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Plant Resistance

Differential Reactions of Soybean Isolines With Combinations of Aphid Resistance Genes *Rag1*, *Rag2*, and *Rag3* to Four Soybean Aphid Biotypes

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Abstract

With the discovery of the soybean aphid (*Aphis glycines* Matsumura) as a devastating insect pest of soybean (*Glycine max* (L.) Merr.) in the United States, host resistance was recognized as an important management option. However, the identification of soybean aphid isolates exhibiting strong virulence against aphid resistance genes (*Rag* genes) has highlighted the need for pyramiding genes to help ensure the durability of host resistance as a control strategy. In this study, soybean isolines with all possible combinations of the resistance and susceptibility alleles at *Rag1*, *Rag2*, and *Rag3* were evaluated for their effectiveness against the four characterized soybean aphid biotypes. All soybean isolines, including the susceptible check carrying none of the resistance alleles (S1/S2/S3), were infested with each biotype in no-choice greenhouse tests, and the aphid populations developed on each isoline were enumerated 14 d after infestation. All gene combinations, with the exception of *Rag3* alone, provided excellent protection against biotype 1. Isolines with *Rag2* alone or in combination with *Rag1* and *Rag3* had greater levels of resistance to biotype 2 than those with either *Rag1* alone, *Rag3* alone, or the *Rag1/3* pyramid. For biotype 3, the *Rag1/3* and *Rag1/2/3* pyramided lines significantly reduced aphid populations compared with all other gene combinations, while the *Rag1/2/3* pyramid provided the greatest protection against biotype 4. Overall, the *Rag1/2/3* pyramided line conferred the greatest protection against all four biotypes.

Key words: *Aphis glycines*, biotype, soybean

The soybean aphid, *Aphis glycines* Matsumura, is a destructive insect pest of soybean (*Glycine max* (L.) Merr.). Although native to Asia, its first occurrence in the United States dates back to 2000 (Hartman et al. 2001), and its distribution has since expanded to almost all soybean-growing regions in the United States and Canada (Venette and Ragsdale 2004). The agronomic and economic impacts of the soybean aphid on soybean are significant. Feeding injury can result in stunting, leaf distortion, and reductions in the number of seed pods (Ragsdale et al. 2007, Sun et al. 1990). In addition, the photosynthetic potential of infected plants may be significantly impacted by the colonization of sooty molds on soybean leaves covered with honey dew excreted by feeding aphids (Gomez et al. 2006; Macedo et al. 2003). Furthermore, yield losses, which have been associated with premature pod abscission due to insect feeding during R1 through R4 growth stages (Fehr et al. 1971), can be as high as 50% in the United States (Ragsdale et al. 2006) or even greater in other parts of the world (He et al. 1991, Wang et al. 1994). The indirect impacts of soybean aphids are evident in their ability to efficiently vector plant viruses, including *Soybean mosaic virus* (Hartman et al. 2001, Hill et al. 2001, Domier et al. 2003).

Timely foliar insecticide applications, especially when the economic threshold of 250 aphids per plant has been reached and >80% of plants have become infested (Ragsdale et al. 2006, 2007), can prevent yield losses (Hartman et al. 2011). However, given the increase in production costs associated with insecticide use (Ragsdale et al. 2007), the threat of insecticides to beneficial insects (Theiling and Croft 1988, Desneux et al. 2007) and the environment (van der Werf 1996), as well as the potential for insecticide resistance with repeated applications, host resistance continues to serve as the most important and environmentally sound control tactic.

In North America, several sources of aphid resistance have been identified in accessions from the USDA Soybean Germplasm Collection (Hill et al. 2004, Mensah et al. 2005, Mian et al. 2008a). Resistance has been characterized, using choice and no-choice experiments, as antibiosis, which affects the insect's biology by interfering with its growth and reproductive ability; antixenosis, which affects the insect's behavior and is expressed as a nonpreference for a specific host; or as tolerance, which confers the ability to withstand devastating insect populations (Smith 2005).

The genetic basis of resistance has been investigated, and a number of aphid resistance genes have been named (Hill et al. 2012). Resistance in the soybean cultivars Jackson and Dowling to an Illinois aphid isolate was found to be inherited as single dominant genes, and were named *Rag* and *Rag1*, respectively (Hill et al. 2006a,b). Both genes were later mapped to the same chromosomal location [chromosome 7; linkage group (LG) M] (Li et al. 2007), suggesting they carry resistance at the same locus or at different closely linked loci (Hill et al. 2012). The identification and mapping of the second aphid resistance gene coincided with the discovery of a new soybean aphid biotype from Wooster, OH. Previous studies showed that the Illinois aphid isolate was unable to colonize plants with *Rag1* (Hill et al. 2004, 2006a), but the Wooster isolates were found to densely colonize plants with *Rag1* (Kim et al. 2008); hence, the Illinois isolate was named biotype 1, while the Ohio isolate was designated biotype 2 (Hill et al. 2009).

Resistance to both biotype 1 and 2 was identified in Plant Introduction (PI) lines PI 243540 (Kang et al. 2008; Mian et al. 2008a,b) and PI 200538 (Hill et al. 2009), and the underlying resistance gene from the two sources was mapped to the same location on chromosome 13 (LG F) and was named *Rag2*. Unfortunately, SF-55, an aphid isolate recovered from Springfield Fen, Indiana, was found to colonize plants with *Rag2*, leading to the designation of biotype 3 (Hill et al. 2010). SF-55 is highly virulent on soybean genotypes with *Rag2* and can also colonize plants with *Rag1* and *Rag1*/*Rag2* in choice and no-choice experiments, indicating that a stronger antibiosis-type resistance is needed for the long-term management of this biotype (Hill et al. 2010). Unfortunately, no resistance gene with complete antibiosis or antixenosis-type resistance has been reported for this biotype. More recently, a soybean aphid from Lomira, WI, was designated biotype 4 (Alt and Ryan-Mahmutagic 2013). This biotype was found to be highly virulent on plants with *Rag1*, *Rag2*, and the *Rag1*/*Rag2* pyramid (Alt and Ryan-Mahmutagic 2013). Similar to biotype 3, sources of resistance and *Rag* genes specific to biotype 4 have not been reported.

With the identification of different aphid biotypes, additional aphid resistance loci were sought, and a third aphid resistance gene, *Rag3*, was mapped in PI 567543C (Zhang et al. 2010), a soybean accession that was reported to express antixenosis-type resistance against aphid isolates found near East Lansing, MI (Mensah et al. 2005). Five additional soybean aphid resistance genes have been further characterized. Of these, *Rag3b* (Zhang et al. 2012) and *Rag5* (Mian et al. 2008a, Jun et al. 2012) are dominant, while the remaining three, *rag1b* (Mensah et al. 2007, Bales et al. 2013), *rag1c* (Zhang et al. 2010), and *rag4* (Zhang et al. 2012), are recessive. While there are no reports of the results from testing these additional genes with the four characterized aphid biotypes, they expand the range of resistance genes available to breeders for developing aphid-resistant soybean cultivars. Of the eight known aphid resistance genes, only *Rag1* and the *Rag1*/*Rag2* pyramid are currently deployed in commercial soybean cultivars marketed as having resistance to the soybean aphid (Caspers-Simmet 2008, McCarville et al. 2012). Given the virulence diversity in the population of soybean aphids in North America (Cooper et al. 2015), the variability in aggressiveness among isolates of any one biotype (Pawlowski et al. 2015), and the ability of aphids to move large distances, durable resistance may be best achieved by pyramiding multiple *Rag* genes. As the arsenal of deployable aphid resistance genes with antibiosis and antixenosis-type resistance continues to expand, the options available to breeders are enormous, but the efficacy of gene combinations against the four known biotypes in North America has not been thoroughly investigated. In light of this knowledge gap, the objective of our research was to evaluate the differential reaction of soybean isolines carrying different combinations of the genes *Rag1*, *Rag2*, and *Rag3* to the four aphid biotypes identified in North America.

Materials and Methods

Aphid Culture Maintenance and Plant Materials

Isolates of biotypes 1, 2, and 3 are clonal descendants of the original isolates from Illinois (Hill et al. 2004, 2006a,b), Ohio (Kim et al. 2008), and Indiana (Hill et al. 2010), respectively. Biotype 4 was obtained from Michael Crossley at the University of Wisconsin (Madison, WI). Although a different isolate from the Lomira isolate reported by Alt and Ryan-Mahmutagic (2013), the biotype 4 isolate was collected from a site near where the Lomira isolate was identified, and our greenhouse assays confirmed similar virulence patterns as the Lomira isolate. Colonies of the four biotypes have been continuously maintained in an apterous state in isolated growth chambers after the original collections were made, which would be at least several hundred generations devoid of sexual reproduction. These biotypes have also been periodically cloned and tested to confirm virulence spectrums.

Separate pilot choice tests were initially set up to confirm the identity of each biotype and to subsequently monitor the virulence expression of each biotype on differential hosts on which they had been maintained. Briefly, for biotypes 1 and 2, two plants each for soybean genotypes Williams 82 (no *Rag* gene), LD10-5903a (*Rag1*), and LD08-12435a (*Rag2*) were sown in a triangular pattern into 15-cm plastic pots containing a soilless potting medium (Sunshine Mix, LC1, Sun Gro Horticulture Inc., Bellevue, WA) and placed in a greenhouse maintained at 25°C. As the plants approached VC growth stage (Fehr et al. 1971), plants were thinned to one of each genotype per pot. A detached leaf from a previously infected plant containing multiple life stages was placed in the middle of the pot, and the levels of aphid colonization on each genotype were monitored after 14 d. A similar design was adopted for biotypes 3 and 4, but in this case, Williams 82 was replaced by LD12-12734a, a soybean breeding line with the *Rag1*/*Rag2* pyramid. Williams 82 was excluded from the virulence confirmation experiment for these biotypes, as previous work had shown that *Rag2* was as susceptible as Williams 82 when infested with biotype 3 (Hill et al. 2010) and because biotype 4 is virulent on all *Rag* gene combinations (Alt and Ryan-Mahmutagic 2013). Confirmation of virulence for the four biotypes was by visual observation of the number of aphids on each genotype.

Eight soybean breeding lines differing in *Rag* gene combinations (Table 1) were used in the main experiment. These lines were developed through four backcrosses using markers to select for the resistance genes during each generation of backcrossing. LD02-4485 served as the recurrent parent, while Dowling was the donor of *Rag1*, PI 200538 the donor of *Rag2*, and PI 567543C the donor of *Rag3*.

Table 1. Soybean genotypes evaluated in no-choice experiment and their corresponding Rag gene combination

Soybean isoline	Resistance gene
LD14-8001	*Rag1/Rag2* (*Rag1/2*)
LD14-8002	*Rag2*
LD14-8003	*Rag1/Rag2/Rag3* (*Rag1/2/3*)
LD14-8004	*Rag1*
LD14-8005	*Rag1/Rag3* (*Rag1/3*)
LD14-8006	*Rag3*
LD14-8007	S1/S2/S3
LD14-8008	*Rag2/Rag3* (*Rag2/3*)

Experimental Set-up and Statistical Analysis

To determine the interaction between the eight soybean isolines and the four soybean aphid biotypes, no-choice tests were conducted as a factorial experiment arranged in a completely randomized design (CRD). Protocols for infestation were similar to those described by Hill et al. (2010). Briefly, two seeds of each isoline were sown into 15-cm plastic pots filled with soilless potting medium (Sunshine Mix, LC1, Sun Gro Horticulture Inc.) in a greenhouse maintained at a 14-h photoperiod with temperatures ranging between 22 and 25°C. Plants in each pot were thinned to one as all plants approached VC stage. Using a damp script liner brush (Royal and Langnickel, Munster, IN), 10 aphid nymphs (2nd to 3rd instar) were carefully placed on the adaxial side of one of the expanding unifoliate leaves at the VC stage. To prevent migration of aphids from infested plants, each pot was covered with a 100- by 300-mm plastic cylindrical cage having a 4-mm wall thickness and two 80- by 180-mm side windows of dimensions sealed with a silk fabric material with 0.1-mm apertures (Hill et al. 2010). Each infested plant represented an experimental unit, and each unit was replicated three times to give a total of 96 experimental units (8 isolines × 4 biotypes × 3 replications). Aphid colonization was evaluated 14 d postinfestation by counting the number of aphids on each plant. The no-choice experiment was repeated in a second trial in the same greenhouse and under the same environmental conditions as the first trial.

The genotype of each isoline was confirmed by conducting a TaqMan assay. For this analysis, two representative seedling samples of each isoline that were randomly selected from the thinned plants prior to aphid infestation were transplanted into a soilless potting medium in the greenhouse maintained at 25°C. At the V2 to V3 growth stage, the uppermost fully expanded trifoliate leaves were sampled for each isoline, and DNA was extracted using the CTAB method as described by Keim and Shoemaker (1988). SNP marker analysis was carried out as described by Kaczorowski et al. (2008) using a LightCycler 480 System (Roche Diagnostics, Indianapolis, IN). Williams 82, Dowling, PI 200538, and E10005 served as reference genotypes for no *Rag* gene, *Rag1*, *Rag2*, and *Rag3*, respectively. SNP markers used for genotyping include 22289 (*Rag1*) (Kim et al. 2010a), KS12 (*Rag2*) (Kim et al. 2010b), and MSUSNP16-10 (*Rag3*) (Zhang 2012, Bales et al. 2013).

Aphid count data for each trial were log transformed to ensure normal distribution and homogeneity of residuals before analysis in SAS (PROC GLM, SAS Institute 2001, Cary, NC). Normality of the data was confirmed after transformation by using the *p*-value obtained from the Shapiro–Wilk normality test and by visual observation of the Q-Q plots. A Brown–Forsythe test of homogeneity of variance was conducted to confirm homogeneity of variance for the residuals after transformation and to determine if trials could be pooled before analysis. Contrast statements were used to determine significant differences among the eight soybean isolines for each of the four aphid biotypes. Although nontransformed data are presented, mean separations reported are from the analysis of the transformed values.

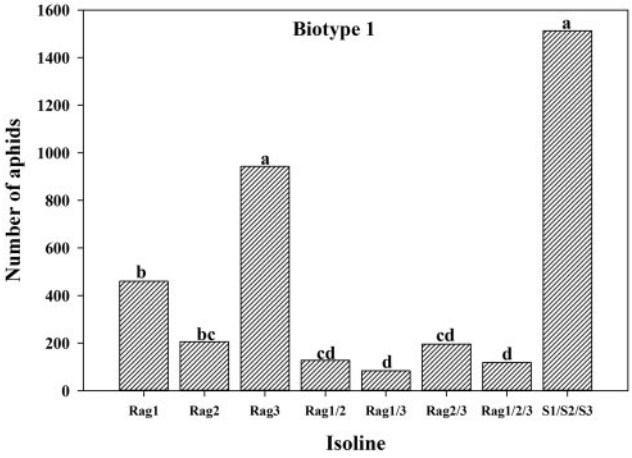

Fig. 1. Differential colonization of the eight soybean isolines with combinations of *Rag* genes by soybean aphid biotype 1 across trials 1 and 2. Means with the same letter are not significantly different ($\alpha = 0.05$). Mean separations are from $\log_{10}$ transformed data.

To determine the similarity in the reaction of the four soybean aphid biotypes to the different gene combinations, aphid count data were subjected to hierarchical cluster analysis in R (R Core Team 2015). For analysis, distance matrix was computed using the Euclidean metric in the dist function (*stats* package), and the Ward's minimum variance method (ward.D2) was selected for agglomerative clustering in the hclust function (*stats* package).

Results and Discussion

The phenotypes of the soybean genotypes tested in the biotype confirmation pilot study were consistent with expectations (Table 2). Homogeneity of variance test for trial revealed a common variance for the residuals ($F = 2.01$; df = 1, 189; $P = 0.16$); therefore, the two trials were pooled for analyses. From the analysis of variance for the mean number of aphids, the main effects of trial, isoline, and biotypes were significant (Table 3). Significant interactions between isoline and biotype, and between trial and biotype were also detected (Table 3). All other interaction effects were not significant. The observed significant interaction between trial and biotype resulted from the mean number of aphids observed being greater in trial 2 than trial 1, except for biotype 3 when a significantly greater numbers were observed in trial 1 than trial 2 (Table 4). The significant interaction between the soybean isolines and the aphid biotypes indicates a differential colonization of the isolines by the four aphid biotypes, thus confirming that the four aphid isolates used in this experiment were different biotypes. Multiple degree of freedom contrasts for the interactive effect of isoline by biotype showed that the differences in colonization among isolines for each biotype were highly significant ($P < 0.0001$); therefore, the differential reaction of the four biotypes to the eight isolines was reported for each biotype separately. Figures 1 to 4 provide a summary of the mean number of aphids recorded for each isoline across the two trials.

Table 2. Phenotypic expression of differential soybean genotypes after infestation with the four soybean aphid biotypes in the pilot study

Soybean differentials[a]	Biotypes[b]			
	1	2	3	4
Williams 82 (no *Rag*)	+	+		
LD10-5903a (*Rag1*)	−	+	−	+
LD08-12435a (*Rag2*)	−	−	+	+
LD12-12734a (*Rag1/Rag2*)			−	+

[a] Williams 82 was obtained from the USDA Soybean Germplasm Collection, Urbana, IL; breeding lines are from B. W. Diers' breeding program.

[b] + and − indicate a virulent and avirulent reaction, respectively

Table 3. Analysis of variance for the main and interactive effects of isoline and biotype on the number of aphids 14 d after infestation

Treatment effect	df	*F* value	*P*
Isoline	7	31.72	<0.0001
Biotype	3	58.99	<0.0001
Trial	1	10.69	0.0014
Isoline × biotype	21	6.03	<0.0001
Trial × isoline	7	0.7	0.6744
Trial × biotype	3	6.94	0.0002
Trial × isoline × biotype	21	0.41	0.9895

Table 4. Mean aphid counts by the four biotypes in the two trials

	Trial 1	Trial 2	*P* value of mean difference
Biotype 1	343	568	0.0059
Biotype 2	628	1,089	0.0046
Biotype 3	1,063	785	0.0225
Biotype 4	1,023	1,553	0.0021

Biotype 1

Our results (Fig. 1) agree with those from previous studies that evaluated the differential response of soybean genotypes with *Rag1*, *Rag2*, and the *Rag1/2* to infestation by this biotype. Biotype 1 has been reported to be avirulent against *Rag1*, *Rag2*, or the *Rag1/2* gene combination (Hill et al. 2004, 2006a,b, 2012). When compared to S1/S2/S3, *Rag1* produced significantly lower aphid numbers, which was not significantly different from that produced on *Rag2*. The aphid numbers recorded on *Rag3* alone were not significantly different from that on S1/S2/S3, suggesting that *Rag3* is ineffective against this biotype; however, when present in combination with *Rag1*, the aphid population was significantly reduced compared to *Rag1* alone. Contrary to what was observed when combined with *Rag1*, *Rag3* did not significantly improve the resistance conferred by *Rag2*. The lowest aphid number was recorded on the *Rag1/3* pyramid; however, the number wasn't significantly different from the aphid populations recorded on *Rag1/2*, *Rag2/3*, and *Rag1/2/3* stacks. These results showed that in all the gene combinations evaluated, the presence of *Rag1* or *Rag2* ensured protection against biotype 1.

Biotype 2

Aphid populations were highest on S1/S2/S3, but the value was not significantly different from those obtained on *Rag1*, *Rag3*, and the *Rag1/3* pyramids (Fig. 2). The colonization of *Rag1* by this biotype agrees with previous findings (Kim et al. 2008); however, the differential colonization of this biotype on *Rag3* in no-choice tests was previously unknown. Our results showed the ineffectiveness of *Rag3* against this biotype when deployed only with *Rag1*. *Rag2* has been previously reported to provide strong antibiosis-type protection against biotype 2 (Kim et al. 2008), and this was confirmed in our test. Only the *Rag2* isoline or those with stacks that include this gene had less colonization than the susceptible line. The *Rag1/2/3* pyramid produced the lowest number of aphids for this biotype, but it was not significantly different than *Rag2* alone.

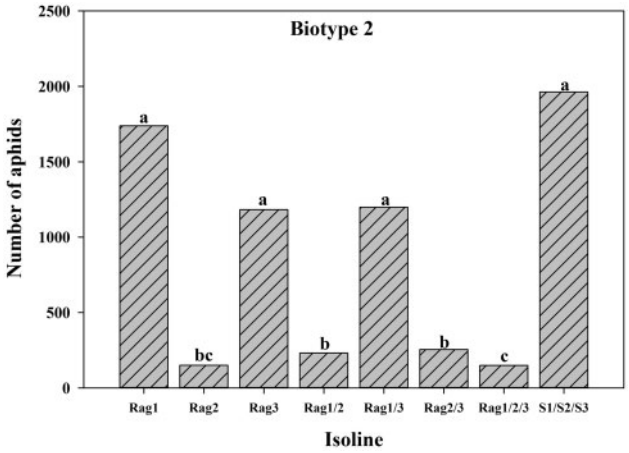

Fig. 2. Differential colonization of the eight soybean isolines with combinations of *Rag* genes by soybean aphid biotype 2 across trials 1 and 2. Means with the same letter are not significantly different ($\alpha = 0.05$). Mean separations are from $\log_{10}$ transformed data.

Biotype 3

Biotype 3 has been reported to overcome the resistance conferred by *Rag2* but is only able to colonize *Rag1* minimally (Hill et al. 2010, Alt and Ryan-Mahmutagic 2013, Pawlowski et al. 2015), and our results are in agreement with those findings (Fig. 3). The number of aphids observed on S1/S2/S3, although numerically higher, was not significantly different from those obtained on *Rag2* and *Rag3*. Aphid colonization on *Rag1* was significantly reduced when compared to the line with no *Rag* gene. We also observed that aphid colonization on *Rag1* alone or on the *Rag1/2* gene combination was numerically lower but not significantly different from the populations observed on *Rag2* and *Rag3*. The *Rag1/3* and *Rag1/2/3* pyramids provided the greatest protection against this biotype based on the significantly lower number of aphids observed.

Biotype 4

Compared to other biotypes, resistance genes showed the least effectiveness in controlling biotype 4. Aphid numbers produced on S1/S2/S3 were not significantly different from those observed on *Rag1*, *Rag2*, *Rag3*, *Rag1/2*, and *Rag1/3* (Fig. 4). Although the number of aphids produced on the *Rag1/2/3* pyramid was the lowest numerically, the value was not significantly different from those obtained from all resistance genes containing isolines except the one carrying *Rag1*. The threefold decrease in aphid population observed on the triple pyramided line when compared to S1/S2/S3 suggests that the combination of all the three dominant genes provides the best protection against this biotype.

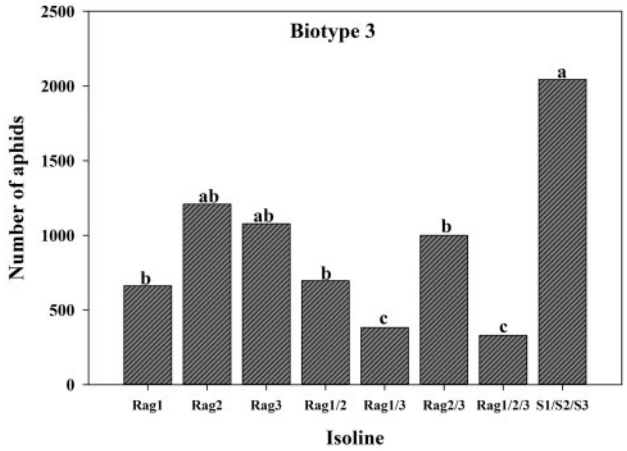

Fig. 3. Differential colonization of the eight soybean isolines with combinations of *Rag* genes by soybean aphid biotype 3 across trials 1 and 2. Means with the same letter are not significantly different ($\alpha = 0.05$). Mean separations are from $\log_{10}$ transformed data.

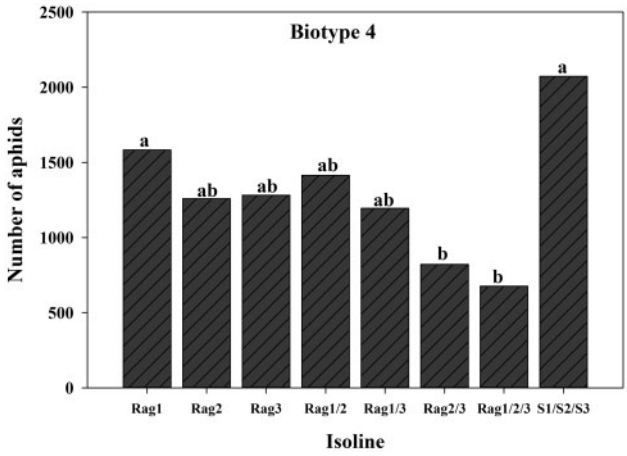

Fig. 4. Differential colonization of the eight soybean isolines with combinations of *Rag* genes by soybean aphid biotype 4 across trials 1 and 2. Means with the same letter are not significantly different ($\alpha = 0.05$). Mean separations are from $\log_{10}$ transformed data.

While the frequencies and distribution of soybean aphid biotypes across North America is unknown at present, the possibility of finding more than one aphid biotype within a geographical location cannot be completely ruled out. For instance, in eastern South Dakota, results from field evaluations of soybean genotypes with *Rag1* or *Rag2* under natural aphid infestations suggested the presence of at least two different biotypes (Bhusal et al. 2013). Different soybean aphid biotypes have also been reported in Michigan (Mensah et al. 2007). A 2-yr study evaluating the geographic distribution of soybean aphid biotypes in the United States and Canada found considerable variability across states and years (Cooper et al. 2015). These observations across different soybean-growing states in North America highlight the importance of stacking aphid resistance genes to ensure the durability of host resistance as a management option for the soybean aphid control. Several field studies have evaluated the durability of lines carrying *Rag1*, *Rag2*, or the *Rag1/2* pyramid to naturally occurring field isolates of the soybean aphid (McCarville and O'Neal 2012, Wiarda et al. 2012, McCarville et al. 2014). While the exact biotypic profiles of the aphid populations were unknown, there was consistency in the observation that a combination of *Rag1* and *Rag2* improved protection over either gene being deployed alone. This suggests the presence of multiple biotypes, especially biotypes 1, 2, and 3. Our results indicate that the *Rag1/2* pyramid provides excellent protection against biotype 1 and biotype 2. Even though the colonization of biotype 3 on *Rag1/2* was statistically comparable to that of *Rag2*, biotype 3 was only able to infest *Rag1/2* minimally as it did *Rag1*. Hill et al. (2010) found a similarity in the response of soybean genotypes with *Rag1* and *Rag1/2* to colonization by biotype 3; however, the aphid populations on these genotypes were significantly lower than that observed on *Rag2*. While we are certain about the purity of the biotype 3 isolate used in this study, the comparable reaction of *Rag2* and *Rag1/2* to this biotype may be due to the significantly lower number of aphids produced by this biotype in the second trial (Table 4). It also is possible that the *Rag2* plants were environmentally stressed, which would have impeded the population growth of biotype 3.

Our results also provide evidence for the effectiveness of the *Rag1/3* gene combination against biotype 1 and biotype 3 but not against biotype 2 or biotype 4. Interestingly, the *Rag1/2/3* pyramided line provided the broadest range of protection against all four soybean aphid biotypes. For example, for biotypes 1 and 2, aphid populations were kept below the economic threshold level of 273 ± 38 aphids per plant (Ragsdale et al. 2007) throughout the 14-d period the aphids were allowed to feed; however, higher aphid numbers were recorded for biotypes 3 (~329 aphids) and 4 (~677 aphids), albeit below the economic injury level of 674 ± 95 aphids per plant (Ragsdale et al. 2007). These findings suggest that in locations with very high frequencies of biotype 3 and biotype 4, yields of soybean lines carrying the *Rag1/2/3* pyramid could be threatened with rising populations of biotypes 3 and 4, which indicate the need for the identification of aphid resistance genes with stronger resistance against biotype 3 and biotype 4.

Because the goal of this study was to evaluate the effects of different aphid-resistant gene combinations to the four known aphid biotypes, it was set up as a no-choice (factorial) experiment to allow us to take a closer evaluation at the interaction between these two factors (soybean isolines and soybean aphid biotypes). Generally, choice and no-choice tests are usually designed to identify antixenosis and antibiosis, respectively, even though a clear distinction between both resistance categories may not always be possible (Smith 2005). Antibiosis-type resistance has been identified as the primary resistance modality for *Rag1* (Hill et al. 2004, Li et al. 2004) and *Rag2* (Mian et al. 2008b, Hill et al. 2009), although a few soybean genotypes with *Rag1* have been found to also express antixenosis-type resistance (Diaz-Montano et al. 2006, Hesler et al. 2007, Hesler and Dashiell 2011). *Rag3* was identified in PI 567543C, a soybean genotype that expresses predominantly antixenosis-type resistance based on the results obtained in no-choice test (Mensah et al. 2005). Therefore, the setup of this study might have prevented the *Rag3* expression of antixenosis when used alone. Even though *Rag3* did not appear to be effective against all four biotypes when used alone, in combination with the *Rag* genes effective against each respective biotype, it provided a stronger antibiosis-type resistance. For example, colonization of biotype 1 on *Rag1/3*, *Rag2/3*, and *Rag1/2/3* were lower than on *Rag1* or *Rag2* alone. Similarly, the combination of *Rag1* with *Rag3* provided a stronger protection against biotype 3 than just *Rag1* alone.

The observed ineffectiveness of *Rag3* alone against the four biotypes is not surprising, as the resistance of PI 567543C to biotype 2 and two unidentified aphid isolates from Michigan was only confirmed in choice tests (Mensah et al. 2005, Mian et al. 2008b,

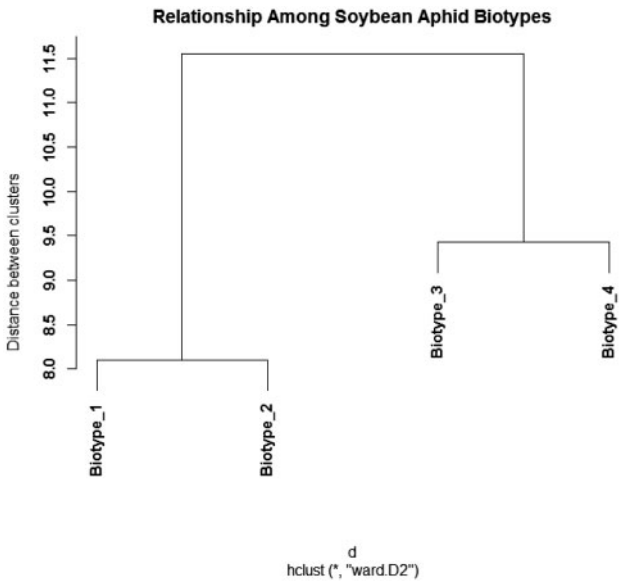

Fig. 5. Cluster dendrogram showing the relationship of the reactions of the soybean aphid biotypes to the different *Rag* gene combinations. Cluster analysis was conducted using the dist and hclust functions from the *stats* package in R.

Zhang et al. 2010). In no-choice experiments conducted by Mensah et al. (2005), PI 567543C also did not provide the same level of protection as Jackson, a soybean genotype that is resistant to biotype 1. Because we did not conduct a choice test, an evaluation of the antixenosis-type resistance of *Rag3* alone and in combination with other *Rag* genes is not possible. Our observations with *Rag3* highlight the importance of conducting preliminary tests to determine the biotype profile of any soybean isolate used in identifying resistance sources or mapping aphid resistance genes.

Results from hierarchical clustering (Fig. 5) provided information about the relationship among the four aphid biotypes in their reaction to the different combinations of *Rag* genes evaluated. Cluster analysis produced two clades; clade 1 consisted of biotype 1 and biotype 2, while clade 2 comprised biotype 3 and biotype 4. These results imply that biotypes 1 and 2 exhibited similarity in their virulence to some or all of the eight soybean isolines carrying different combinations of *Rag* genes. Previous studies have indicated the inability of both biotypes 1 and 2 to colonize *Rag2* (Hill et al. 2010), and from our study, only gene combinations with *Rag2* proved effective against both biotypes. The similarity in avirulence against *Rag2* might explain the grouping of both biotypes within the same clade. The virulence expression of biotype 3 was comparable to that of biotype 4. Moreover, biotype 3 and biotype 4 both exhibited variability in their virulence patterns, which might explain the grouping of both biotypes within the same clade. Biotype 3 and biotype 4 are able to colonize *Rag1*, *Rag2*, and the *Rag1/2* pyramid (Hill et al. 2010, Alt and Ryan-Mahmutagic 2013), and it is unclear how they are different from each other. Compared to biotype 3, we observed higher populations of biotype 4 on all isolines, suggesting that the true differences between these two biotypes may be more related to their aggressiveness.

In conclusion, our results indicate that a three-gene pyramid has the potential for improved soybean aphid management and can thereby reduce the need for insecticide applications. Chandrasena et al. (2015) recently reported the effectiveness of *rag*3 + *rag*1c gene combination against field isolates from Michigan, which, based on the phenotypes of differential hosts, were assumed to comprise both biotypes 3 and 4. Future work should be aimed at evaluating, under field conditions, the differential response of the *Rag1/2/3* pyramid to naturally infesting soybean aphid populations across field locations in North America, as well as the effect of *Rag1/2/3* pyramid on yield and other agronomic traits. Finally, a molecular explanation for the stronger antibiosis-type resistance provided by *Rag3* against a specific biotype when used in combination with *Rag* genes effective against that biotype would improve our understanding of the interaction between *Rag* genes and their potential for the management of different soybean aphid biotypes.

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