Differential Reactions of Soybean Isolines With Combinations of Aphid Resistance Genes \textit{Rag1}, \textit{Rag2}, and \textit{Rag3} to Four Soybean Aphid Biotypes

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Abstract

With the discovery of the soybean aphid (\textit{Aphis glycines} Matsumura) as a devastating insect pest of soybean (\textit{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 (\textit{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 \textit{Rag1}, \textit{Rag2}, and \textit{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 isolate were enumerated 14 d after infestation. All gene combinations, with the exception of \textit{Rag3} alone, provided excellent protection against biotype 1. Isolines with \textit{Rag2} alone or in combination with \textit{Rag1} and \textit{Rag3} had greater levels of resistance to biotype 2 than those with either \textit{Rag1} alone, \textit{Rag3} alone, or the \textit{Rag1/3} pyramid. For biotype 3, the \textit{Rag1/3} and \textit{Rag1/2/3} pyramided lines significantly reduced aphid populations compared with all other gene combinations, while the \textit{Rag1/2/3} pyramid provided the greatest protection against biotype 4. Overall, the \textit{Rag1/2/3} pyramided line conferred the greatest protection against all four biotypes.

Key words: \textit{Aphis glycines}, biotype, soybean

The soybean aphid, \textit{Aphis glycines} Matsumura, is a destructive insect pest of soybean (\textit{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 \textit{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 (Theling 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 (Alt and Ryan-Mahmutagic 2013). This accession was highly resistant to biotypes 1 and 2 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.
Experimental Setup 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 isolate were sown into 15-cm plastic pots filled with soilless potting medium (Sunshine Mix, L1, 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 isolate was confirmed by conducting a TaqMan assay. For this analysis, two representative seedling samples of each isolate 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 isolate, and DNA was extracted using the CTAB method as described by Kaczorowski et al. (2008) using a LightCycler 480 System (Roche Diagnostics, Indianapolis, IN). Williams 82, Dowling, PI 200538, and E10005 were sampled for each isolate, 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 were 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.

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, isolate, and biotype were significant (Table 3). Significant interactions between isolate 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 isolate 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 isolate across the two trials.

| Soybean isolate | 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) |

Table 1. Soybean genotypes evaluated in no-choice experiment and their corresponding Rag gene combination.
Table 2. Phenotypic expression of differential soybean genotypes after infestation with the four soybean aphid biotypes in the pilot study

| Soybean differentials⁵ | Biotypes⁴ | 1 | 2 | 3 | 4 |
|------------------------|-----------|---|---|---|---|
| Williams 82 (no Rag)    | +         | + | + | + |   |
| LD10-5903a (Rag1)      | –         | + | – | + |   |
| LD08-12435a (Rag2)     | –         | – | + | + |   |
| LD12-12734a (Rag1/Rag2)| –         | + |   |   |   |

⁵ Williams 82 was obtained from the USDA Soybean Germplasm Collection, Urbana, IL; breeding lines are from B. W. Diers’ breeding program.
⁴ + 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

| Biotypes | 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   | 1,533   | 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...
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 (Rhusal 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 Dashiel 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,
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 rag3 + rag1c 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 antixenosis-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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