data tested with biotype 2
PI 587663	277	189	200	189	184
PI 5587677	174	69	148	40	69
PI 587685	99	60	26	5	59
PI 587972	154	51	97	51	51
PI 594592	279	47	35	36	48

Table 4 Results of Mendelian genetic analyses of the segregation of resistant and susceptible F_2 plants in five F_2 populations, derived from crossing five multi-biotype resistant plant introductions with susceptible soybean genotypes, after infestation with either soybean aphid biotype 1 or biotype 3

Resistant plant introduction (PI)	Maturity group	Origin	Biotype tested	Susceptible parent	F_2 observed R:S[a]	Expected R:S	χ^2 test P value
PI 587663	VII	China	1	LD02-5320	200:77	3:1	0.28
PI 587677	VII	China	3	LD03-6566	137:37	3:1	0.26
PI 587685	VII	China	3	LD03-10504	93:26	3:1	0.43
PI 587972	VI	China	1	LD03-10504	120:34	3:1	0.40
PI 594592	VII	China	3	LD02-5320	171:108	9:7	0.10

[a] R resistant (aphid colonization rating 1 or 2), S susceptible (aphid colonization rating 3 or 4)

of the segregation of $F_{2:3}$ family classes failed to reject the expected 1 all resistant: 2 segregating:1 all susceptible ratio of family classifications, which supported evidence of single, dominant genes for resistance against soybean aphid biotypes 1, 2, or 3 for the five PIs tested.

Associations between F_2 aphid colonization and $F_{2:3}$ family resistance segregation classifications

Correlations of the aphid colonization ratings of biotypes 1 or 3 on F_2 plants with the $F_{2:3}$ family resistance segregation classifications when challenged against biotypes 2 or 3 are listed in Table 5. For the PI 587663-derived population, biotype 1 colonization ratings on F_2 plants were negatively correlated with $F_{2:3}$ family resistance segregation against biotype 2. This showed that resistance expressed against biotype 1 in parental F_2 plants was also expressed against biotype 2 in their progeny plants because a higher number of $F_{2:3}$ families segregating biotype 2-resistant plants tended to be associated with lower F_2 biotype 1 colonization ratings. PI 587663 $F_{2:3}$ family resistance segregation between biotypes 2 and 3 was also correlated but positive, indicating that parental F_2 plants that were resistant to biotype 2 produced progeny that were susceptible to biotype 3. Similarly, the PI 587972 population biotype 1 colonization ratings on F_2 plants were negatively correlated with $F_{2:3}$ family resistance segregation against biotype 2, but also against biotype 3, while $F_{2:3}$ family resistance segregation

between biotypes 2 and 3 were positively correlated. $F_{2:3}$ family segregation between biotypes 2 and 3 was also positively correlated for the PI 587677 population. There were significant negative correlations between aphid colonization ratings on F_2 plants derived from PI 594592 against biotype 3 and $F_{2:3}$ family resistance segregation classifications against biotype 2 and against biotype 3. For the population derived from PI 587685, the correlations between biotype 3 F_2 plant aphid colonization ratings and biotype 3 $F_{2:3}$ family resistance classifications, and between biotype 2 and biotype 3 $F_{2:3}$ family resistance classifications, could not be calculated by JMP due to insufficient sample size of the biotype 3 $F_{2:3}$ family resistance classifications, resulting from limited quantity of $F_{2:3}$ seed from PI 587685 F_2 plants available for the biotype 3 progeny testing (only F_2 plants that produced a minimum of 12 seeds were used).

Genotyping and marker associations

Results of the BSA analysis with the Illumina GoldenGate assays of genomic DNA from PI 587663, PI 587685, PI 587972, and bulks of DNA from resistant and susceptible F_2 plants indicated that all three PIs have a resistance gene mapping to the *Rag2* region. Bulked segregant analysis was not done for the populations derived from PI 587677 and PI 594592.

Table 6 summarizes the non-parametric analyses of the effects of genetic regions associated with aphid-resistant PI

Table 5 Results of correlation analyses between F$_2$ soybean aphid colonization ratings and classifications of F$_{2:3}$ families after infestation with soybean aphid biotypes 1, 2 and 3 in populations derived from crossing five multi-biotype resistant plant introductions (PI) with susceptible soybean genotypes

Population Parental PI	Generation	Aphid Biotype	F$_{2:3}$ family biotype 2 resistance classification[a]	F$_{2:3}$ family biotype 3 resistance classification
587663	F$_2$[b]	1	−0.19*[c]	−0.07
	F$_{2:3}$	2		0.20**
587677	F$_2$	3	−0.06	−0.07
	F$_{2:3}$	2		0.57***
587685	F$_2$	3	−0.18	
	F$_{2:3}$	2		
587972	F$_2$	1	−0.98***	−0.57***
	F$_{2:3}$	2		0.60***
594592	F$_2$	3	−0.47***	−0.52**
	F$_{2:3}$	2		0.12

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

[a] F$_{2:3}$ family resistance classification with 0 = all plants in the family were susceptible, 1 = family had both resistant and susceptible plants, and 2 = all plants in the family were resistant

[b] Ordinal aphid colonization rating 1, 2, 3, or 4, with increasing rating indicating increasing aphid colonization and plant damage, with 1 = few solitary live aphids, often with dead aphids; 2 = several transient aphids present along with some viviparous aptera surrounded by a few nymphs, but without established colonies; 3 = dense aphid colonies; and 4 = dense colonies accompanied by plant damage such as leaf distortion and stunting

[c] Spearman's ρ statistic

[d] Marker genotypes of F$_2$ parents not determined

marker alleles on aphid colonization on F$_2$ plants and on F$_{2:3}$ family resistance segregation classifications against biotypes 1, 2, or 3. There were significant correlations found for regions associated with aphid-resistant PI parent alleles for *Rag1, Rag2, Rag3,* and *rag4.*

PI 587663

There were significant positive correlations between *Rag1* region markers Satt245 and Satt567 with F$_{2:3}$ family resistance segregation when infested with biotype 3 (Table 6), indicating an association between the aphid-resistant PI *Rag1* marker alleles with higher segregation of resistant plants against biotype 3. The effect of the region associated with the PI 587663 Satt245 allele on F$_{2:3}$ family resistance segregation was partially dominant over the susceptible parent allele because the non-parametric comparisons 1 (heterozygous genotype) versus 0 (homozygous for susceptible parent alleles), and 2 (homozygous for resistant parent alleles) versus 1 were non-significant, while 2 versus 0 was significant. The allele effect was dominant for the region associated with the PI 587663 Satt567 allele because the 1 versus 0 and the 2 versus 0 comparisons were significant, while the 2 versus 1 was non-significant.

Significant negative correlations between PI 587663 Satt114, Satt335, and SNP2 marker alleles in the *Rag2* region and F$_2$ colonization rating against biotype 1 were found (Table 6), indicating that lower biotype 1

colonization was associated with PI 587663 alleles at those marker loci. Biotype 1 resistance in F$_2$ plants associated with PI 587663 alleles at Satt114, Satt335, and SNP2 was partially dominant. Correlations between the PI 587663 alleles at Satt114, Satt335 and SNP2, with F$_{2:3}$ family classification against biotypes 2 and 3, were positive and significant, indicating that PI 587663 alleles at those marker loci were associated with higher segregation of resistant plants when challenged against biotypes 2 and 3. Biotype 2 resistance associated with PI 587663 alleles at Satt114, Satt335, and SNP2 was additive against biotype 2, whereas for the biotype 3 resistance associated with the three markers, there was no effect, a recessive effect, or a partial dominance effect, respectively.

There was a significant correlation between the PI 587663 Satt285 marker allele in the *Rag3* region and F$_{2:3}$ family classification against biotype 3, indicating an association between the PI 587663 allele and biotype 3 resistance in F$_{2:3}$ families. The effect of the allele was partially dominant.

PI 587677

A significant negative correlation between the PI 587677 *Rag1* Satt540 allele and F$_2$ plant biotype 3 colonization rating was found (Table 6). Biotype 3 resistance associated with that allele was dominant. There was a significant positive correlation between the PI 587677 Satt540 allele

Table 6 Effects of alleles of soybean aphid resistance genes on aphid colonization found in five multi-biotype resistant plant introduction (PI) accessions and their genetic associations with *Rag* region molec-ular markers after infestation with soybean aphid biotypes 1, 2 and 3 in populations derived from crossing with susceptible soybean geno-types

Resistance source	Generation tested	Biotype tested	Significant *Rag* regions	Significant markers	Linkage Group	Chromosome	Soybean consensus map position (cM)	Resistant parent allele effect	Spearman's correlations
PI587663	F2	1	*Rag2*	Satt114	F	13	43.9	Partially dominant	−0.28***
				SNP2 (ss4969648, Kim et al. 2010)			49.924	Partially dominant	−0.30***
				Satt335			61.05	Partially dominant	−0.28***
	F2:3	2	*Rag2*	Satt114	F	13	43.9	Additive	0.33***
				SNP2 (ss4969648, Kim et al. 2010)			49.924	Additive	0.37***
				Satt335			61.05	Additive	0.33***
	F2:3	3	*Rag1*	Satt567	M	7	32.746	Dominant	0.31***
				Satt245			49.031	Partially dominant	0.16*
			Rag2	Satt114	F	13	43.9	Inconclusive	0.15*
				Satt335			61.05	Recessive	0.20**
				SNP2 (ss4969648, Kim et al. 2010)			49.924	Partially dominant	0.15*
			Rag3	Satt285	J	16	24.171	Partially dominant	0.16*
PI587677	F2	3	*Rag1*	Satt540	M	7	34.27	Dominant	−0.50***
	F2:3	2	*Rag2*	Satt335	F	13	61.05	Additive	0.72***
			rag4	Satt586	F	13	33.7	Partially dominant	0.30*
	F2:3	3	*Rag1*	Satt540	M	7	34.27	Homozygote advantage	0.33*
			Rag2	Satt335	F	13	61.05	Dominant	0.55**
			rag4	Satt586	F	13	33.7	Recessive	0.45**
PI587685	F2	3	*Rag1*	Satt540	M	7	34.27	Dominant	−0.30*
				Satt463			46.27	Dominant	−0.37**
	F2:3	2	*Rag2*	Satt114	F	13	43.9	Additive	0.67***
				Satt335			61.05	Additive	0.67***
				SNP1485 (ss4969627, Kim et al. 2010)			Unconfirmed	Additive	0.67***
PI587972	F2	1	*Rag2*	Satt114	F	13	43.9	Dominant	−0.53***
				Satt510			55.596	Partially dominant	−0.39**
	F2:3	2	*Rag2*	Satt114	F	13	43.9	Dominant	0.54***
				Satt510			55.596	Partially dominant	0.42**
	F2:3	3	*Rag2*	Satt114	F	13	43.9	Additive	0.79***
				Satt510			55.596	Partially dominant	0.54***
PI594592	F2	3	*Rag1*	Satt540	M	7	34.27	Dominant	−0.52***
			Rag3	Satt654	J	16	41.96	Partially dominant	0.31*
	F2:3	2	*Rag2*	Satt114	F	13	43.9	Additive	0.58***
			Rag3	Satt654	J	16	41.96	Homozygote disadvantage	−0.30*

Table 6 (continued)

Resistance source	Generation tested	Biotype tested	Significant *Rag* regions	Significant markers	Linkage Group	Chromosome	Soybean consensus map position (cM)	Resistant parent allele effect	Spearman's correlations
		3	*Rag1*	Satt540	M	7	34.27	Additive	0.65***

and biotype 3 resistance segregation in $F_{2:3}$ families; however, the result of the analysis on the effect of the resistance associated with that allele was unclear.

Significant positive correlations were found between $F_{2:3}$ family biotype 2 and biotype 3 resistance segregation classification and PI 587677 alleles at Satt335 in the *Rag2* region and the Satt586 marker in the *rag4* region. The effects of the biotype 2 and 3 resistance associated with the PI 587677 alleles varied from dominant to recessive, depending on the marker locus.

PI 587685

There were significant negative correlations found between F_2 biotype 3 colonization ratings and two *Rag1* region markers (Table 6) and the effects of the biotype 3 resistance associated with the PI 587685 alleles were dominant.

Additionally, there were significant positive correlations between $F_{2:3}$ family biotype 2 resistance segregation classification and the PI 587685 alleles at markers Satt114, Satt335, and SNP1485 in the *Rag2* region. The effects of the biotype 2 resistance associated with the PI 587685 *Rag2* region Satt114 and Satt335 marker alleles were additive.

PI 587972

Significant negative correlations were found between F_2 biotype 1 colonization ratings and PI 587972 alleles at three markers in the *Rag2* region (Table 6). There were also significant positive correlations between the PI 587972 alleles at the same *Rag2* region markers and $F_{2:3}$ family biotype 2 and 3 resistance segregation classifications. The effects of the biotype 1, 2, and 3 resistance associated with the PI 587972 alleles at Satt114 in the *Rag2* region were completely dominant, except for $F_{2:3}$ biotype 3 resistance segregation classification associated with the Satt114 allele, which was additive. The effects of resistance against all three biotypes associated with the PI 587972 Satt510 allele in the *Rag2* region were partially dominant.

PI 594592

F_2 biotype 3 colonization ratings were significantly negatively correlated with the PI 594592 allele associated

with marker Satt540 in the *Rag1* region, with a dominant resistance effect, and with the allele at Satt654 in the *Rag3* region, with a partially dominant resistance effect (Table 6). There was also a significant correlation between biotype 3 $F_{2:3}$ resistance segregation classification and the PI 594592 Satt540 allele, with an additive effect of the resistance associated with the marker allele. A significant positive correlation between $F_{2:3}$ family biotype 2 resistance segregation classification and the PI 594592 allele at Satt114 in the *Rag2* region, with additive resistance expression, was found. The PI 594592 allele at Satt654 in the *Rag3* region was negatively correlated with $F_{2:3}$ family segregation for resistance to biotype 2.

Discussion

Resistance against soybean aphid biotypes 1, 2, 3 and 4 in three germplasm sources, PI 587663, PI 587677, and PI 587685, were reported for the first time. In addition, PI 437696, previously identified to have two aphid-resistance genes mapping in the *Rag1* and *Rag2* regions (Fox et al. 2014), was also found to be resistant to all four biotypes. A recent report (Bansal et al. 2013) that named two PI accessions, PI 606390A and PI 340034, as resistant to biotypes 1, 2, and 3, did not test for resistance to biotype 4.

Mendelian, correlation, and resistance allele effect analyses showed that PI 587663 had a single, dominant gene mapping in the *Rag2* region, with a partially dominant effect that controlled resistance to biotype 1. That gene also controlled resistance to biotypes 2 and 3, with variable allele effects. The results of Golden Gate assay of bulked DNA samples of resistant or susceptible F_2 plants confirmed there was a resistance gene in the *Rag2* region. Correlation and resistance allele effects analyses also showed that there were resistance genes mapping in the *Rag1* and in the *Rag3* regions that contributed to resistance against biotype 3, with partially or completely dominant effects. Therefore, PI 587663 had three non-allelic, independently inherited soybean aphid resistance genes. Evidence showing an association between *Rag1* region marker alleles and biotype 3 resistance in $F_{2:3}$ plants, but no association between the marker alleles and biotype 1 in F2 plants, suggested that the gene in the *Rag1* region controlled resistance to biotype 3 but not biotype 1. That result could mean

that there was a different allele at that locus than the allele found in Dowling, the original source of *Rag1* (Hill et al., 2006; Li et al. 2007) or that biotype 1 was virulent against the PI 587663 gene, while biotype 3 was not. The gene mapping in the *Rag2* region gave resistance to all three biotypes, including biotype 3, suggesting it could be a new allele at the *Rag2* locus providing resistance to biotype 3, whereas the original *Rag2* allele found in PI 200538 (Hill et al. 2009) did not. The gene mapping in the *Rag3* region that provided resistance to biotype 3 may be the same gene as reported in PI 567543C (Zhang et al. 2010) and may also provide resistance against biotype 4 because this PI was also found to be resistant to all four biotypes in this study.

As above, Mendelian, correlation and resistance allele effects analyses showed that a dominant gene in PI 587677, mapping in the *Rag1* region, controlled resistance to biotype 3 and that genes mapping in the *Rag2* and *rag4* regions with variable allele effects controlled resistance to both biotypes 2 and 3. Either of these genes may be responsible for resistance to biotype 1. It is possible that the genes mapping in the *Rag1, Rag2,* and *rag4* regions in PI587677 collectively controlled resistance to all four biotypes.

A gene in PI 587685 located in the *Rag1* region, with dominant effects, controlled resistance to biotype 3. Also, a gene located in the *Rag2* region, which was identified by the GoldenGate assay, controlled resistance to biotype 2 with additive effects. It is probable that either of these two genes controlled resistance to biotype 1 and it is also likely that there are either new alleles at the *Rag1* or *Rag2* loci, or closely linked genes, that control resistance to biotype 4. Testing the F_2 population against biotype 1 could reveal the presence of duplicate dominant genes.

Resistance in PI 587972 to biotypes 1, 2 and 3 was clearly controlled by a gene located in the *Rag2* region (also identified by the GoldenGate assay) that had dominant, partially dominant, or additive effects on resistance, depending on the marker and biotype tested. Because the gene controlled resistance to biotype 3, it is unlikely the same *Rag2* allele found in PI 200538 (Hill et al. 2009) or PI 243540 (Mian et al. 2008), which were susceptible to biotype 3 (Hill et al. 2010; Pawlowski et al. 2014) and is more likely a new *Rag2* allele or closely linked gene in the *Rag2* region.

Genetic and marker association analyses showed that resistance to biotype 3 in PI 594592 was controlled by a gene in the *Rag1* region, with dominant or additive effects. Additionally, a gene with partially dominant effects mapping in the *Rag3* region contributed to resistance against biotype 3 and also was associated with resistance to biotype 2. Based on a higher coefficient of correlation, most of the resistance to biotype 2 appeared to be explained by a gene with additive effects mapping in the *Rag2* region. The complementary, epistatic interaction (9R:7S ratio) for

resistance to biotype 3 found in the Mendelian analysis of the resistance segregation in the F_2 population indicated that two dominant alleles were interacting with each other resulting in complementary gene action. A dominant allele at the gene in the *Rag2* region inherited from PI 594592 likely controlled resistance to biotype 2. Any of these resistance genes probably could control resistance to biotype 1 if they were challenged against it. Resistance phenotyping of the F_2 population with biotype 1 could reveal more than one major gene segregating if biotype 1 was avirulent against the genes.

While seed production by F_2 plants determined populations sizes used for evaluating marker genotype–aphid colonization phenotype associations, there was no obvious association between seed production and aphid colonization. The resistance genetics conclusions stated above are bolstered by the use of more than one soybean aphid biotype in the study. This demonstrates the utility of using multiple soybean aphid biotypes with variable virulence to determine soybean aphid resistance genetics in soybean cultivars previously identified (Kim et al. 2014). This is especially important if a completely avirulent biotype is not available for use.

Genetic allelism tests and fine mapping work will be required to discern between potential allelism at the *Rag* loci or if there are new, closely linked loci mapping within the *Rag* regions in these five aphid resistance sources. In any event, markers flanking these loci will be useful in marker-assisted selection procedures to transfer the resistance genes in the resistant PI accessions into elite, high-yielding soybean germplasm.

Results in this study showed that PIs 587663, 587677, 587685, and 594592 are natural multi-resistance gene pyramids. Other PIs with natural soybean aphid resistance gene pyramids have been reported (Bales et al. 2013; Chen et al. 2006; Fox et al. 2014; Jun et al. 2012; Kim et al. 2014). Recently, it was shown that a resistance gene pyramid, developed by our research group using marker-assisted selection to transfer and combine *Rag1* and *Rag2* from different sources, provided stronger field resistance and yield protection against soybean aphids than soybean lines with either resistance gene alone (McCarville et al. 2014; Wiarda et al. 2012). That result suggested the *Rag1, Rag2* pyramid was resistant to multiple aphid biotypes.

In conclusion, the sources of resistance and the added information on the acting resistance genes reported in this study (Table 7) will be useful to soybean breeders developing new soybean aphid-resistant soybean cultivars. The strategy to develop and use resistance gene pyramids to manage soybean aphids in soybean has been discussed (McCarville et al. 2014), and results of the multi-biotype resistance expressed in natural resistance gene pyramids found in this study would complement this effort. Although

Table 7 Gene regions of five plant introductions (PI) with resistance to *Aphis glycines* (*Rag*)[a]

Resistant PI	Probable resistance gene regions
PI587663	*Rag1, Rag2, Rag3*
PI587677	*Rag1, Rag2, rag4*
PI587685	*Rag1, Rag2*
PI587972	*Rag2*
PI594592	*Rag1, Rag2, Rag3*

[a] Based on no-choice tests from the literature, *Rag1* and *Rag2* provide resistance to biotype 1, *Rag2* provides resistance to biotype 2, *Rag1* provides resistance to biotype 3, and none of the single genes provide resistance to biotype 4 (also see Ajayi-Oyetunde et al. 2016). The spectrum of resistance of plants with *rag4* against the four aphid biotypes is not known. The gene found in PI587972 is in the *Rag2* region, and is an allele or a linked gene to *Rag2*. The genes found in the other PIs may also be different alleles or linked genes to the known *Rag* genes

it is challenging for soybean breeders to develop multiple resistance gene pyramids in elite, high-yielding genetic backgrounds, there is much potential therein to increase resistance gene durability similar to resistance gene pyramids used to combat plant diseases (Crute and Pink 1996; Djian-Caporalino et al. 2014; Mundt 1991). There is also the possibility that pyramids would be more attractive to soybean producers (McCarville et al. 2014), which validates current and future efforts to combine soybean aphid resistance genes into pyramids.

Author contribution statement CBH and GLH conceived the project; CBH, DS, and CF conducted the research; CBH completed the data analyses and drafted the manuscript; and all authors contributed to the final submission.

Compliance with ethical standards

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

Ethical standards The authors state that the experiments comply with the current laws of the country in which they were performed (USA).

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