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ORIGINAL PAPER

Genetic linkage mapping of the soybean aphid resistance gene in PI 243540

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Abstract The soybean aphid (*Aphis glycines* Matsumura) is a pest of soybean [*Glycine max* (L.) Merr.] in many soybean growing countries of the world, mainly in Asia and North America. A single dominant gene in PI 243540 confers resistance to the soybean aphid. The objectives of this study were to identify simple sequence repeat (SSR) markers closely linked to the gene in PI 243540 and to position the gene on the consensus soybean genetic map. One hundred eighty-four F_2 plants and their $F_{2:3}$ families from a cross between the susceptible cultivar Wyandot and PI 243540, and the two parental lines were screened with the Ohio biotype of soybean aphid using greenhouse choice tests. A SSR marker from each 10-cM section of the consensus soybean map was selected for bulked segregant analysis (BSA) to identify the tentative genomic location of the gene. The BSA technique was useful to localize the gene to a genomic region in soybean linkage group (LG) F. The entire F_2 population was then screened with polymorphic SSR markers from this genomic region and a linkage map with nine SSR markers flanking the gene was constructed. The aphid resistance gene was positioned in the interval between SSR markers Satt334 and Sct_033 on LG F. These SSR markers will be useful for marker assisted selection of this gene. The aphid resistance gene from PI 243540 mapped to a different linkage group than the only named soybean aphid resistance gene, *Rag*1, from 'Dowling'. Also, the responses of the two known biotypes of the soybean aphid to the gene from PI 243540 and *Rag*1 were different. Thus, the aphid resistance gene from PI 243540 was determined to be a new and independent gene that has been named *Rag*2.

Trade and manufacturers' names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by the USDA implies no approval of the product to the exclusion of others that may also be suitable.

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Abbreviations

BSA	Bulked segregant analysis
QTL	Quantitative trait loci
SSR	Simple sequence repeats

Introduction

The soybean aphid was first reported in the northern soybean growing region of the USA in 2000 (Hartman et al. 2001). By 2004, 80% of U.S. soybean fields were infested by the aphid (Venette and Ragsdale 2004). The soybean aphid is native to southeastern and eastern Asia, and it has long been a pest of soybean in many Asian countries, including China, Japan, South Korea, The Philippines, Indonesia, Malaysia, Thailand, Vietnam, and Russia (Wu

et al. 2004). In addition to the USA, the soybean aphid was also found in Canada and Australia in recent years (OMAFRA 2002; Fletcher and Desborough 2000).

Severe aphid infestations of soybean can result in a number of visible symptoms, including curling, wilting, yellowing, and premature dropping of leaves (DiFonzo and Hines 2002). Significant yield loss can occur directly from aphid feeding damage. Yield losses of soybean attributed to the aphid were reported to be greater than 50% in Minnesota (Ostlie 2002) and up to 52% in China (Wang et al. 1994). In addition to reducing seed-yield the aphid can reduce seed quality (e.g., discoloration, deformation, etc), which is a major concern for producers and consumers of food-grade soybean. Besides the yield loss of soybean from direct feeding damage, aphids can transmit certain plant viruses such as *alfalfa mosaic virus*, *soybean dwarf virus*, and *soybean mosaic virus* to soybean (Iwaki et al. 1980; Hartman et al. 2001; Hill et al. 2001).

Currently available U.S. soybean cultivars are all susceptible to the soybean aphid, and producers depend on insecticides for controlling the aphid. Insecticides can cost between $30 and $35 per hectare, and an estimated $84–$105 million was spent in 2003 alone to control the aphid in the North Central soybean growing region of the USA (Paul 2004). Additionally, spraying soybean fields with insecticides can kill beneficial insects, and may cause environmental pollution (Sun et al. 2000). Use of synthetic chemicals for controlling aphids is also unacceptable to the producers and consumers of organic soybean products.

Host plant resistance often is the most environmentally sound and economically feasible approach for controlling the aphid. The first step in breeding a resistant cultivar is to identify sources of resistance. Genetic resistance to aphids in soybean germplasm has been reported in China (Fan 1988; Sun et al. 1991). Several research groups in the USA have recently identified aphid resistant soybean germplasm (Hill et al. 2004; Mensah et al. 2005; Diaz-Montano et al. 2006; Mian et al. 2008).

The aphid resistance in crop plants is often qualitative (controlled by one or two genes) rather than quantitative (controlled by many genes or quantitative trait loci) (Klingler et al. 2005). The aphid resistance in each of the two soybean cultivars Dowling and Jackson is controlled by a single dominant gene (Hill et al. 2006a,b). The gene in Dowling was designated as *Rag*1 and the gene in Jackson remained unnamed. Li et al. (2007) have mapped *Rag*1 and the unnamed gene from Jackson to the same genomic region on soybean linkage group (LG) M, indicating that these two resistance genes may be allelic. In contrast, initial reports indicate that resistance in PI 567541B is controlled by quantitative trait loci (QTL) and that resistance in PI 567598B is controlled by two recessive genes (Chen et al. 2006; Mensah et al. 2006).

Until recently it was believed that there was no biotype diversity of soybean aphid in North America (Hill et al. 2006a). However, Kim et al. (2008) have confirmed the existence of two biotypes of the aphid in North America and that the Ohio biotype of soybean aphid was able to break the resistance provided by the *Rag*1 and *Rag* (Jackson) genes. Mian et al. (2008) have reported that PI 243540 showed resistance against both the Ohio and Illinois biotypes of the aphid. Research on the inheritance of aphid resistance in PI 243540 indicates that resistance in this soybean accession is controlled by a single dominant gene (Kang et al. 2008). The objectives of this study were to identify simple sequence repeat (SSR) markers closely linked to the aphid resistance gene in PI 243540 and to position the gene on the consensus soybean genetic map.

Materials and methods

Mapping population and evaluation of aphid resistance

One hundred and eighty-four F_2 plants and their $F_{2:3}$ families from a cross of 'Wyandot' × PI 243540 and the two parental lines were used for this study. Wyandot is a high yielding maturity group II, food-grade soybean cultivar developed in Ohio, and it is highly susceptible to the soybean aphid (Mian et al. 2008). PI 243540 is a maturity group IV cultivated accession from Japan that was received at the National Plant Germplasm System (NPGS) on Nov 06, 1957 (http://www.ars-grin.gov/npgs/; accessed April 30, 2008). The unverified Japanese name of PI 243540 is 'Sennari'. The observations on a number of morphological and agronomic traits of PI 243540 are listed at the above mentioned NPGS website.

The parents and the F_1 hybrids, segregating F_2 plants and the $F_{2:3}$ families were evaluated for their response to the Ohio biotype of soybean aphid in greenhouse choice tests in Wooster, OH. The source of the aphids and greenhouse conditions were the same as described by Mian et al. (2008). Each plant was grown in a 15-cm × 4-cm diameter plastic cone-tainer (Stuewe and Sons, Inc., Corvallis, OR, USA). At the V1-stage (Fehr and Caviness 1977) 20–30 aphids of all developmental stages were placed on each plant as described by Mian et al. (2008). Ten cone-tainers (each with three to four seedlings) of each of the two parents were placed among the F_2 plants at regular intervals as checks. Three weeks after infestation, the plants were evaluated for resistance to the aphid on a 1–5 scale, where 1 = no aphids present; 2 = few (<25) solitary live or dead aphid bodies present; 3 = some aphids (25–100) with some viviparous aptera surrounded by few nymphs present; 4 = dense colonies on the upper half of the stem, underside of most leaves, and near the growing point of the plant with more

than 300 aphids; and 5 = similar or more severe aphid infestation as in score 4, accompanied by visible plant damage (e.g., curled and/or yellow leaves, stunted growth, etc.). These rating criteria were modified from the ones used by Hill et al. (2006b). After scoring, each plant was transplanted in a 7.6-l size plastic pot filled with soil media and the plant was sprayed with an insecticide. The aphid susceptible plants were given special care with nutrient and water to make sure they recovered from the aphid damage and were able to set seeds. Each plant was kept under long day light hours (>15 h) until a full recovery and lush vegetative growth had occurred. Then plants were placed under a 13-h day light period to initiate flowering. Plants were grown to maturity and the $F_{2:3}$ seeds from each F_2 plant were harvested in an envelope.

To confirm the aphid resistance phenotypes of the F_2 plants, the corresponding $F_{2:3}$ families were screened in a greenhouse choice test with the Ohio biotype of the aphid. A minimum of 12 seedlings from each $F_{2:3}$ family were screened for segregation of aphid resistance. The greenhouse screening protocols were the same as described for the F_2 plants, except that six to eight seedlings were grown in each 3.8-l plastic pot. Ten pots (each with six to eight seedlings) of each parent were also placed randomly among the progeny pots. Three weeks after infestation, each plant in a $F_{2:3}$ family was assigned a score based on the 1–5 scores described earlier. An F_2 plant producing all-susceptible F_3 plants (aphid scores of 3, 4 or 5) was confirmed as susceptible, while an F_2 plant with a family of all resistant plants (scores of 1 or 2) or a combination of resistant and susceptible plants was confirmed as resistant. Since the aphid resistance in PI 243540 is known to be controlled by a single dominant gene, the aphid resistance scores of the progeny were converted to qualitative scores of 'a' for a susceptible F_2 line, and 'c' for a resistant (homozygous or heterozygous) F_2 line for mapping the dominant aphid resistance gene.

DNA extraction and SSR marker genotyping

Young expanding leaf tissue was collected in 2-ml tubes, immediately frozen in liquid nitrogen, and stored in a −80 C freezer until lyophilized in a freeze drier. The dry tissues were ground to fine powder using a Mixer Mill (Model MM 301; Retsch, Hannover, Germany). The DNA was extracted using a CTAB protocol (Saghai-Maroof et al. 1984). Evenly distributed SSR primer pairs were selected from the integrated soybean map of Song et al. (2004) to get a genome wide coverage of a marker in every 10-cM. Particular priority was placed on soybean LG M with the *Rag*1 gene, and other LGs with known disease and insect resistance genes, i.e., LGs D1b, E, F, G, J and N. The primer sequences were obtained from the SoyBase website (http://soybase.org/resources/ssr.php; accessed on April 30, 2008). The primers were synthesized by Integrated DNA Technologies, Inc (Coraville, IA, USA). The PCR reactions were performed using a modified version of the protocols of Diwan and Cregan (1997). The PCR cycles consisted of initial denaturation at 94 C for 3 min, followed by 32 cycles of 45 s denaturation at 94 C, 45 s annealing at 47–59 C (depending on the optimum annealing temperature for each primer pair), and 45 s extension at 72 C followed by a 8 min final extension at 72 C on a thermal cycler (model TC-512; Techne, Princeton, New Jersey, USA). The PCR products along with a 50 base pair size-standard were resolved by horizontal gel electrophoresis using 4% super-fine resolution agarose (Ameresco, Solon, OH, USA). The gels were stained with ethidium bromide and the gel images were captured with Genesnap (V.6.08) using the Gene Genius Bioimaging System (SYNGENE, Cambridge, UK). The SSR bands were scored manually from the gel images. The SSR markers polymorphic between the two parents were scored as 'a', 'b' or 'h', where a = only band(s) of the susceptible parent present, 'b' = only band(s) of the resistant parent present, and h = bands from both parents present. For SSR markers with polymorphic bands differing by less than 10 bp between the two parents, the PCR reactions were performed using forward primers with a dye-labeled M13 tail, following the principle of Schuelke (2000). The tailed primer PCR method for CEQ™ 8800 of Beckman Coulter (http://www.lsbi.mafes.msstate.edu/TailedPrimers.pdf, accessed on April 30, 2008) were used with some modifications. PCR reaction mixture contained 50–100 ng of soybean genomic DNA, 2.5 mM Mg^{2+}, 200 µM dNTP, 50 nM of M13 forward and 100 nM reverse primers, 100 nM of M13 dye, 1× PCR buffer, and 1.0 unit DNA polymerase in a total volume of 20 µl. Specifically for three markers—Sat_234, Sct_033 and Sat_375—dNTP content was increased to 250 µM. PCR cycles consisted of initial denaturation at 94 C for 4 min, followed by 30 cycles of 40 s denaturation at 94 C, 40 s annealing at a temperature between 47 and 59 C (depending on the optimum annealing temperature for each primer pair), and 1 min extension at 72 C, followed by 8 cycles of 40 s denaturation at 94 C, 40 s annealing at 53 C, and 1 min extension at 72 C. The PCR was ended with a 12 min final extension at 72 C on a thermal cycler (Techne, model TC-512). Fragment analysis and allele calling was done using the CEQ 8800 genetic analyzer and software, respectively (Beckman Coulter, Fulerton, CA, USA).

Bulked segregant analysis

The bulked segregant analysis (BSA) was performed following the protocol described by Michelmore et al. (1991). Two resistant bulks were formed by pooling the DNA from 15 F_2 plants with an aphid score of 1. Equal amounts of DNA were pooled from eight plants to form the resistant

bulk 1, and the resistant bulk 2 was formed by pooling DNA from seven plants. Similarly, the susceptible bulks 1 and 2 were formed with DNA from eight and seven F_2 plants with aphid score of 5, respectively. The resistant and susceptible DNA bulks and DNA from the two parents were amplified with the selected SSR primers to identify SSR markers linked to the aphid resistance gene.

Data analysis and genetic mapping

The SSR marker data and the aphid phenotypes of the F_2 lines were analyzed to construct a linkage map with JoinMap 4.0 (Van Ooijen 2006) using the Kosambi mapping function. A logarithm (base 10) of odds (LOD) score of $\geq$3.0 was used to identify linked loci. At each locus, the segregation ratio of alleles was determined by χ^2 goodness of fit to identify if the locus met the expected 3:1 or 1:2:1 ratio using JoinMap 4.0 with a significance threshold of $P = 0.05$.

Results

Segregation of the mapping population for aphid resistance

The aphid scores for all PI 243540 plants were 1 or 2, and the aphid scores for most Wyandot plants were 4 or 5, with few plants (<5%) having a score of 3.0. The progeny with scores of 1 or 2 were considered resistant, and those with scores of 3, 4, or 5 were considered susceptible because PI 243540 plants always had scores of $\leq$2.0. Most F_2 plants had aphid scores of 1, 2, 4, or 5 and only four plants had a score of 3. These four plants were confirmed as susceptible in the F_3 generation with all-susceptible progeny in the corresponding $F_{2:3}$ families. The individual F_2 progeny and the corresponding $F_{2:3}$ families segregated for aphid resistance as expected for a single dominant gene. Of the 184 F_2 individuals, 138 were resistant and 46 were susceptible, with an exact fit to a 3:1 ratio for segregation of a single dominant gene model for aphid resistance (Table 1). The $F_{2:3}$ families had 46 homozygous susceptible, 57 homozygous resistant and 81 heterozygous resistant families which fit the expected 1:2:1 ratio ($P = 0.14$) for the segregation of a single dominant gene (Table 1). However, the number of homozygous resistant plants (57) was higher than the expected number (46). The reason for this segregation distortion towards the homozygous resistant families is not clear. It is possible that screening of more individuals from these homozygous resistant families will reveal additional segregating families. Due to a limited number of $F_{2:3}$ seeds, screening of more plants from these families was not possible. Due to this slight distortion in segregation of aphid resistance in the F_3 generation, we mapped aphid resistance as a dominant as well as a co-dominant trait.

Genetic map construction

The BSA identified two SSR markers—Satt362 and Sct_188—on linkage group F to be linked to the aphid

Table 1 χ^2-test of segregation ratio for the aphid resistance gene (*Rag*2) and nine SSR markers among 184 F_2 plants from a 'Wyandot' × PI 243540 mapping population

Locus	Number of F_2 lines in each category[b]				χ^2 1:2:1[c]	*P*	χ^2 3:1[d]	*P*-value
	a	b	h	–				
Sat_229	49	27	105	3	9.99	0.01	0.41	0.52
Sat_234	50	47	84	3	1.03	0.60	0.66	0.41
SOYHSP176	43	53	87	1	1.54	0.46	0.22	0.64
Satt334	43	57	81	3	4.16	0.12	0.15	0.70
Sct_033	47	52	77	8	3.03	0.22	0.27	0.60
Satt362	51	46	86	1	0.93	0.63	0.80	0.37
Sct_188	45	45	94	0	0.09	0.96	0.03	0.86
Sat_375	43	45	94	2	0.24	0.89	0.18	0.67
Satt490	37	49	96	2	2.13	0.34	2.12	0.15
*Rag*2[a]	46	57	81	0	3.95	0.14	0.00	1.00

[a] The *Rag*2 data were collected by screening the F_2 lines and corresponding $F_{2:3}$ families

[b] *a* homozygous aphid susceptible genotype or homozygous for the marker allele from the susceptible parent (Wyandot), *b* homozygous aphid resistant genotype or homozygous for the marker allele from the resistant parent (PI 243540), *h* heterozygous for aphid resistance or the marker, – missing data

[c] Expected segregation = 1:2:1 = a:h:b

[d] Expected segregation = 3:1 = [b + h]:a

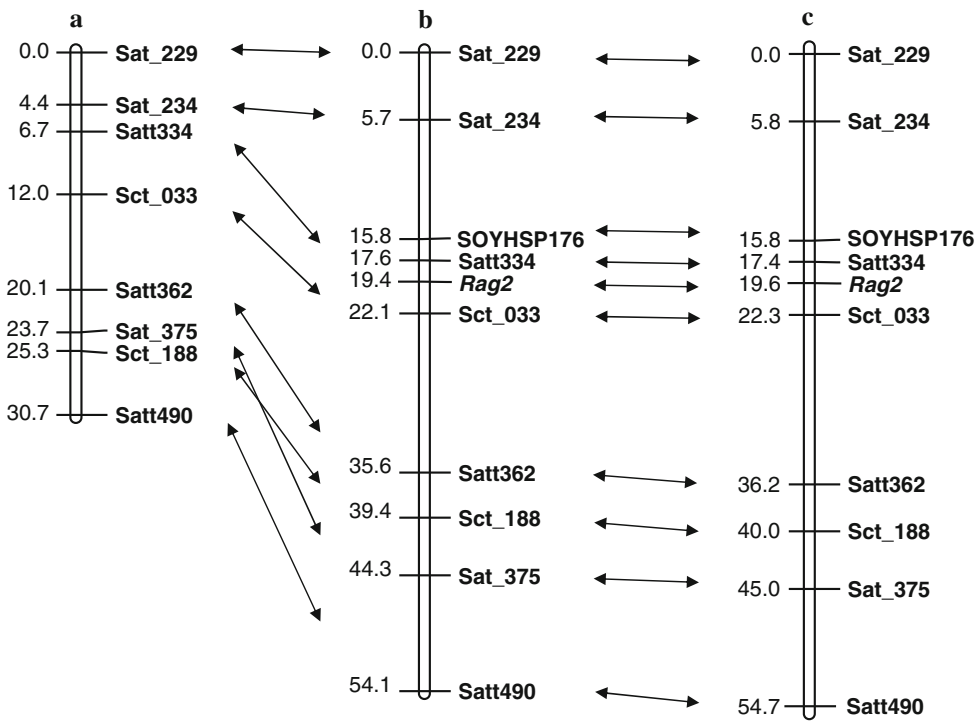

Fig. 1 **a** The relevant segment of the Choi et al. (2007) soybean linkage group (LG) F, **b** the map positions and map orders of the *Rag*2 gene on soybean linkage F when *Rag*2 was mapped as a dominant gene, and **c** the map position and map order of *Rag*2 when it was mapped as a co-dominant gene

resistance gene in this population. After finding the linkage of the two SSR markers from LG F with the gene, all SSR markers within ±30 cM of the two markers on the Choi et al. (2007) soybean linkage map were then screened for polymorphism between the two parents using a universal dye labeled PCR (Schuelke 2000) and a Beckman CEQ 8800 genetic analyzer. Seven additional SSR markers from the region were identified as polymorphic between the parents. The 184 F_2 plants were then screened with the nine SSR markers. Data from all markers except Sat_229 fit the 1:2:1 segregation ratio expected for co-dominant markers, and Sat_229 fit the 3:1 ratio of a dominant marker (Table 1). Sat_229 data were used as dominant markers (scores of 'a' and 'c') while the remaining SSR data were used as co-dominant markers (scores of 'a', 'b' and 'h') for the purpose of map construction. The locus for aphid resistance in PI 243540 has been named *Rag*2. When *Rag*2 was mapped as a dominant gene, the linkage map was 54.1 cM in length, and *Rag*2 was placed between Satt334 and Sct_033 with distances of 1.8 and 2.7 cM from these markers, respectively (Fig. 1). When mapped as a co-dominant gene, *Rag*2 was placed in the same interval between Satt334 and Sct_033 with a very minor change in the map position of the gene (Fig. 1).

Discussion

*Rag*1 is the only soybean gene for soybean aphid resistance that has been officially named prior to this work (Hill et al. 2006a). *Rag*1 from the cultivar Dowling and the unnamed *Rag* gene from the cultivar Jackson, which provide resistance to the Illinois biotype of soybean aphid, were mapped on soybean LG M (Li et al. 2007). The resistance of the genes from these two sources is not effective against the aphid biotype from Ohio (Kim et al. 2008; Mian et al. 2008). Mian et al. (2008) have reported PI 243540 to have strong antibiosis resistance against the Ohio biotype of soybean aphid. PI 243540 was also resistant against the Illinois biotype of the aphid in a choice test (Mian et al. 2008). The aphid resistance in PI 243540 was determined to be controlled by a single dominant gene (Kang et al. 2008). In this study we have mapped this gene on soybean linkage group F with closely linked SSR markers and clearly demonstrated that the aphid resistance gene in PI 243540 is independent from the *Rag*1 gene in Dowling. Using the convention of the Soybean Genetics Committee, the new gene identified in PI 243540 has been named *Rag*2, with 'ag' standing for *Aphis glycines* and the number '2' indicating that this was the second soybean aphid resistance gene recognized by the Committee.

The total map distances of 54–55 cM of the linkage map constructed in this study are considerably larger than the corresponding map distance of 31 cM in the consensus soybean linkage map of Choi et al. (2007). The main reason for the overall expansion of the map distance in this map compared to the consensus maps may be that we have only 10 markers on the map while the Choi et al. (2007) map has 61 markers in the same genomic section. Additionally, map position and distances between loci on genetic maps

constructed for different populations can vary for other reasons such as insertions, deletions, translocations, or other chromosomal modifications in one or both parents that could change the distances between loci as well as their relative map orders (Stam 1993).

The map orders for the nine SSR markers are in agreement with the Choi et al. (2007) map, except that two closely linked markers Sct_188 and Sat_375 on the consensus map have interchanged their positions in our map (Fig. 1). This minor anomaly in map positions of these two markers, however, can be ignored because they are nearly 20 cM away from the gene of interest. We recommend two SSR markers, Satt334 and Sct_033, to be used for marker assisted selection for this gene. If neither of these two markers is polymorphic in a cross, SOYHSP176 which is the third closest marker to the gene can be used. More than 10,000 single nucleotide polymorphism (SNP) markers will be mapped on the Choi et al. (2007) soybean genetic map by the end of 2008 and more SNP markers will be added to the map in 2009 (Dr P.B. Cregan, personal communication, 2008). Thus, fine mapping of *Rag*2 with SNP markers and selection of SNP markers for use in marker assisted breeding for *Rag*2 should be considered.

A number of R-genes and QTL for resistance to biotic and abiotic stresses of soybean have been mapped within ±20 cM of the *Rag*2 gene on LG F (www.soybase.org, accessed on April 30, 2008; Choi et al. 2007). The recently published soybean transcript map of Choi et al. (2007) was constructed by mapping one SNP in each of 1,141 genes in one or more of three recombinant inbred line mapping populations. The bacterial blight resistance gene (*Rpg*1) caused by *Pseudomonas syringae* was positioned between Satt334 and Sct_033 in the same interval with the *Rag*2 gene. This region also is known for an R-gene cluster which include *Rpg*1-b and *Rpg*1-r for resistance to *Pseudomonas syringae* (Ashfield et al. 1998), *Rps*3 for resistance to *Phytophthora sojae* (Diers et al. 1992), *Rsv*1 for resistance against the *soybean mosaic virus* (Yu et al. 1994; Gore et al. 2002) and *Rpv*1 for resistance against *peanut mottle virus* (Roane et al. 1983; Gore et al. 2002). In addition, a number of QTL for stress resistance have been mapped to this genomic region, including QTL associated with peanut root-knot nematode resistance (Ma 1-2, 1-4, 1-5, 2-1) (Tamulonis et al. 1997a), javanese root-knot nematode resistance (Mj 2-1,1-4,1-6, 1-7) (Tamulonis et al. 1997b), corn earworm resistance (CEW 2-1) (Rector et al. 2000), and aluminum tolerance (Al tol 1-4) (Bianchi-Hall et al. 2000).

Several QTL associated with physiological traits have also been mapped to this genomic region (www.soybase.org). These include QTL for plant height (Pl ht 5-6, 7-2, 11-3) (Lee et al. 1996), lodging (Ldge 5-5, 5-6, 6-2) (Mansur et al. 1996; Orf et al. 1999), canopy width (canopy width 1-2) (Mian et al. 1998), and seed weight (sd wt 6-6) (Orf et al. 1999). Thus, the *Rag*2 gene is located in a region of the soybean genome on LG F that is rich in stress resistance genes and QTL, as well as in QTL for other important traits. The *Rag*2 gene warrants further molecular characterization in order to reveal possible interactions of this gene with other important genes located in the same genomic region. Particular emphasis should be placed on investigating any possible interaction of *Rag*2 with the nematode (Ma and Mj) resistance QTL in the region. The first cloned aphid resistance gene (Mi-1.2) in tomato (*Lycopersicon esculentum* L.) also confers resistance to a biotype of the potato aphid (*Macrosiphum euphorbiae*) and to root-knot nematode (*Meloidogyne incognita*) (Rossi et al. 1998).

PI 243540 is an old cultivar (named Sennari) from Japan and its agronomic appearance is more like a cultivar than a 'wild looking' PI. However, yield drag is expected when this PI is used as the donor parent, as is the case with most PIs from other countries. Use of molecular markers to select for *Rag*2 and to remove undesirable portions of the PI genome from progeny lines will enhance introgression of *Rag*2 in elite cultivars.

In summary, a new gene for strong antibiosis type resistance to the Ohio biotype of the soybean aphid has been identified and mapped on soybean linkage group F using nine linked SSR markers. Three of the SSR markers that flank the gene are located within 2-4 cM of the gene and these markers will be useful for marker assisted selection of this gene. This gene is an independent locus from *Rag*1 and has been named *Rag*2. Due to the presence of multiple biotypes of the soybean aphid in North America, genes with resistance to each biotype and pyramiding of genes with resistance to different biotypes will be needed for developing soybean lines with broad resistance to the soybean aphid. The *Rag*2 gene and the SSR markers found closely linked to the gene should be useful in breeding for soybean aphid resistance.

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