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Pyramiding different aphid-resistance genes in elite soybean germplasm to combat dynamic aphid populations

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Abstract The soybean aphid (*Aphis glycines* Matsumura), an invasive species, has posed a significant threat to soybean [*Glycine max* (L.) Merr.] production in North America since 2001. Use of resistant cultivars is an effective tactic to protect soybean yield. However, the variability and dynamics of aphid populations could limit the effectiveness of host-resistance gene(s). Gene pyramiding is a promising way to sustain host-plant resistance. The objectives of this study were to determine the prevalent aphid biotypes in Michigan and to assess the effectiveness of different combinations of aphid-resistance genes. A total of 11 soybean genotypes with known resistance gene(s) were used as indicator lines. Based on their responses, Biotype 3 was a major component of Michigan aphid populations during 2015–2016. The different performance of *Rag*-"Jackson" and *Rag1*-"Dowling" along with the breakdown of resistance in plant introductions (PIs) 567301B and 567324 may be explained by Biotype 3 or an unknown virulent biotype establishing in Michigan. With the assistance of flanking markers, 12 advanced breeding lines carrying different aphid-resistance gene(s) were developed and evaluated for effectiveness in five trials across 2015 to 2017. Lines with *rag1c*, *Rag3d*, *Rag6*, *Rag3c* + *Rag6*, *rag1b* + *rag3*, *rag1c* + *rag4*, *rag1c* + *rag3* + *rag4*, *rag1c* + *Rag2* + *rag3* + *rag4*, and *rag1b* + *rag1c* + *rag3* + *rag4* demonstrated strong and consistent resistance. Due to the variability of virulent aphid populations, different combinations of Rag genes may perform differently across geographies. However, advanced breeding lines pyramided with three or four Rag genes likely will provide broader and more durable resistance to diverse and dynamic aphid populations.

Keywords Soybean aphid · Biotype · Rag · Marker-assisted selection · Gene pyramiding · Advanced breeding line

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Introduction

Soybean [*Glycine max* (L.) Merr.] is one of the most important crops in North America because of its multiple uses as an animal feed, cooking oil, biofuel, and human protein source. In 2016, the USA ranked first in world soybean production (117.3 million metric tons) with 55.3 million metric tons exported (SoyStats 2016). However, soybean production in North America has been threatened by the soybean aphid, *Aphis glycines*

Matsumura (Hemiptera: Aphididae), an invasive species native to Asia (Wu et al. 2004).

Soybean aphid has aggressively dispersed to all major soybean producing areas in the USA and Canada (Ragsdale et al. 2011) since its discovery in southern Wisconsin in 2000 (Alleman et al. 2002). The direct aphid stylet-feeding on plant sap is the most prominent damage that can cause up to 40% soybean yield loss (Ragsdale et al. 2007). Under heavy infestations, soybean foliage can be stunted, wrinkled, distorted, and wilted; yield components, such as seed size and number, are also reduced (Wu et al. 2004). Transmissions of plant viruses by soybean aphids lead to further yield loss in soybean production (Hill et al. 2001; Clark and Perry 2002). In addition, honeydew secreted by aphids promotes growth of sooty mold on leaves, impairing soybean photosynthesis by blocking sunlight and causing additional yield loss (Malumphy 1997; Lemos Filho and Paiva 2006).

Currently, insecticides are widely used to manage soybean aphids. However, this control method increases production cost, the risk of environmental contamination, and the mortality of beneficial insects (e.g., natural enemies and pollinators) (Ohnesorg et al. 2009; Lundin et al. 2015). The formation of insecticide resistance in soybean aphid populations is also an increasing concern. A more cost-effective and environmentally friendly way to managing soybean aphids is to utilize the native host-plant resistance present in soybean germplasm. Extensive screening of different soybean germplasm pools has identified ~30 plant introductions (PIs) and cultivars with antibiosis (affecting insect biology or reproduction) or antixenosis (non-preference) resistance (Hill et al. 2004; Li et al. 2004; Yang et al. 2004; Mensah et al. 2005; Hesler et al. 2007; Mian et al. 2008a; Fox et al. 2014).

Despite the high number of PIs and cultivars identified as resistant to soybean aphid, many share same resistance genes or alleles; this might be due to the genetic bottleneck of soybean in North America (Hyten et al. 2006). Aphid-resistance QTLs identified in North America are designated as Rag (Resistance to Aphis glycines); different resistance alleles have been uncovered at six loci, *Rag1* to *Rag6*. The dominant antibiosis-resistant *Rag1*/*Rag* (Hill et al. 2006a, b; Li et al. 2007), the recessive antibiosis-resistant *rag1c* (Zhang et al. 2009), and *rag1b* (Bales et al. 2013) were mapped to chromosome 7 between markers Satt463 and Satt567. Additionally, *Rag1* was fine-mapped to a 115-kb interval between markers SNPKS9-3 and SNPKS5 (Kim et al. 2010a). The dominant *Rag2* (Mian et al. 2008b; Hill et al. 2009) and *Rag5* (Jun et al. 2012) were mapped to a genomic region between Satt334 and Sct_033 on chromosome 13, but they confer different resistance modality (antibiosis vs. antixenosis) (Michel et al. 2010; Jun et al. 2012). *Rag2* later was refined to a 54-kb interval between markers SNP46169.7 and SNP21A (Kim et al. 2010b). Aphid resistance in 20 PIs is associated with *Rag2*, indicating *Rag2* may be a major aphid-resistance source in the USDA soybean germplasm collection (Fox et al. 2014). The recessive antibiosis *rag4* was mapped to a different location (between Satt649 and Satt348) on chromosome 13 (Zhang et al. 2009). Jun et al. (2013) identified two major QTLs (QTL_13_1 and QTL_13_2) near *Rag2* and *rag4*, and a minor QTL (QTL_6_1) on chromosome 6; these three QTLs suggested PI 567324 has oligogenic antixenosis resistance to soybean aphids. Six aphid-resistance QTLs/alleles were detected in a region between markers Satt285 and Satt654 on chromosome16, and designated *Rag3* (antixenosis), *Rag3b* (antibiosis), *rag3* (antibiosis), *Rag3c* (antibiosis), *Rag3d* (antibiosis), and *Rag3e* (antixenosis) (Zhang et al. 2010, 2013; Bales et al. 2013; Du 2016; Zhang et al. 2017a). Additionally, *Rag3c* was delimited to a 150-kb interval between markers Gm16-3 and Gm16-5 (Zhang et al. 2017b). The antibiosis-resistance gene *Rag6* was refined to a 49-kb interval between markers Gm08-15 and Gm08-17 on chromosome 8 (Zhang et al. 2017a, b).

The biggest concern of employing host-plant resistance is the breakdown of single resistance genes by virulent biotypes. To date, four different soybean aphid biotypes have been discovered in North America. Biotype 1 is avirulent to all Rag genes (Hill et al. 2004). Biotype 2 can reproduce on soybean plants with *Rag1* (Kim et al. 2008). Biotype 3 readily colonizes soybeans with *Rag2*; it also reproduces on soybeans with *Rag1* in choice tests (Hill et al. 2010). A recent multi-year study reported that the occurrence of soybean aphid biotypes was highly variable across both locations and years in the Midwestern USA (Cooper et al. 2015). The variability and dynamics of aphid populations could limit the durability of effectiveness of a single resistance gene. In this study, PI 567541B (a natural pyramid of *rag1c*/*rag4*) and PI 567598B (a natural pyramid of *rag1b*/*rag3*) demonstrated the widest spectrum of resistance to aphids across locations and years (Cooper et al. 2015). Similarly, other studies showed that soybean

lines with artificial pyramids of *Rag1/Rag2* had significantly lower aphid colonization than lines with the *Rag1* or *Rag2* gene alone (Wiarda et al. 2012; McCarville et al. 2014). However, Alt and Ryan-Mahmutagic (2013) reported a new soybean aphid biotype, Biotype 4, capable of colonizing PI 567541B, PI 567598B, and soybean lines with the pyramid of *Rag1*/*Rag2*. There are likely more virulent biotypes not yet discovered. Therefore, integrating cultivars with multiple resistance genes, particularly with different modes of action, is important to achieve a broader and more durable resistance against different aphid populations.

The Soybean Breeding and Genetics Program at Michigan State University (MSU) has identified seven soybean accessions carrying resistant alleles at four resistance loci, including *Rag1*, *Rag3*, *Rag4*, and *Rag6* (Bales et al. 2013; Zhang et al. 2009, 2010, 2013; Zhang et al. 2017a; Du 2016). Zhang et al. (2017b) refined *Rag6* to a 49-kb interval between markers Gm08-15 and Gm08-17, and *Rag3c* to a 150-kb interval between markers Gm16-3 and Gm16-5. Fine mapping studies of five other aphid-resistance QTLs (*rag1b*, *rag1c*, *rag3*, *Rag3d*, and *rag4*) refined their genomic locations and identified closely linked SNP markers (unpublished data). With the assistance of these SNP markers, a pool of improved soybean germplasm with different combinations of aphid-resistance genes was developed. The objectives of this study were to (1) assess the introgression of aphid-resistance gene(s) using the Illumina Infinium SoySNP6K iSelect BeadChip, (2) determine the prevalence of soybean aphid biotypes in Michigan, and (3) assess the effectiveness of different Rag gene combinations against Michigan aphid populations.

Materials and methods

Plant materials

A total of 11 resistant soybean genotypes, including "Jackson," LD05-16060 (*Rag1*-"Dowling"), PI 243540, PI 567543C, PI 567585A, PI 567597C, PI 567598B, PI 567541B, PI 567301B, E08934 (derived from *G. soja* 85-32) (Zhang et al. 2017a), and PI 567324, were used as indicator lines to screen for aphid biotypes in field-cage trials during the summers of 2015 and 2016. LD05-16060 was an advanced breeding line carrying the *Rag1* gene from "Dowling" and was developed by Dr. Brian Diers at University of Illinois Urbana-Champaign (UIUC).

In total, 12 advanced breeding lines (Table 1) carrying different Rag gene(s) were developed through marker-assisted selection (MAS) with markers flanking the initial-mapped or fine-mapped regions (Li et al. 2007; Hill et al. 2009; Kim et al. 2010a, b; Zhang et al. 2017a, b; unpublished data). LD05-16657a with *Rag1* and LD08-12430a with *Rag2* were developed by Dr. Brian Diers at UIUC while "E" lines were developed at MSU in East Lansing, Michigan, with different combinations of *rag1b*, *rag1c*, *Rag2*, *Rag3c*, *Rag3d*, *rag3*, *rag4*, and *Rag6* (Hill et al. 2009; Zhang et al. 2009; Bales et al. 2013; Du 2016; Zhang et al. 2017a) (Table 1). E00003 has been consistently susceptible to Michigan aphids over the years (Zhang et al. 2017a, b), and it served as a susceptible check in this study.

DNA extraction and the Illumina Infinium SoySNP6K iSelect BeadChip genotyping analyses to assess the effectiveness of MAS

Leaf tissue was collected from a seedling of each advanced breeding line. Genomic DNA from each sample was extracted using the modified CTAB protocol described by Kisha et al. (1997), and genotyped using the Illumina Infinium SoySNP6K iSelect BeadChip (Illumina, San Diego, CA), which consists of 5403 single nucleotide polymorphisms (SNPs) selected from the Illumina Infinium SoySNP50K iSelect BeadChip (Song et al. 2013). The genome-wide SNP distribution of the Illumina Infinium SoySNP6K iSelect BeadChip was visualized with R (R Development Core Team 2016) (Supplementary Fig. 1). Genotypes were called using the program GenomeStudio (1.9.4 version, Illumina, San Diego, CA). Each SNP was coded based on the standard codes for nucleotides derived from the International Union of Pure and Applied Chemistry. The quality of each SNP was checked as previously reported (Yan et al. 2010). SNPs with call rate < 80% across all samples were removed from the dataset. The genome-wide SNP data of each advanced breeding line was compared to that of the original aphid-resistance-gene(s) donor, mined from the public SoySNP50K iSelect BeadChip data on SoyBase (Grant et al. 2010) except for E12901. Graphic representation of genomic regions of interest from each sample was drawn with the program FlapJack (Milne et al. 2010). SNP markers that are monomorphic between the original donor line and the

Table 1 Pedigree information of advanced breeding lines integrated with different Rag genes

Line	Rag gene(s)	Pedigree information
E00003	None	C95001 (AP1995) x C94043 (PIO 9281)
LD05-16657a	*Rag1*	Dwight (3) x (Dowling x Loda)
E14922	*rag1c*	[E00003 x (SDX00R-039-42 x PI 567541B)] x E00003
LD08-12430a	*Rag2*	LD02-4485(2) x (Ina x PI 200538)
E11950	*rag3*	(Titan x PI 567598B) x LD05-16060
E12904	*Rag3d*	(Skylla x PI 567585A) x Skylla
E14923	*Rag6*	(Skylla x LD01-7323) x [E00003 x (Jiyu 71 x *G.soja* 85-32)]
E14912	*rag1b, rag3*	[LD01-5907 x (Titan x PI 567598B)] x LD02-4485
E13369	*rag1c, rag4*	E07051 x {[E00003 x (SDX00R-039-42 x PI 567541B] x E00003)}
E14902	*Rag3c*, *Rag6*	(Skylla x LD01-7323) x [E00003 x (Jiyu 71 x *G.soja* 85-32)]
E13901	*rag1c, rag3*, *rag4*	{(Skylla x PI 567598B) x [Skylla x (SDX00R-039-42 x PI 567541B)]} x E07051
E13903	*rag1c*, *Rag2, rag3*, *rag4*	{[Skylla x PI 567598B] x [Skylla x (SDX00R-039-42 x PI 567541B)]} x LD08-12430a
E14919	*rag1b*, *rag1c, rag3*, *rag4*	[E00003 x (SDX00R-039-42 x PI 567541B)] x [LD01-5907 x (Titan x PI 567598B)]

*Donors of aphid-resistance genes are indicated with underlines

elite parental line were filtered. At each SNP of the advanced breeding line, the allele same as that of the original donor was assigned with the black color, and the alternative allele was assigned with the gray color.

Evaluation for soybean aphid resistance

Indicator lines and the advanced breeding lines were evaluated in choice tests in field-cage trials (Mensah et al. 2005) during the summers of 2015 and 2016. All the lines were planted in a randomized complete block design with three replications in a 12.2 × 18.3 m aphid- and predator-proof polypropylene cage (Redwood Empire Awning Co., Santa Rosa, CA) on the Agronomy Farm of MSU, East Lansing, Michigan. In each replication, 15 seeds from each line were planted in a single 60 cm long plot with 60 cm row spacing.

The advanced breeding lines were also evaluated in the greenhouse choice tests (Mensah et al. 2005) in the Plant Sciences greenhouse at MSU during fall 2015, spring 2016, and spring 2017. Eight seeds from each line were planted in a 125-mm deep, 105-mm-diameter plastic pot. All the lines were arranged in a randomized complete block design with three replications. The greenhouse was maintained at 26/15 °C day/night with supplemental light (14 h/day) provided by sodium vapor lights.

Soybean aphids were collected from multiple locations across Michigan in the early summer of each testing year and maintained on susceptible soybean plants (E00003) in field cages or the greenhouse. In each trial, each plant was artificially infested with two wingless aphids at the soybean V_2 stage (Fehr and Cavinese 1977). Each plant was visually rated for aphid resistance using a 0–4 scale (Mensah et al. 2005) when the susceptible check reached rating of 3.0 (usually 3 weeks after the initial infestation). Criteria of the 0–4 scale are as follows: 0 = no aphids; 0.5 = fewer than 10 aphids; 1 = 11–100 aphids; 1.5 = 101–150 aphids; 2 = 151–300 aphids; 2.5 = 301–500 aphids; 3 = 501–800 aphids, leaves and stems are covered with aphids, leaves appear slightly curly and shiny; 3.5 = more than 800 aphids, the plant appears stunted with curled yellow leaves, the plant is covered with few cast skins, no sooty mold; 4 = more than 800 aphids, the plant appears stunted with severely curled yellow leaves, the plant is covered with cast skins and sooty mold (Mensah et al. 2005). A damage index (DI) for each replication of each line was calculated as DI (%) = ∑ (rating value × no. of plants in the category) / (4 × total no. of plants) × 100 (Mensah et al. 2005). The DI ranged from 0% (no infestation) to 100% (most severe infestation). In each trial, the average DI of each line from three replications was analyzed with one-way analysis of variance (ANOVA) at a significance level of 0.05 followed by paired wise comparisons using the PROC GLM function in SAS 9.4 (SAS Institute, Cary, NC). Lines with DI less than 37.5% were considered as aphid resistant (Zhang et al. 2017a, b).

Results and discussion

Data from the Illumina Infinium SoySNP6K iSelect BeadChip verified the successful introgressions of all targeted aphid-resistance genes

As shown in Fig. 1, genomic regions inherited from the original donor are indicated with the black color whereas genomic regions from the elite germplasm are presented in gray color. Targeted aphid-resistance genes with their published genomic locations (Glyma.Wm82.a1) were listed for each advanced breeding line. Unpublished fine-mapped regions of some Rag genes (including *rag1b*, *rag1c*, *rag3*, *rag4*) were indicated with rectangle boxes. When inspecting the regions of interest, all targeted aphid-resistance genes were successfully integrated into these advanced breeding lines, which verified the different Rag gene combination in each of the advanced breeding lines. The original genome-wide SNP data of each advanced breeding line along with E12901 (the donor of *Rag6* and *Rag3c*) were presented in Supplementary Table 1 and shared publicly.

Indicator lines suggested Biotype 3 and undescribed virulent biotype(s) prevailing in Michigan

In both the 2015 and 2016 field-cage trials, LD05-16060 (*Rag1*), PI 243540, PI 567301B, and PI 567324 were heavily colonized by aphids collected from Michigan fields and their DIs (ranging from 61.7 to 79.2%) were not significantly different from the susceptible check, E00003 (DIs ~ 79.2 to 83.3%) (Fig. 2 and Table 2). PI 567585A was moderately resistant in 2016 (DI of 43.3%), although it performed better in 2015 (Fig. 2 and Table 2). The remaining soybean genotypes including "Jackson" showed strong resistance (DIs ranging from 12.5 to 33.3%) to the same aphid populations in both field trials (Fig. 2 and Table 2).

"Dowling"(*Rag1*) and "Jackson"(*Rag*) were reported as overcome by Biotype 2 in both choice and no-choice tests (Kim et al. 2008). Biotype 3 aphids readily colonized *Rag2* soybeans in choice and no-choice tests as well as *Rag1* soybeans in choice tests (Hill et al. 2010). Alt and Ryan-Mahmutagic (2013) discovered a new biotype, Biotype 4, capable of colonizing PI 567541B and PI 567598B. In our study, the *Rag1* (LD05-16060) and *Rag2* (PI 243540) lines were readily colonized by aphids; in contrast, the *Rag* line ("Jackson"), PI 567541B, and PI 567598B maintained strong resistance. This suggests that Biotype 3 aphids were a major component of the collected aphid populations in Michigan during 2015 and 2016.

The response of "Jackson" to Biotypes 3 or 4 is unknown as it was not included in the previous aphid biotype studies by Hill et al. (2010) and Alt and Ryan-Mahmutagic (2013). In our study, "Jackson" performed differently than LD05-16060 (carrying *Rag1*-" Dowling') in both years; it showed a strong resistance in 2015 and a very strong resistance in 2016 whereas LD05-16060 was consistently as susceptible as E00003. In a regional investigation conducted by Cooper et al. (2015), "Jackson" was characterized as resistant in multiple states (SD, IA, MI, and OH) whereas "Dowling" was susceptible in all ten participating states in the year of 2010. Zhang et al. (2017a) also observed that "Jackson' was resistant whereas "Dowling" was susceptible in Michigan during 2010. Combining the evidences from Cooper et al. (2015) and Zhang et al. (2017a), the different reactions of these two varieties to aphid populations in some years (2010, 2015, and 2016) suggested that *Rag* and *Rag1* themselves are likely different, despite being mapped to a similar genomic region (Li et al. 2007). They could be allelic at a same locus or different QTLs located closely. "Jackson" showed strong resistance to aphid populations that were primarily Biotype 3 in our field trials during 2015 and 2016, which suggests Biotype 3 is likely not able to overcome the resistance in "Jackson." Further study on the response of "Jackson" to Biotype 3 is needed to exam this hypothesis. It is also possible that the different performance of *Rag1* and *Rag* in the present study was due to an undescribed aphid biotype capable of colonizing *Rag1* but not *Rag* soybeans. Single clones of Michigan aphids will be tested on "Dowling" and "Jackson" to explore this possibility.

Mian et al. (2008a) reported that PI 567301B had strong antixenosis resistance to Biotypes 1 and 2, controlled by a major QTL (*Rag5*) and a minor QTL on chromosome 8 (Jun et al. 2012). Similarly, Mian et al. (2008a) reported that PI 567324 showed moderate antixenosis resistance to Biotype 1 and strong resistance to Biotype 2, contributed by QTL13_1 mapped closely to *Rag2*, QTL13_2 mapped closely to *rag4*, and a minor QTL_6_1 on chromosome 6 (Jun et al. 2013). Jun et al. (2013) suggested that the oligogenic resistance in PI 563724 would provide broader and more durable aphid resistance compared to lines with a single aphid-

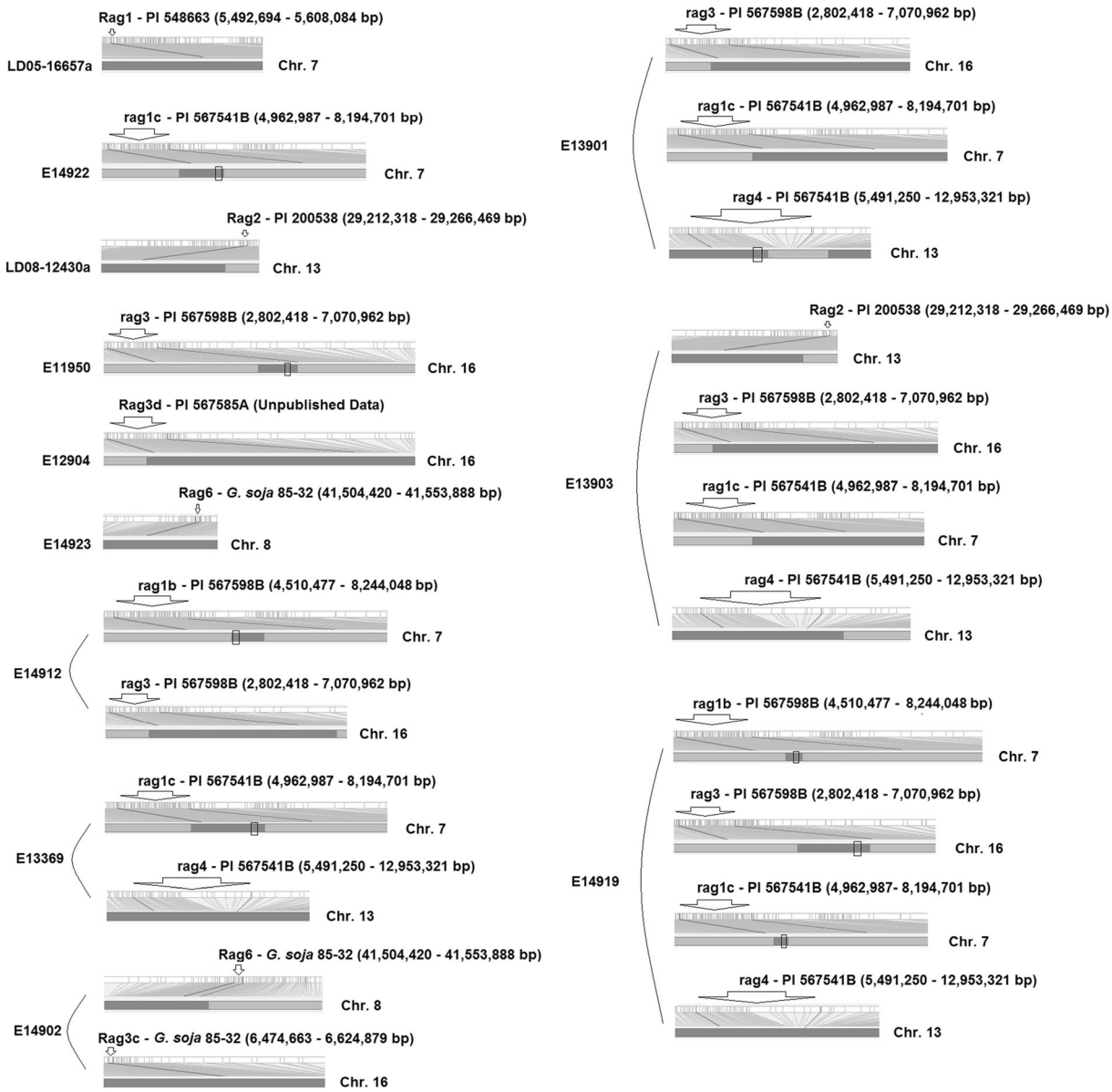

Fig. 1 Graphic representation of genomic region(s) of interest for each advanced breeding line. Genomic regions inherited from the original donor(s) of the aphid-resistance gene(s) are presented in black while genomic regions from the susceptible elite background are presented in gray. Targeted aphid-resistance genes with their published genomic locations are listed for each advanced breeding line. Unpublished fine-mapped regions of some Rag genes (including *rag1b*, *rag1c*, *rag3*, *rag4*) are indicated with rectangle boxes. The genomic locations are according to Glyma.Wm82.a1 on SoyBase (Grant et al. 2010)

resistance gene. However, in our field trials during 2015 and 2016, both PI 567301B and PI 563724 were heavily colonized by aphids. Although the reaction of these PIs to other biotypes has not been tested, their high damage indices (ranging from 68.3 to 75%) in our study could be explained by their susceptibility to Biotype 3 aphids which appeared to predominate the aphid population in 2015 and 2016; it also could be due to an undescribed virulent biotype in Michigan. PI 567301B and PI 563724 will be tested with Biotype 3 and/or single clones isolated from Michigan aphid populations to further investigate the hypotheses.

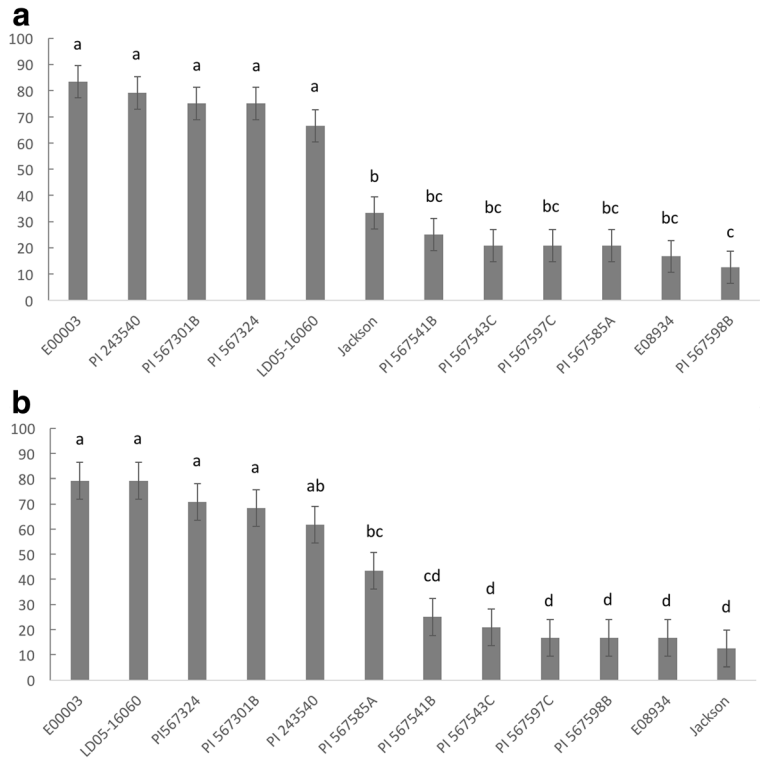

Fig. 2 Aphid damage indices (%) of a susceptible check (E00003) and indicator lines used to screen for soybean aphid biotypes in **a** 2015 and **b** 2016 field-cage trials. Bars with same letter(s) are not significantly different at $P < 0.05$ in each trial

Lines with rag1c or Rag3d or Rag6 or pyramided Rag genes showed strong and broad resistance

As shown in Fig. 3, several soybean lines with a single aphid-resistance gene were readily colonized by aphids in our study. LD05-16657a with *Rag1* and LD08-12430a with *Rag2* had severe aphid damages (DI ~ 66.8 to 88.3%) in all trials across 2015–2017 (Table 3), which was consistent with the performance of indicator lines, LD05-16060 (*Rag1*) and PI 243540 (*Rag2*). E11950 with *rag3* showed strong resistance in all the greenhouse trials but had moderate aphid damages (DI ~ 42.2 to 60%) in the field trials (Table 3), whereas the original donor, PI 567598B, had very strong

Table 2 Aphid damage indices (%) of indicator lines in field trials in Michigan, 2015–2016

Line	Rag genes	Mean soybean aphid damage index (%)*	
		Field 2015	Field 2016
E00003	None	83.3a	79.2a
LD05–16060	*Rag1*	66.7a	79.2a
PI 243540	*Rag2*	79.2a	61.7ab
PI 567301B	*Rag5* + *QTL_8*	75a	68.3a
PI 567324	*Rag2'* + *rag4'* + *QTL_6_1*	75a	70.8a
Jackson	*Rag*	33.3b	12.5d
PI 567541B	*rag1c* + *rag4*	25bc	25cd
PI 567543C	*Rag3*	20.8bc	20.8d
PI 567597C	*Rag3e*	20.8bc	16.7d
PI 567585A	*Rag3d*	20.8bc	43.3bc
E08934	*Rag6* + *Rag3c*	16.7bc	16.7d
PI 567598B	*rag1b* + *rag3*	12.5c	16.7d

*DI (%) followed by same letter(s) are not significantly different at $P < 0.05$ in each trial

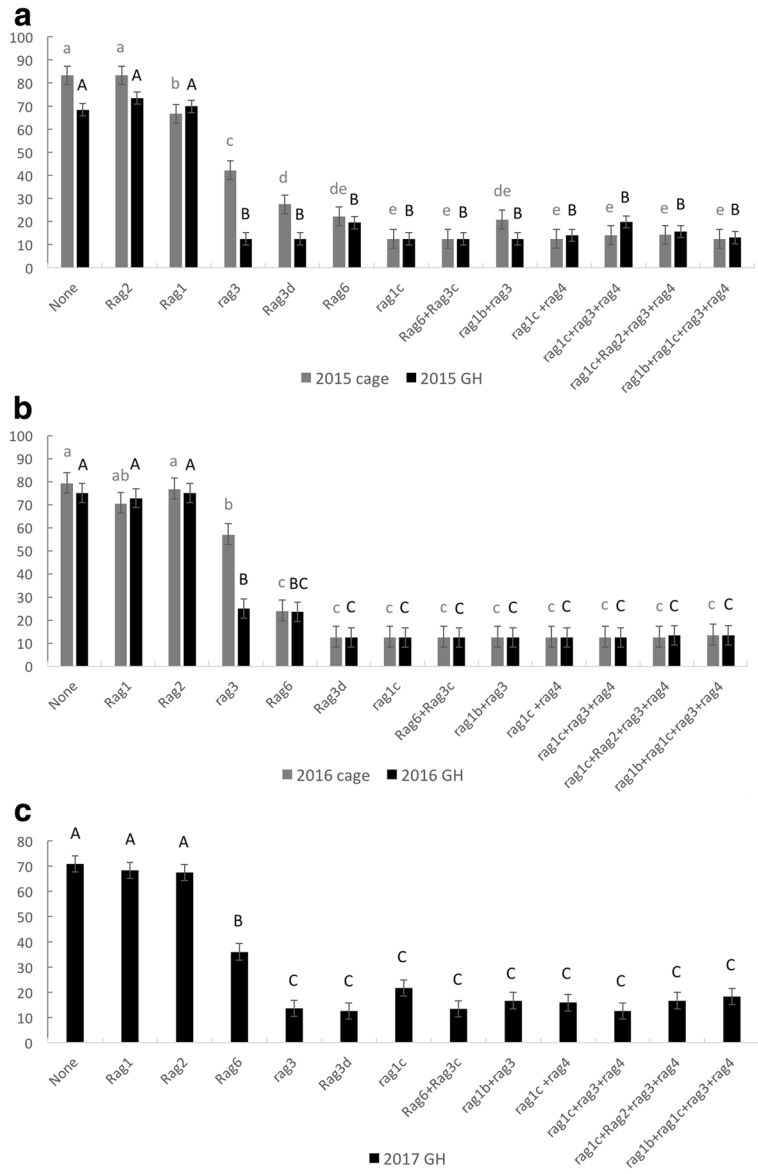

Fig. 3 Aphid damage indices (%) of a susceptible check (E00003) and the advanced breeding lines with different combinations of aphid-resistance gene(s) in **a** field-cage and greenhouse trials in 2015, **b** field-cage and greenhouse trials in 2016, and **c** a greenhouse trial in 2017. Damage indices from the field-cage trial were presented with gray bars followed by lowercase letters in **a** and **b**. Damage indices from the greenhouse trial were presented with black bars followed by uppercase letters in **a** and **b**. Within each trial, bars with same letter(s) are not significantly different at $P < 0.05$

resistance in the field trials (DI ~ 12.5 to 16.7%) (Table 2). PI 567598B also had the lowest frequency (18%) of aphid colonization across 11 locations during 2008–2010 (Cooper et al. 2015). Combining the results from Cooper et al. (2015) and the present study, the pyramid of *rag1b*/*rag3* is critical to provide soybean with broad and durable resistance.

E14923 with *Rag6* alone was highly resistant (DI ~ 19.5 to 23.9%) to aphids across all trials during 2015–2016. However, its damage index (36.0%) in 2017 greenhouse trial was slightly below the resistance threshold (DI ~ 37.5%), and it was statistically greater than those of the remaining resistant lines (Fig. 3c and Table 3). The original donor, E08934 (*Rag6* + *Rag3c*), and the advanced breeding line, E14902 (*Rag6* + *Rag3c*), exhibited very strong and consistent resistance (DI ~ 12.5 to 16.7%) across all trials (Tables 2 and 3). Collectively, *Rag6* alone offers a strong resistance; however, the pyramid of *Rag6*/*Rag3c* provides a stronger and more durable resistance.

E12904 with *Rag3d* appears to have a consistent stronger resistance compared to its original donor, PI 567585A. It displayed a strong resistance (DI ~ 12.5 to 27.4%) across all five trials during 2015–2017 (Fig. 3

Table 3 Aphid damage indices (%) of advanced breeding lines in field and greenhouse trials in Michigan, 2015–2017

Line	Rag genes	Mean soybean aphid damage index (%)*				
		Field 2015	Greenhouse 2015	Field 2016	Greenhouse 2016	Greenhouse 2017
E00003	None	83.3a	68.5A	79.2a	75A	70.8a
LD05-16657a	*Rag1*	66.8b	70A	70.5ab	72.8A	68.3a
LD08-12430a	*Rag2*	83.3a	73.5A	76.7a	75A	67.5a
E11950	*rag3*	42.2c	12.5B	60.0b	25B	13.5c
E14923	*Rag6*	22.2de	19.5B	23.9c	23.6BC	36.0b
E12904	*Rag3d*	27.4d	12.5B	12.5c	12.5C	12.5c
E14922	*rag1c*	12.5e	12.5B	12.5c	12.5C	21.7c
E14902	*Rag3c* + *Rag6*	12.5e	12.5B	12.5c	12.5C	13.3c
E14912	*rag1b* + *rag3*	20.8de	12.5B	12.5c	12.5C	16.7c
E13369	*rag1c* + *rag4*	12.5e	14.1B	12.5c	12.5C	15.8c
E13901	*rag1c* + *rag3* + *rag4*	14.1e	19.8B	12.5c	12.5C	12.5c
E13903	*rag1c* + *Rag2* + *rag3* + *rag4*	14.2e	15.6B	12.5c	13.3C	16.7c
E14919	*rag1b* + *rag1c* + *rag3* + *rag4*	12.5e	13.0B	13.3c	13.3C	18.2c

*DI (%) followed by same letter(s) are not significantly different at $P < 0.05$ in each trial

and Table 3). However, PI 567585A had moderate aphid damage (DI ~ 43.3%) in 2016 field trial even though it had a lower damage index (20.8%) in 2015 field trial (Fig. 2 and Table 2). The consistent strong resistance effect of *Rag3d* in E12904 may be attributed to the elite genetic background; some background gene(s) may up-regulate the expression of *Rag3d*.

Across all five trials during 2015–2017, E14922 with *rag1c* showed a consistent strong resistance (DI ~ 12.5 to 21.7%) whereas LD05-16657a with *Rag1* was consistently susceptible (DI ~ 66.8 to 72.8%) (Fig. 3 and Table 3). The strong resistance provided by *rag1c* alone suggested that *rag1c* is a different gene or allele from *Rag1* even though they were mapped in close proximity (Li et al. 2007; Zhang et al. 2009; Kim et al. 2010a). This conclusion is consistent with the genotypic evidence collected by Zhang et al. (2009); the band patterns of SSR markers flanking *rag1c* were distinctive between PI 567541B and "Dowling."

Among the resistant soybean genotypes tested by Cooper et al. (2015), PI 567541B and PI 567598B demonstrated the widest spectrum of resistance to aphid populations across North America during 2008–2010; the broad resistance was deduced contributed by the natural pyramids of two resistance genes in these two PIs. However, PI 567541B and PI 567598B were later found fully colonized by Biotype 4 (Alt and Ryan-Mahmutagic 2013). In our study, E14912 (*rag1b* + *rag3* from PI 567598B) and E13369 (*rag1c* + *rag4* from PI 567541B) showed very strong resistance across 2015 to 2017 (Fig. 3 and Table 3); however, their resistance might be limited in geographic regions that have a higher pressure of Biotype 4 or other undescribed virulent biotypes.

rag1c and *rag3* are the two major genes controlling aphid resistance in PI 567541B and PI 567598B, respectively (Zhang et al. 2009; Bales et al. 2013). Additionally, Chandrasena et al. (2015) detected a significant additive × additive interaction between *rag1c* and *rag3*, contributing up to 24% of the phenotypic variation in aphid resistance. To achieve a broader and more durable resistance, additional aphid-resistance gene(s) were pyramided with *rag1c* + *rag3*. Advanced breeding line E13901 was pyramided with three aphid-resistance genes, including *rag1c*, *rag3*, and *rag4*. Compared to E13901, E13903 has one more aphid-resistance gene, *Rag2*, to provide additional resistance. E14919 has all four genes from PI 567541B and PI 567598. All these advanced breeding lines (E13901, E13903, and E14919) pyramided with multiple aphid-resistance genes had very strong and consistent resistance to aphid populations in Michigan across 2015–2017 (Fig. 3 and Table 3), and they are expected to be strong and durable when combating diverse and dynamic aphid populations across geographic regions.

Conclusion

The utilization of host-plant resistance is an effective way to control soybean aphids. However, the aphid resistance provided by *Rag1* soybeans, PI 243540 (*Rag2*), PI 567301B (*Rag5*), and PI 567324 (*Rag2'* + *rag4'* + *QTL_6_1*) was overcome by aphids in our field trials during 2015 and 2016. The high damage indices of PI 567301B and PI 567324 could be explained by their susceptibility to Biotype 3 aphids which appeared to be prevalent in our field trials. In contrast to the susceptibility of *Rag1* soybeans, "Jackson" maintained strong resistance in the field trials during 2015 and 2016. Coupled with the similar evidences from Cooper et al. (2015) and Zhang et al. (2017a), *Rag1* and *Rag* are likely different loci or alleles, which may be distinguished by Biotype 3. In addition, it is possible that an undescribed virulent biotype prevalent in our field trials caused the susceptibility of PI 567301B and PI 567324 and the different responses from *Rag1* soybeans and "Jackson." Biotype 3 and single isolates of Michigan aphids will be tested on these soybean genotypes to further exam the hypotheses.

Advanced breeding lines with single aphid-resistance genes, such as *rag1c*, *Rag3d*, and *Rag6*, showed very strong resistance to Biotype 3 across trials during 2015–2017. The strong resistance provided by *rag1c* suggested that it is a different locus or allele from *Rag1* even though they were mapped closely. According to a regional study by Cooper et al. (2015), soybean aphids have a high degree of virulence diversity in North America, which means the effectiveness of a single aphid-resistance gene is likely limited by soybean aphid virulence variability.

Advanced breeding lines pyramided with two aphid-resistance genes, such as *rag1b* + *rag3*, *rag1c* + *rag4*, and *Rag3c* + *Rag6*, demonstrated strong resistance in Michigan. Although Biotype 3 dominated in our trials, there is variability in soybean aphid populations from year-to-year across the Midwest, and undescribed biotypes are likely yet to be identified. Lines with multiple Rag genes, such as *rag1c* + *rag3* + *rag4*, *rag1c* + *Rag2* + *rag3* + *rag4*, and *rag1b* + *rag1c* + *rag3* + *rag4*, likely will provide broader and more durable resistance to diverse and dynamic aphid populations. The advanced breeding lines with different combinations of Rag genes developed in this study are significant resources for breeders to develop varieties to combat different aphid populations across many geographies.

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Compliance with ethical standards

This work complies with the current laws of the USA.

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

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