SSR MARKERS AND SOYBEAN APHID RESISTANCE IN A CAGED FIELD TRIAL OF F_3-DERIVED SOYBEAN LINES

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ABSTRACT

The soybean aphid has recently become an economically important pest of soybean in South Dakota and the surrounding region. Aphid resistance and associated molecular markers have been identified in the cultivar Dowling and other lines. Our objective in this work was to confirm the utility of simple sequence repeat (SSR) markers that can be used to assist in introgressing the aphid resistance gene Rag1 from the cultivar Dowling into South Dakota soybeans. F_3-derived lines from selected F_3 plants from two populations of the 3-way cross (SDX00R-039-42XPI71506)XDowling and one population of the 3-way cross (SD01-5RXPI71506)XDowling were evaluated for resistance to soybean aphid in a field cage trial in 2006, and SSR markers flanking the *Rag1* gene on the M linkage group were characterized in each line. Satt540 and Satt245 showed the segregation ratios expected for these linked loci, and aphid resistance was significantly associated with the Dowling alleles at these SSR markers.

INTRODUCTION

Genes for resistance to soybean aphid from the cultivars Dowling and Jackson have been localized to linkage group M, and associated markers have been described (Hill et al. 2004, 2005; Li et al. 2007). Additional sources of aphid resistance have also been identified in the soybean germplasm (Diaz-Montano et al. 2006; Mensah et al. 2005; Hesler et al. 2007). QTLs associated with resistance to other insects have also been localized to M and other linkage groups (Narvel et al. 2001;Terry et al. 2000; Walker et al. 2004; Zhu et al. 2006).

The objectives of the work described here were to identify lines with aphid resistance from Dowling as well as potential aphid resistance from PI71506, to confirm the utility of the simple sequence repeat (SSR) markers identified by Li et al. (2007) for selection of *Rag1* gene resistance, and to determine whether aphid resistance from PI71506 was associated with the Rag1 gene or other genetic loci.

MATERIALS AND METHODS

Plant material. There were three 3-way cross populations, produced from crosses among 4 lines. The lines used were two SD soybean breeding lines, SDX00R-039-42 and SD01-5R; a Plant Introduction (PI) line, PI71506; and the aphid resistant variety Dowling (Hill et al. 2005). Crosses of SDX00R-039-42 X PI71506 and SD01-5R X PI71506 were made in summer 2004 in the field. The F_1 seeds were planted in the greenhouse during winter 2004-2005, and the resulting F1 plants were crossed to Dowling. The resulting 3-way F_1 plants were selfed to produce 3-way cross F_2 plants. These were grown in the greenhouse in winter 2005-06 and bioassayed for aphid resistance in controlled greenhouse infestations. There were 3 F_2 plants on which aphids did not survive: 2 plants from the cross (SDX00R-039-42XPI71506)XDowling, and one plant from the cross (SD01-5RXPI71506)XDowling. Seeds were harvested from these 3 selected plants, and the resulting F_3 families were grown in the greenhouse in winter 2005-06, and bioassayed for aphid antibiosis in the greenhouse and for aphid antixenosis by lab assays. Plants that did not support aphid survival and which were not preferred by aphids were selected, and seed from these plants were composited as F_3-derived lines, which were grown in the following caged aphid trial in summer 2006.

Caged field trial. The F_3-derived lines were grown in 3-foot rows in a 64' x 42' cage that was designed to keep aphids in and exclude predators, in the field at the SDAES research farm near Aurora SD. Each F_3-derived family was grown as a separate block. The F_3-derived lines from the first and second plants derived from SDX00R-039-42XPI71506)XDowling were grown as Families 1 and 2 in blocks 1 and 2, respectively. Family 3, comprising the F_3-derived lines from the cross (SD01-5RXPI71506)XDowling, was planted in block 3. Each block also included the control lines, which included the parents if available, another susceptible line (519-R5, Syngenta), and resistant lines with the *Rag1* gene introgressed from Dowling. Each line was grown in a 3-foot un-replicated row.

Five aphids (collected from a field near Brookings SD in 2006 and maintained in the greenhouse on susceptible soybeans) were placed on every plant in each row, on 11 July 2006. Aphids were counted on 2, 8, 16, and 23 August 2006, on 5 plants in each row, including the first tagged plant, and 4 other randomly selected plants (not necessarily tagged plants). Plant growth stage was recorded at each counting.

SSR markers. In each row, 4 plants were randomly selected and tagged for DNA samples. Unexpanded leaflets (up to 1 cm in length) were collected from each of these plants and pressed onto FTA cards (Whatman Inc.) for DNA extraction. DNA was extracted according to the manufacturer's protocol, and amplified by PCR in 25 µl reaction volumes, using 1.5 mM $MgCl_2$, 0.200 mM of each dNTP (Sigma), 2 µM of each primer (IDT), in 1X reaction buffer, with 0.625 to 1.5 units Taq polymerase (New England Biolabs). SSR markers included Satt435 and/or Satt540, Satt463 and/or Satt245, Satt536, and Satt250. Primer sequences were obtained from SoyBase. PCR amplifications were conducted with an initial denaturation at 94 or 95 C for 2 min, then 40 cycles of

92 C for 30 sec, 45 C to 50 C for 30 to 40 sec, and 68 C for 30 to 60 sec, with a final extension at 68-72 C for 2 to 7 min. The reaction products were separated by electrophoresis on 3.75% or 4.5% agarose gels, at 90 to 137 V, on ice, for 3 to 9 hours.

SSR genotypes were characterized for each of the 4 tagged plants per plot. In each plot, aphids were counted on the first tagged plant and on 4 other plants randomly chosen on each counting date. Data were analyzed by general linear model (GLM) analysis of variance (SAS Institute, 1982). Where aphid counts were made on tagged plants of known genotype, data were analyzed on an individual plant genotype basis. For analysis of aphid numbers in whole plots, we used the combined allele composition of all 4 plants characterized within a plot as the plot (line) genotype. Mean aphid numbers per plot were then analyzed in relation to the plot/line genotype, which was thus composed of 8 possible alleles per SSR marker.

RESULTS

Aphid numbers varied significantly depending on the date on which they were counted ($p<0.0001$), block ($p<0.0001$), the line evaluated ($p<0.0001$), and interactions among these effects ($R^2=0.86$ for the model, $p<0.0001$).

There were 9 parent or control lines in this trial. These reference lines were known to be resistant or susceptible to the soybean aphid, and were analyzed separately to confirm whether the expected differences in aphid resistance were observed. The model was significant ($R^2=0.87$, $p<0.0001$), with variation attributable to count date, block, line and interactions highly significant ($p<0.0001$ each). However, differences among lines diminished by mid-August. Variation attributable to lines accounted for 44% of the variation in aphid numbers on August 2, and 53% on August 8. By August 16, the R^2 for lines had diminished to 0.16; and by August 23, the model was not significant.

SSR markers flanking the R gene were chosen based on proximity and detectable polymorphism. Satt435 (M_38.94 cM) and Satt463 (M_50.097 cM), designated as selectable markers for the Dowling *Rag1* gene by Hill et al. (2005), showed little variation in allele size between some of the parent lines used in these crosses, so the flanking markers Satt540 (M_35.85) and Satt245 (M_53.54) were used instead for the SDX005-039-42 families, and Satt540 and Satt435 were used for the SD01-5R family. Satt435 and Satt463 are approximately 11 cM apart; Satt540 and Satt245 are approximately 18 cM apart. Allele sizes for the the SSR markers Satt540 and Satt245 in parent and control lines are given in Table 1.

In general, the susceptible control lines 519-R5 (Syngenta) and SD01-76R supported significantly higher numbers of aphids than the resistant control lines, e.g., the LD05 and LDXG series, and PI71506 (Table 1). However, by August 16, all lines had an average of over 500 aphids per plant.

The change in aphid populations over time differed among parent and control lines (Fig 1), with the fastest increase in the susceptible lines (519-5R and SD01-76R), and delayed population buildup in the lines carrying the *Rag1* gene

Table 1. SSR genotypes and mean number of aphids per plant, of parent and control lines, averaged over all count dates.

	GENOTYPE		
Line	**Satt245**	**Satt540**	**Aphids[1]**
519-R5 (Syngenta)	215215	145145	2,270 a
SDX00R-039-42	215215	145145	
SD01-76R	215215	160160	2,223 a
SD01-5R	215215	160160	
LD05-16106	195195	164164	668 b
LDXG-1	195215	150175	663 b
LDXG-3	195215	164164	375 bc
LD05-16094	195195	164164	339 bc
LD05-16066	195195	164164	280 c
PI71506	215215	155155	221 c
LD05-16143	195195	164164	124 c
Dowling	195195	164164	

[1]Means followed by the same letter do not differ by Student Newman-Keuls test, ($p<0.05$)

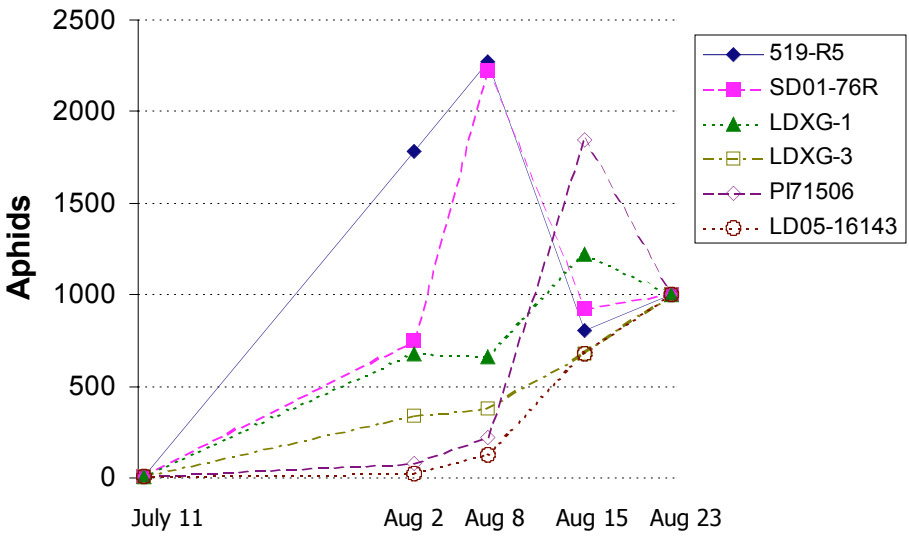

Figure 1. Mean number of aphids on parent and control lines over time.

from Dowling. PI71506 displayed a similarly slow rate of increase in aphids initially, but reached aphid numbers equivalent to those of the susceptible lines by August 16. By August 23 there were no differences in aphid numbers among these lines.

Previous analyses had shown that each of the individual 3-way F_2 parent plants (of the F_3-derived lines) was heterozygous for both of the flanking SSR markers. Therefore, the F_3s and F_3-derived lines were expected to segregate for these markers also.

The date when aphids were counted and family (block) effects were significant in the overall analysis of F_3-derived lines. There were fewer aphids in Family 2 throughout the trial (Fig 2). Each F_3-derived family comprised different lines, and the SSR allele composition and effects differed among families, which were therefore analyzed separately. Within each family, the number of aphids differed signficantly depending on the line, date counted, and their interaction. Representative aphid means by line within each family on August 8 (the time at which variation among lines was most significant) are given in Table 2.

On tagged plants whose individual genotype was determined for the *Rag1*-flanking SSR markers, aphid numbers varied in relation to the date counted, Satt540 alleles, and Satt245 alleles ($p<0.0017$, $R^2=0.248$). Aphid numbers were up to 3 times higher on plants with the susceptible alleles for both Satt540 and Satt245 than on plants that were homozygous for the Dowling *Rag1* gene alleles at both flanking markers (Table 3). Plants with the Dowling parental genotype at both SSR loci supported significantly fewer aphids than plants with either of the other parental genotypes, or that were heterozygous for one or both SSR markers (Table 4). The combined heterozygotes were intermediate in Family 1, i.e., dominance did not appear to be complete at the *Rag1* locus.

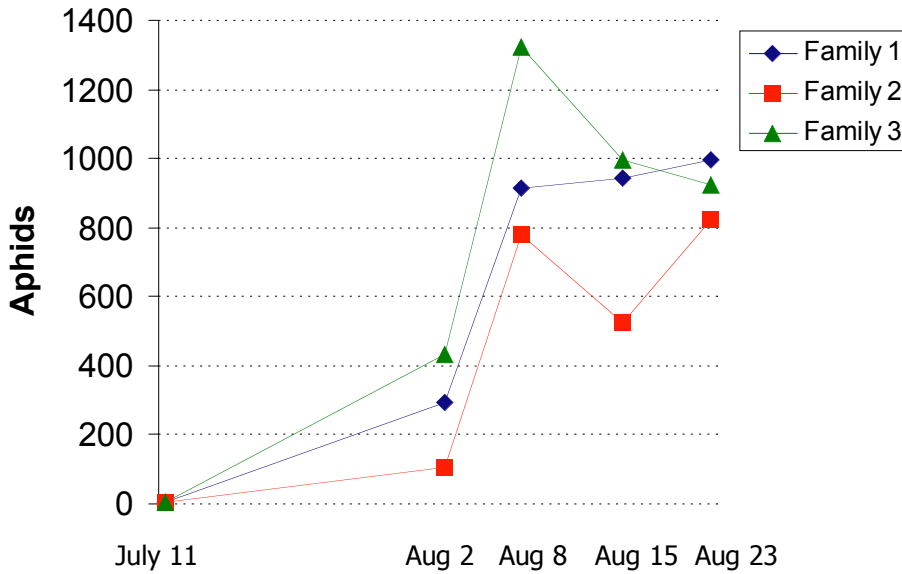

Figure 2. Mean number of aphids in the 3 families of F_3-derived lines, over time.

Table 2. Mean number of aphids counted August 8 on representative lines from Family 1 ((SDX00R-039-42XPI71506)XDowling), Family 2 ((SDX00R-039-42XPI71506)XDowling), and Family 3 ((SD01-5RXPI71506)XDowling). Family 1: p_{model}<0.0001, R^2=0.88); Family 2: p_{model}=0.0001, R^2=0.87); Family 3: p_{model}=0.0001, R^2=0.95).

FAMILY 1		FAMILY 2		FAMILY 3	
Line	Aphids[1]	Line	Aphids[1]	Line	Aphids[1]
SDX04R AP 1-31	3,700 a	SDX04R AP 2-10	3,500 a	SDX04R AP 3-07	4,800 a
SDX04R AP 1-25	3,500 ab	SDX04R AP 2-18	2,200 b	SDX04R AP 3-01	3,500 b
SDX04R AP 1-67	3,200 abc	SDX04R AP 2-32	1,160 cd	SDX04R AP 3-56	2,500 cd
SDX04R AP 1-74	2,500 cd	SDX04R AP 2-38	980 cdef	SDX04R AP 3-13	2,000 d
SDX04R AP 1-35	1,912 def	SDX04R AP 2-20	686 def	SDX04R AP 3-04	1,090 ef
SDX04R AP 1-68	1,400 efgh	SDX04R AP 2-36	311 def	SDX04R AP 3-20	750 efgh
SDX04R AP 1-49	1,000 fghi	SDX04R AP 2-30	227 def	SDX04R AP 3-34	512 efgh
SDX04R AP 1-78	785 ghi	SDX04R AP 2-26	188 ef	SDX04R AP 3-48	242 fgh
SDX04R AP 1-28	584 ghi	SDX04R AP 2-27	153 f	SDX04R AP 3-50	101 h
SDX04R AP 1-05	331 ghi	SDX04R AP 2-23	49 f	SDX04R AP 3-15	65 h
SDX04R AP 1-73	179 hi	SDX04R AP 2-21	23 f	SDX04R AP 3-47	32 h
SDX04R AP 1-76	92 i	SDX04R AP 2-01	11 f	SDX04R AP 3-09	21 h
SDX04R AP 1-82	39 i				
SDX04R AP 1-83	9 i				

[1]Means followed by the same letter, within each column, do not differ by Student Newman-Keuls test, p<0.05.

Table 3. Individual [tagged] plants of Family 1 F_3-derived lines. Mean number of aphids (averaged over all countdates) per genotypic class of Satt245 and Satt540 SSR markers flanking the* Rag1 *gene.

GENOTYPE			
Satt540	Satt245	Number of Plants	Aphids
164 164	195 195	14	397 a
164 164	195 215	7	407 a
164 164	215 215	4	588 ab
164 145	195 215	6	635 ab
145 145	195 215	4	670 ab
164 145	195 195	7	682 ab
145 145	195 195	3	940 ab
145 145	215 215	24	960 ab
164 145	215 215	3	1,304 b

[1]Means followed by the same letter do not differ by Student-Newman-Keuls test, p<0.05.

Table 4. Individual plants [tagged] of Family 1 and Family 2 F_3-derived lines. Least squares mean number of aphids (adjusted for change over dates counted) per parental and combined heterozygous genotypes.

	FAMILY 1			FAMILY 2		
Genotype	Number of Plants	Aphids[2]	Stderr	Number of Plants	Aphids[2]	Stderr
Dowling	14	398 a	82	20	209 a	102
Heterozygotes	34	643 b	68	51	427 a	64
SDX00R-039-42	24	971 c	107	11	763 b	139

[2]Means followed by the same letter do not differ by LSmeans test.

Line genotypes were determined from characterization of flanking marker alleles of 4 plants per plot (line). Both of the *Rag1*-flanking SSR markers, Satt245 and Satt540, were significantly associated with variation in aphid numbers ($p<0.001$) in Families 1 and 2, and Satt245 was significant in Family 3.

In Family 1, segregation of the F_3-derived lines did not differ significantly from that expected assuming the parent was heterozygous and Satt540 and Satt245 are linked at a distance of ca. 18 cM ($X^2=13.35$, $p>0.10$) (Table 5). In Family 1, the Satt540 and Satt245 alleles corresponded to those of Dowling and the SDX00R-039-42 parent; alleles from PI 71506 were not observed in this family. Aphid numbers differed significantly among genotypes and date counted. Lines that were homozygous for both the 195 bp (Dowling) allele of Satt245 and the 164 bp (Dowling) allele of Satt540 had fewer aphids than other lines. Aphid means by genotypic class for the combined Rag1 flanking markers, Satt540 and Satt245, over the length of the trial, are shown in Fig 3.

Table 5. Segregation of Satt540 and Satt245 alleles in Family 1 F_3-derived lines.

GENOTYPE		Number of		
Satt540	Satt245	Lines(O)	Expected (E)	$(O-E)^2/E$
164 164	195 195	8	(0.168) 12	1.30
164 164	195 215	4	(0.037) 3	0.33
164 164	215 215	0	(0.008) 1	1.00
164 145	195 195	5	(0.074) 5	0
164 145	195 215	37	(0.352) 25	5.76
164 145	215 215	7	(0.074) 5	0.80
145 145	195 195	0	(0.008) 1	1.00
145 145	195 215	2	(0.074) 5	1.80
145 145	215 215	8	(0.168) 12	1.33
		71		
X^2				13.32 (ns, $p>0.10$)

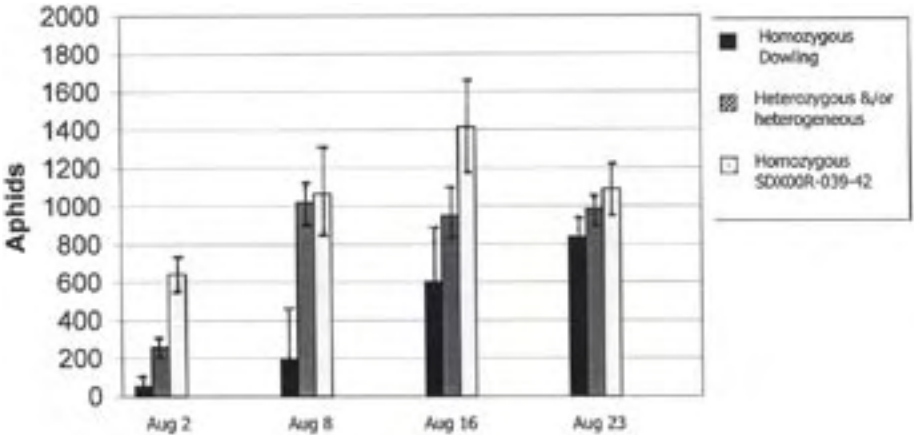

Figure 3. Mean number of aphids per plant by SSR genotypic class (Satt540 and Satt245) of F_3-derived lines of Family 1.

Family 2 also segregated for alleles of Satt245 and Satt540, and did not deviate from the expected linkage between these markers (Table 6). The allele composition of this family differed from that of the Family 1, i.e., the Satt245 and Satt540 alleles from Dowling and PI71506 were present in Family 2, and the corresponding alleles from SDX00R-039-42 were not. Aphid numbers were significantly lower in lines that carried both the Satt245 195 bp allele (from Dowling) and the Satt540 164 bp allele (from Dowling). Aphid means at each date counted, by genotypic class for the combined *Rag1* flanking markers, Satt540 and Satt245, are shown in Fig 4.

Table 6. Family 2 F_3-derived lines. Segregation of Satt540 and Satt245 genotypic classes.

GENOTYPE		Number of		
Satt540	**Satt245**	**Lines (O)**	**Expected (E)**	**$(O-E)^2/E$**
164 164	195 195	5	(0.168) 4.7	0.36
164 164	195 213	2	(0.037) 1.0	1.00
164 164	213 213	0	(0.008) 0.2	0.20
164 155	195 195	2	(0.074) 2.1	0.01
164 155	195 213	9	(0.352) 9.9	0.08
164 155	213 213	2	(0.074) 2.1	0.01
155 155	195 195	1	(0.008) 0.2	3.20
155 155	195 213	3	(0.074) 2.1	0.39
155 155	213 213	3	(0.168) 4.7	0.61
		28		
X^2				5.86 (ns, $p>0.50$)

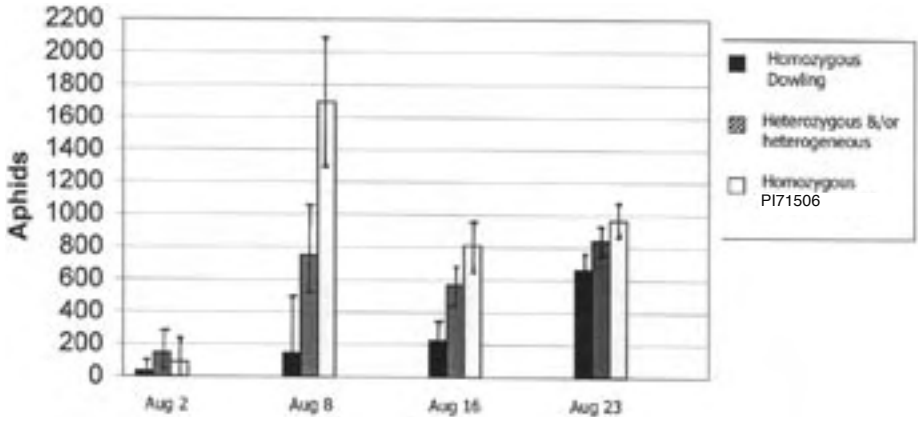

Figure 4. Mean number of aphids per plant by SSR genotypic class of F_3-derived lines of Family 2.

In Family 3, Satt245 alleles segregated 1:2:1 for the Dowling: heterozygotes: SD01-SR alleles (X^2=0.125, p>0.90), and aphid numbers varied significantly depending on Satt245 genotype until August 23. Aphid numbers were over 4 times higher on plants with the SDX00R-039-42 genotype than on those with the Dowling alleles for Satt245. We were unable to reliably and consistently determine the alleles for either Satt540 or Satt435 in many of theplants in the F_3-derived lines, due to lack of resolution of the small differences among the allele variants, and apparent interference from some of the extracts, as well as sample degradation.

Aphid numbers significantly decreased in relation to the number of 164 bp (Dowling) alleles for Satt540, and to a lesser extent the number of 195 bp (Dowling) alleles for Satt245, present in Family 1 throughout the trial. In Family 2, aphid numbers also declined with increasing numbers of both 164 bp alleles for Satt540 and 195 bp alleles for Satt245.

Aphid numbers differed significantly among allele classes for each of the individual SSR loci flanking the *Rag1* gene. For example, in Family 1, the presence of 2 copies of the 195 bp (Dowling) allele of Satt245 reduced aphid numbers (averaged over the entire trial) to less than 50% of the number of aphids on plants that were homozygous for the 215 bp allele of Satt245, and the presence of 1 copy of the 195 bp allele reduced aphid numbers by about one-third (p_{model}<0.0001, R^2=0.22).

The reliability of Satt540 and Satt245 alleles as predictors of resistance or susceptibiity in these lines was evaluated by characterizing alleles in each line, and comparing the predicted response to actual aphid counts. A line was characterized as susceptible if all plants in the line were homozygous for the susceptible parent alleles at both Satt540 and Satt245, and as resistant if all plants within the line were homozygous for the Dowling alleles at both Satt540 and Satt245.

Using this method, 10 lines in Block 1 were characterized as susceptible. On August 8, 90% of these lines had over 500 aphids per plant. Also in Block 1, 9

lines were predicted to be resistant, and 78% of these had less than 300 aphids per plant on August 8. In Block 2, 4 lines were predicted to be susceptible; however, 3 out of these 4 plants had less than 300 aphids per plant on August 8. Six plants in Block 2 were characterized as resistant, and none of these had more than 300 aphids per plant on August 8.

In Block 3, only Satt245 alleles were available to predict aphid resistance. Of 12 lines characterized as susceptible, 2 (17%) had less than 500 aphids per plant on August 8. Of 13 lines identified as resistant, 5 (38%) had over 500 aphids per plant on August 8.

DISCUSSION

For a given locus (diploid), a single progeny plant resulting from a 3-way cross can be expected to carry one allele from the third parent plus one allele from either the first parent or the second parent, but not both [$(a^1a^1 \times a^2a^2) \times a^3a^3 = ½ (a^1a^3) + ½ (a^2a^3)$]. This was observed in the 2 families derived from the 3-way cross with SDX00R-039-42, each of which was derived from a single individual plant. The *Rag1*-flanking SSR marker allele composition of Family 1 differed from that of Family 2, reflecting the probability that the original F_1 parents of the 3-way cross differed, in that although both carried the Dowling alleles (a^3 in the previous example) for Satt540 and Satt245, one carried as well the Satt540 and Satt245 alleles from the susceptible parent (SDX00R-039-42), and the other carried the Satt540 and Satt245 alleles from PI 71506.

In both families, aphid resistance was associated with the Dowling alleles of the SSR markers flanking the *Rag1* gene. Both of the Dowling alleles for Satt540 and Satt245 were required in homozygous condition for optimum aphid resistance in these lines, suggesting that in our populations, *Rag1* resistance was not completely dominant. It also appears that in these lines, aphid numbers may overcome the *Rag1*-associated resistance within a few weeks.

The Satt540 and Satt245 alleles from PI71506 were present in Family 2 and appeared to be associated with susceptibility to aphids. However, Family 2 generally supported fewer aphids than Family 1; thus, other genes or QTLs that may be associated with the M linkage group or perhaps elsewhere in the genome may contribute to aphid resistance or non-preference. Narvel et al. (2001) have identified a major insect-resistance QTL in the M linkage group, south of the Rag1 gene. However, our analyses of one marker, Satt536, in the region of this QTL displayed little or no polymorphism among Dowling, PI71506, and SDX00039-42.

In conclusion, aphid resistance derived from the Rag1 gene of Dowling can be selected using the flanking markers Satt540 and Satt245. However, this resistance may not always be dominant in some populations; and resistance may not hold up for extended periods or under heavy aphid infestation. Aphid resistance that may be derived from PI71506 does not appear to be associated with the Satt540 to Satt245 region.

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