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ORIGINAL PAPER

Fine mapping of the soybean aphid-resistance gene *Rag2* in soybean PI 200538

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Abstract The discovery of biotype diversity of soybean aphid (SA: *Aphis glycines* Matsumura) in North America emphasizes the necessity to identify new aphid-resistance genes. The soybean [*Glycine max* (L.) Merr.] plant introduction (PI) 200538 is a promising source of SA resistance because it shows a high level of resistance to a SA biotype that can overcome the SA-resistance gene *Rag1* from 'Dowling'. The SA-resistance gene *Rag2* was previously mapped from PI 200538 to a 10-cM marker interval on soybean chromosome 13 [formerly linkage group (LG) F]. The objective of this study was to fine map *Rag2*. This fine mapping was carried out using lines derived from 5,783 F_2 plants at different levels of backcrossing that were screened with flanking genetic markers for the presence of recombination in the *Rag2* interval. Fifteen single nucleotide polymorphism (SNP) markers and two dominant polymerase chain reaction-based markers near *Rag2* were developed by re-sequencing target intervals and sequence-tagged sites. These efforts resulted in the mapping of *Rag2* to a 54-kb interval on the Williams 82 8× assembly (Glyma1). This Williams 82 interval contains seven predicted genes, which includes one nucleotide-binding site-leucine-rich repeat gene. SNP marker and candidate gene information identified in this study will be an important resource in marker-assisted selection for aphid resistance and for cloning the gene.

Abbreviations

INDEL	Insertion and deletion
kb	Kilobase pair
MAS	Marker-assisted selection
MCA	Melting curve assay
NBS-LRR	Nucleotide-binding site-leucine-rich repeat
SNP	Single nucleotide polymorphism
STS	Sequence-tagged site

Introduction

Although the soybean aphid (SA: *Aphis glycines* Matsumura) has a short history in the USA, the aphid has caused significant damage and has spread to most soybean growing states since its discovery in the country in 2000 (Voegtlin 2008). There is a need to identify new sources of SA resistance as diversity in SA was shown to occur in North America. Michel et al. (2009) showed the presence of diversity in SA based on simple sequence repeat (SSR) diversity and Kim et al. (2008) showed that at least two different SA biotypes exit in North America. These biotypes are biotype 1, which was collected in Illinois and is controlled by the SA-resistance genes *Rag1* from Dowling and *Rag* in 'Jackson', and biotype 2, which originated from Ohio and can overcome these genes. Evaluations with both SA biotypes 1 and 2 show that resistance in plant introduction (PI) 200538 is not defeated by either SA biotype and thus this PI is a potential source of new, useful SA-resistance gene(s) (Kim et al. 2008). Recently, a new SA

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biotype was collected from the overwintering host glossy buckthorn (*Frangula alnus*) that can readily colonize plants with *Rag2* (Hill et al. 2010). This has been named biotype 3 and it demonstrates the continued necessity to identity new SA-resistance genes and to stack the genes in soybean cultivars.

Several research groups have identified soybean genotypes with SA resistance and have mapped resistance genes onto soybean chromosomes. Hill et al. (2004a, b) first discovered nine SA-resistant genotypes. Of the nine genotypes, Dowling (PI 548663), Jackson (PI 548657), and 'Sugao Zarai' (PI 200538) were later shown as having SA resistance characterized as antibiosis by Li et al. (2004). Hill et al. (2006a, b) identified that a SA-resistance gene named *Rag1* was present in Dowling and a gene named *Rag* was present in Jackson. These were both dominant resistance genes and they were mapped to the same region between Satt463 and Satt435 on soybean chromosome 7 [linkage group (LG) M] (Li et al. 2007).

Mensah et al. (2008) identified two PIs with antibiosis resistance and two PIs with antixenosis resistance to SA. Zhang et al. (2009) further evaluated PI 567541B, one of the sources of antibiosis resistance, and mapped two recessive quantitative trait loci (QTL) from this source. They mapped one QTL onto chromosome 7 (LG M) and another onto chromosome 13 (LG F). The genetic location of the QTL on chromosome 7 was the same as the location of *Rag1* from Dowling, however, the QTL on chromosome 7 was recessive in contrast to the dominant resistance found for *Rag1*. They also identified a significant interaction between two genes (Zhang et al. 2009).

Mian et al. (2008a) identified that PI 243540, PI 567301B, and PI 567324 are resistant to SA biotype 2 collected in Ohio. Kang et al. (2008) showed that strong antibiosis resistance in PI 243540 was controlled by a single dominant gene that was mapped to chromosome 13 (LG F) by Mian et al. (2008b) and this gene was subsequently named *Rag2*. Hill et al. (2009) recently reported that PI 200538 carries a single dominant gene conferring resistance to both SA biotype 1 and 2 and this gene maps to the same genomic region as the *Rag2* allele from PI 243540. Although it is not known whether the PI 200538 and PI 243540 resistance genes are allelic or are just tightly linked on chromosome 13, the PI 200538 gene will be referred to as *Rag2* in this report.

Previous research mapped *Rag2* to a 10-cM interval from PI 200538 and a 4.5-cM interval from PI 243540 (Hill et al. 2009; Mian et al. 2008b) resulting in large gaps between the gene and markers on these linkage maps. The objective of this study was to fine map the location of *Rag2* from PI 200538 through the identification of additional genetic recombinants close to the gene and the development of single nucleotide polymorphism (SNP) markers by re-sequencing sequence-tagged sites (STSs) and target regions based on the Williams 82 8× draft assembly (Glyma1) (Schmutz et al. 2010). Fine mapping and high-resolution linkage analysis of the region containing *Rag2* will facilitate soybean aphid resistance breeding because SNP markers developed during this process that are closely linked to or within *Rag2* can be used for marker-assisted selection (MAS). In addition, this research will aid efforts to clone *Rag2* by positioning the gene into a small interval containing few candidate genes.

Materials and methods

Plant material

To fine map *Rag2*, three sources of soybean populations were used. The first was a population of 95 $F_{2:3}$ lines from the three-way cross LD02-4485 × (Ina × PI 200538) that was originally used to genetically map *Rag2* (Hill et al. 2009). The population was phenotyped for aphid resistance and genotyped with SSR markers in the F_2 and $F_{2:3}$ generations as described by Hill et al. (2009). PI 200538 (Sugao Zairai) is a maturity group (MG) VIII soybean accession originating from Japan (USDA-ARS Germplasm Resources Information Network, http://www.ars-grin.gov/npgs/; accessed 26 Oct 2009). Ina is a MG IV (relative maturity 4.5) soybean cyst nematode (SCN) (*Heterodera glycines* Ichinohe)-resistant cultivar that is susceptible to SA (Nickell et al. 1999; Hill et al. 2004a). LD02-4485 is a high yielding MG II experimental line developed by the University of Illinois that is SA susceptible and SCN resistant.

The second source of germplasm used in the *Rag2* fine mapping was a set of $BC_2F_{2:3}$, $BC_3F_{2:3}$, and $F_{2:3}$ lines derived from plants selected for having putative genetic recombination in the interval containing *Rag2* (Table 1). These were selected from a total of 3,151 BC_2F_2, BC_3F_2, and F_2 plants segregating for *Rag2* that were grown in the field at Urbana, IL in 2008. These plants were screened with the SSR markers Satt510 and Satt114 (Song et al. 2004), which flanked the gene (Hill et al. 2009). One hundred and eighty-five plants with recombination events between the markers were selected and harvested. These selected recombinant lines were tested with three SNP markers (#1485, #20, and #1) that map close to the *Rag2* region to identify which lines have recombination events near the gene (Tables 2, 3; Fig. 1b). From the marker screening, 12 lines with recombination events in the marker intervals were identified and five were selected for SA resistance testing. Selected lines 18 and 32 are $BC_2F_{2:3}$ lines with the pedigree LD03-6566 (3) × [LD02-4485 × (Ina × PI 200538)] (Table 1). Line 86 is a $F_{2:3}$ line with

Table 1 Pedigree information for 243 recombinant lines identified during the fine mapping of *Rag2* from PI 200538

Line no.	No. of tested recombinant lines	Pedigree of lines	Generation of line
1–8	8	LD02-8782 × LD02-4485 (3) × (Ina × PI 200538)	$F_{2:3}$
9–36	28	LD03-6566 (3) × [LD02-4485 × (Ina × PI 200538)]	BC_2 $F_{2:3}$
37–52	16	LD04-8782 × LD03-6566 (2) × [LD02-4485 × (Ina × PI 200538)]	$F_{2:3}$
53–103	51	LD05-3230 × [LD02-4485 (3) × (Ina × PI 200538)]	$F_{2:3}$
104–156	53	LD05-3230 × LD03-6566 (2) × [LD02-4485 × (Ina × PI 200538)]	$F_{2:3}$
157–185	29	LD02-4485 (4) × (Ina × PI 200538)	BC_3 $F_{2:3}$
K1–K29	29	LD03-6566 (4) × [LD02-4485 × (Ina × PI 200538)]	BC_3 $F_{2:3}$
K30–K48	19	LD03-6566 × [LD02-4485 (4) × (Ina × PI 200538)]	$F_{2:3}$
K49–K58	10	LD02-4485 (5) × (Ina × PI 200538)	BC_4 $F_{2:3}$

the pedigree LD05-3230 × [LD02-4485 (3) × (Ina × PI 200538)]. Lines 162 and 181 are $BC_3F_{2:3}$ lines that both have the pedigree LD02-4485 (4) × (Ina × PI 200538). These last two lines were also tested with all SNP markers in the region except KS9-3 (Table 2). Both LD05-3230 and LD03-6566 are high yielding, SA susceptible experimental lines developed by the University of Illinois soybean breeding program.

The third source of germplasm was a set of 58 lines (K1–K58) selected for having putative genetic recombination in the interval containing *Rag2* (Table 1) from a total of 2,632 BC_3F_2, F_2, and BC_4F_2 plants segregating for *Rag2* that were grown in the field at Urbana, IL in 2009. These plants were first screened with the SNP markers #20 and #1485, which flanked the gene (Table 2; Fig. 1b). Fifty-eight plants with recombination between the markers were selected and screened with SNP markers KS7 and KS12 to identify which plants had recombination events close to the gene (Table 2). From the marker screening, three plants with recombination events between KS7 and KS12 were identified, and lines were derived from the three plants and used in progeny tests. Selected line K16 is a $BC_3F_{2:3}$ line with the pedigree LD03-6566 (4) × [LD02-4485 × (Ina × PI 200538)]. Lines K31 and K37 are in the $F_{2:3}$ generation and have the pedigree LD03-6566 × [LD02-4485 (4) × (Ina × PI 200538)]. Progeny plants from the three recombinant lines were tested with all SNP markers.

Aphid culture

The SA biotype 2 was established at the Ohio Agricultural Research and Development Center (OARDC), Wooster, Ohio during the summer of 2005 by collecting aphids from nearby soybean fields. Biotype 2 was maintained on a continuous supply of plants of LD05-16611, an experimental line developed by the University of Illinois, which has *Rag1* from Dowling. The SAs were maintained in a growth chamber at 22°C and under 16-h irradiation and 70% relative humidity (Hill et al. 2004a).

Soybean aphid biotype 2 resistance evaluation

The 95 $F_{2:3}$ lines from the LD02-4485 × (Ina × PI 200538) population were tested for resistance to SA biotype 2 as described by Hill et al. (2009). Briefly, a minimum of 11 F_3 plants from each line were tested with SA biotype 2 in a greenhouse and rated for aphid colonization on a 1–4 scale with 1 = few solitary live aphids and 4 = dense colonies accompanied by plant damage. The greenhouse was maintained at 22–25°C with 14 h ambient light supplemented by 1,000-W high pressure sodium vapor lamps positioned approximately 2 m above the greenhouse benches (Hill et al. 2004a).

Progeny from the eight selected recombinant plants were tested for SA resistance using choice tests with biotype 2 in order to determine the position of the *Rag2* gene relative to the recombination points in each line. Lines 18 and 32 were the first evaluated for resistance. This was followed by testing lines 86, 162, and 181, followed by testing of K16, K31, and K37. These tests included from 38 to 67 progeny plants from each selected recombinant line (Table 2). In all SA resistance tests, the photoperiod was 14 h and the temperature was between 22 and 25°C. The first SA resistance test was conducted in a plant growth chamber with 30 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR) (Kim et al. 2008). The second SA resistance test was conducted in plant growth chamber with 300 μmol m^{-2} s^{-1} PAR. The third SA resistance test was conducted in a greenhouse under conditions described above.

Individual plants were grown in 60 by 60 by 60-mm plastic 48-pot inserts (Hummert Intl., Earth city, MO, USA) contained inside plastic trays without holes (Hummert Intl.) as described by Kim et al. (2008). Each 48-pot insert included 44 progeny from a line and two replications of the parents PI 200538 and LD02-4485. The 48 plants in an insert were arranged in a completely randomized design. Experiments were inoculated by placing leaves of LD05-16611 that were infested with

Table 2 SNP marker genotypes of eight recombinant lines and their reactions to aphid biotype 2

Physical position (Mb)[a]		29.097	29.104	29.123	29.141	29.182	29.212	29.223	29.247	29.266	29.273	29.293	29.310	29.549	Marker used in F test	Test number[b]	No. of plants tested	Aphid numbers[c]			$P > F$[d]	R^2[e]
Line	Phenotype[f]	#1485	KS16	KS14	KS12	KS10	KS9-3	KS8	KS7	KS5	KS4	KS2	#20	#1				R	H	S		
18	Segregating	H[g]	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	*H*	*S*	#20	1	42	24	22	114	<0.0001	0.91
32	Resistant	R	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	*R*	*H*	#1	1	43	23	25	29	0.22	0.07
86	Segregating	H	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	*H*	*R*	#20	2	38	349	337	2,774	<0.0001	0.97
162	Resistant	R	R	R	R	R	NT	R	*R*	*H*	H	H	H	H	KS5	2	67	74	104	93	0.44	0.02
181	Segregating	R	R	*R*	*H*	H	NT	H	H	H	H	H	H	H	KS12	2	66	107	151	2,252	<0.0001	0.93
K37	Segregating	S	S	S	S	*S*	*H*	H	H	H	H	H	H	H	KS9-3	3	40	54	72	1,088	<0.0001	0.99
K16	Resistant	H	H	H	H	H	*H*	*R*	R	R	R	R	R	R	KS9-3	3	41	61	55	46	0.41	0.04
K31	Resistant	H	H	H	H	H	*H*	*R*	R	R	R	R	R	R	KS9-3	3	40	22	24	23	0.86	0.01

[a] Physical position of the markers based on the Williams 82 8× assembly (Glyma1) available at http://www.phytozome.net (Schmutz et al. 2010). The mega base (Mb) positions of the SNP markers correspond to the locations of each SNP and the positions of the dominant markers are the locations of the end sequences of the reverse primers

[b] SA resistance test. Test 1 was conducted in a plant growth chamber maintained at 22–25°C with 14 h illumination with 30 μmol m^{-2} s^{-1} PAR irradiation, Test 2 was conducted in a plant growth chamber maintained at 22–25°C with 14 h illumination with 300 μmol m^{-2} s^{-1} PAR irradiation, and Test 3 was conducted in a greenhouse maintained at 22–25°C with 14 h illumination with 1,000 W high pressure sodium vapor lights

[c] Mean number of aphids on each plant predicted to be homozygous resistant (R), heterozygous (H), and homozygous susceptible (S) for *Rag2* based on the segregation of the marker listed three columns to the left

[d] Significance level of the marker association

[e] R^2 value of the marker association

[f] Phenotype of the line based on aphid numbers and the marker association test

[g] Marker genotypes of the recombinant plants that formed the recombinant lines. *R* homozygous for the allele from PI 200538, *H* heterozygous, *S* homozygous for the allele from the susceptible parent, *NT* not tested. Italicized letters are placed at the genetic interval containing inferred recombination event

Table 3 Information on the 35 STSs on chromosome 13 (LG F) (Hyten et al. 2010) that were sequenced during the fine mapping of *Rag2*

Marker name	GenBank accession # of STS	NCBI-dbSNP name of SNP located on STS	Consensus Map 4.0 Position (cM)
#1	GF097646	ss4969612	52.261
#2	GF097659	ss4969648	49.924
#3	GF097679	ss4969734	50.395
#4	GF091828	ss107912850	51.666
#5	GF091836	ss107914023	51.936
#6	GF091900	ss107912922	55.317
#7	GF091960	ss107912982	55.858
#8	GF092024	ss107913046	56.031
#9	GF092100	ss107913125	50.103
#10	GF092140	ss107913165	54.149
#11	GF092174	ss107913199	56.171
#12	GF092326	ss107913351	52.389
#13	GF093742	ss107917482	48.349
#14	GF092414	ss107913443	49.424
#15	GF092564	ss107913594	56.94
#16	GF092576	ss107918675	52.338
#17	GF097352	ss107912576	54.374
#18	GF093970	ss107918696	49.932
#19	GF092628	ss107913658	54.078
#20	GF094005	ss107918836	56.609
#21	GF092655	ss107913685	51.598
#22	GF097432	ss107912657	53.202
#23	GF097440	ss107912665	54.922
#24	GF094366	ss107920248	49.32
#25	GF094412	ss107920435	55.989
#26	GF094471	ss107920639	56.243
#27	GF097393	ss107912618	53.202
#28	GF094141	ss107919283	51.602
#29	GF094142	ss107919284	50.707
#30	GF094701	ss107921354	54.078
#31	GF096278	ss107927651	55.781
#32	GF096883	ss107929664	53.202
#33	GF096992	ss107929998	52.218
#34	GF097147	ss107930597	51.896
#1485	GF097621	ss4969627	NA

200–300 aphids of biotype 2 at all life stages on top of V_E-stage seedlings. Ten days after inoculation, resistance was evaluated by counting the total number of aphids on each plant.

DNA extraction

Genomic DNA from the 95 F_2 plants was extracted using young trifoliolate leaves of each plant with the CTAB (hexadecylatrimethylammonium bromide) method described by Honeycutt et al. (1992) with minor modifications (Hill et al. 2009). Because tissue samples were collected during the SA resistance evaluation experiment, aphids were eliminated from leaves with the systemic insecticide imidacloprid prior to sampling.

Genomic DNA from the 3,151 BC_3F_2, BC_2F_2, and F_2 plants in 2008 and 2,632 BC_3F_2, F_2, and BC_4F_2 plants in 2009 was extracted using young trifoliolate leaf tissue from each plant using the quick extraction method (Bell-Johnson et al. 1998). Genomic DNA from 185 recombinant lines selected in 2008 and 58 recombinant plants selected in 2009 was extracted using young trifoliolate leaf tissue pooled from 12 progeny plants from each line using the CTAB method described above.

After the completion of the resistance assays, genomic DNA from each of the 377 individual progeny from the

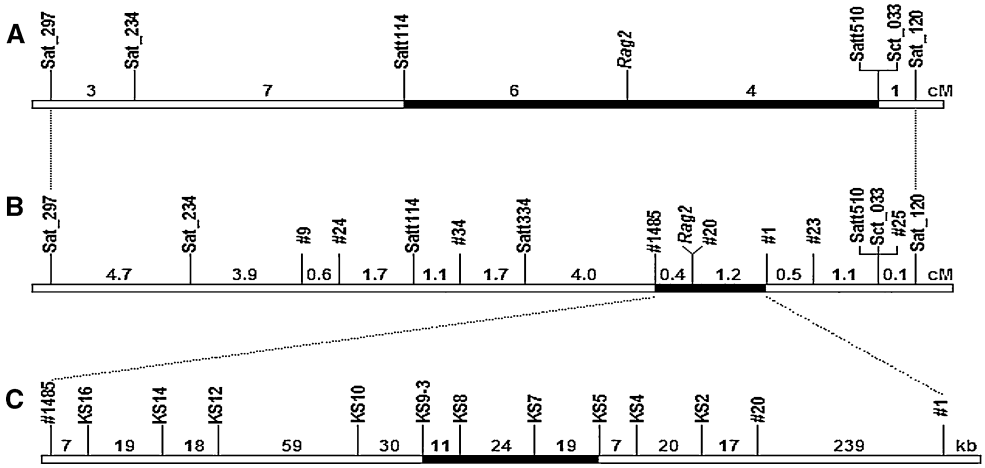

Fig. 1 Genetic and physical maps of the interval *Rag2* is located on soybean chromosome 13 (LG F). **a** Linkage map of the interval *Rag2* is located using the same population of 95 $F_{2:3}$ lines as Hill et al. (2009). The numbers between the markers are the linkage distances in centiMorgans (cM). **b** Linkage map of the interval *Rag2* is located using SNP markers developed by re-sequencing of STSs using the same Hill et al. (2009) population. **c** High-resolution physical map of the interval *Rag2* is located using eight recombinant lines and SNP markers developed by re-sequencing in the target interval. *Numbers* between the markers show physical distances in kilo bases (kb) on the Williams 82 8× assembly (Glyma1) available at http://www.phytozome.net (Schmutz et al. 2010). The interval containing *Rag2* was narrowed down to a 54-kb region between the markers KS9-3 and KS5

eight selected recombinant lines in these assays was extracted using the CTAB method. All CTAB DNA was quantified by ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and diluted to 25 ng μl^{-1} for SSR genotyping and 20 ng μl^{-1} for SNP genotyping.

SSR marker re-screening within the interval containing *Rag2*

All SSR markers previously mapped between Satt510 and Satt114 on chromosome 13 (LG F) were retested to determine if any were polymorphic between PI 200538 and LD02-4485, the two parents of the first mapping population that includes 95 $F_{2:3}$ lines. The primer sequences and location of the SSR markers are available from Soybean Linkage Map-2006 (http://bfgl.anri.barc.usda.gov/cgi-bin/soybean/Linkage.pl; accessed 27 May 2009). Polymerase chain reaction (PCR) was performed according to Cregan and Quigley (1997). PCR reactions consisted of 36 cycles of denaturation at 94°C for 25 s, annealing at 46°C for 25 s, and extension at 68°C for 25 s with a PTC 100 Programmable Thermal Controller (MJ Research Inc., Watertown, MA, USA) with slight modifications according to the specific annealing temperature of the primers. The PCR products were first analyzed in 3% agarose gels (BMA, Rockland, ME, USA) and then retested in 3% metaphor-agarose gels with ethidium bromide staining in 1× Tris–Borate-EDTA buffer.

Re-sequencing of STSs

To develop additional markers and narrow the gene interval, re-sequencing of STSs already mapped to the region on soybean chromosome 13 (LG F) where *Rag2* is located was performed. Information on target amplification primer pairs for the 34 STSs (Table 3) containing SNPs from this region was obtained from Hyten et al. (2010). These STSs were first re-sequenced to determine if the SNPs previously identified were present between the parents of the recombinant lines. A target amplification primer pair for one additional STS, GF097621 (Table 3), was designed using IDT SciTools PrimerQuest^{SM} software (Integrated DNA Technologies, Coralville, IA, USA).

The re-sequencing was done by first PCR amplifying the STSs. Amplification reactions were conducted with 100 ng of parental DNA, 0.25 μM of forward and reverse primer, 1× of buffer (BioLabs Inc., MA, USA), 0.25 mM of each dNTP (Applied Biosystems, Foster City, CA, USA), and 1 U of *Taq* polymerase (BioLabs Inc.), in a total volume of 40 μl. The reaction mixture was denatured at 95°C for 1 min and subjected to 28 cycles of 94°C for 30 s, annealing at 55°C for 40 s, and extension at 68°C for 2 min 20 s, followed by one cycle of 8 min at 68°C using a PTC 100 Programmable Thermal Controller (MJ Research Inc.). PCR products were resolved by gel electrophoresis in 0.9% TAE agarose gels stained with ethidium bromide. The presence of a single PCR product was verified for each primer pair. If primer pairs produce no product or multiple

products, annealing temperatures or PCR cycles were modified to identify the optimum PCR condition for each primer pair (Choi et al. 2007).

When single PCR products from the two parents were produced, these were purified with the QIAquick Gel Extraction Kit (Qiagen, CA, USA). Purified PCR products were then sequenced from both ends using the same primers as for PCR amplification with the ABI BigDye Terminator v3.1 cycle sequencing kit on an ABI PRISM 3730 sequencer (Applied Biosystems) at the University of Illinois Keck Center Core Facility. To detect SNPs between two parents, ABI trace files were analyzed by Sequencher version 4.9 (Gene Codes Corporation, Ann Arbor, MI, USA).

Saturation of the *Rag2* region with additional SNP markers

Once *Rag2* was positioned relative to the SNPs developed from the STSs, direct re-sequencing of target regions was conducted to develop additional SNP markers that could be used to better define the genomic position of *Rag2*. A total of 27 target amplification primer pairs were designed every 10 kb between nucleotides 29,097 and 29,310 kb on chromosome 13 based on the Williams 82 8× draft assembly (Glyma1) (Schmutz et al. 2010). The primers were designed with IDT SciTools PrimerQuest^{SM} software (Integrated DNA Technologies). The uniqueness of each primer pair in the genome was double-checked by BLAST analysis. Sequencing and SNP detection were conducted as described above.

SNP marker and dominant marker genotyping

Target amplification primers and probes for TaqMan assays or melting curve assays (MCAs) were designed for the confirmed SNPs. Target amplification primers and simple probe for MCA were designed by use of the LightCycler® Probe Design Software 2.0 (Roche Diagnostics, Switzerland) and were blasted to Williams 82 8× draft assembly (Glyma1) to check whether there was a single match of the sequences and to verify their position in the soybean genome. If a target amplification primer or simple probe matched multiple regions of the soybean genome, the primer or probe was redesigned until it had a single match in the soybean genome. TaqMan primers and probes were designed by Assays-by-Design Service (Applied Biosystems). SNP marker genotyping using TaqMan assays or MCAs was conducted with the Roche LightCycler® 480 System (Roche Diagnostics, Indianapolis, IN, USA) described by Kaczorowski et al. (2008).

The target sequencing primer pairs KS8 and KS10 produced a desired single PCR product only for the susceptible parent LD02-4485 and were used as dominant markers. These markers could distinguish homozygous resistance alleles from homozygous susceptible alleles, but could not distinguish homozygous susceptible alleles from heterozygous alleles. Therefore, plants having no PCR product were classified as homozygous resistance and those with a product were classified as heterozygous or homozygous susceptible. The forward and reverse primer sequences of KS8 were 5′-TACCCTCAAATGGACTTGGTGCCT-3′ and 5′-TGGCGATGGTGATCTTGACTGTCT-3′, receptively. The forward and reverse primer sequences of KS10 were 5′-TCCCATTACGCCGTTCAGCAAGAT-3′ and 5′-GGTGTACAAGGAAAGCCCAAGACT-3′. PCR condition for these two markers was the same as for the SSR marker and the PCR products were analyzed in 1% agarose gels. All markers for lines with genotypes given on Table 2 were tested on each progeny plant in the aphid assays resulting in 3–13 markers tested on these progeny plants.

Genetic mapping and statistical analysis

Molecular marker and phenotype data were used to construct a genetic map with the 95 $F_{2:3}$ lines from the LD02-4485 × (Ina × PI 200538) population. Linkage analysis was performed with JoinMap 3.0 (Van Ooijen and Voorrips 2001) using the Kosambi mapping function. A logarithm (base 10) of the odds (LOD) score of 5.0 was used as a threshold to group markers into a linkage group.

To test whether *Rag2* was segregating among plants in each of the eight recombinant lines phenotyped for SA resistance, the progeny plants that had been evaluated for SA resistance were tested with a polymorphic marker in each line that maps near *Rag2*. Single factor analysis of variance was used to identify the associations between SA resistance and marker segregation using the PROC GLM procedure of SAS (SAS Institute 2002).

Results

Genetic mapping of *Rag2* using SSR markers and SNP markers developed by STS re-sequencing

The retesting of all SSR markers mapped between Satt114 and Satt510 on chromosome 13 (LG F) revealed that Satt334 was polymorphic between PI 200538 and LD02-4485. The polymorphism between the two soybean genotypes could not be distinguished in 3% agarose gels, so this marker was genotyped with 3% metaphor-agarose gels. Satt334 is located between Satt114 and Satt510 on the genetic map developed by Hill et al. (2009) (Fig. 1a, b).

STSs previously mapped near the *Rag2* locus were re-sequenced to saturate the SSR map and to further

define the location of *Rag2* on chromosome 13. Thirty-four out of the 35 STSs producing a single PCR product in both parents and were sequenced (Table 3). STS #28 was the exception and failed to produce an amplification product. Sixteen of the STSs were found to have 29 SNPs and 33 small insertion/deletions (INDELs). Among these 16 STSs, 8 (#1, #9, #20, #23, #24, #25, #34, #1485) contained SNPs that were appropriate for the development of MCA or TaqMan SNP marker assays (Tables 4, 5).

The eight SNP markers developed from re-sequencing the STSs were incorporated onto the genetic map developed by Hill et al. (2009). All SNP markers were mapped between Satt510 and Sat_234. *Rag2* was mapped between the SNP markers #1 and #1485 and co-segregated with SNP #20 (Fig. 1b). This resulted in narrowing the genetic interval containing the gene from 10 to 1.6 cM in length using the 95 $F_{2:3}$ lines from the LD02-4485 × (Ina × PI 200538) population (Fig. 1a, b).

Fine mapping of *Rag2* using SNP markers developed by re-sequencing

To further define the position of *Rag2*, SNP marker analyses and SA resistance tests were conducted on progeny plants from the five lines (18, 32, 86, 162, and 181) identified in 2008 as having recombination events between SNP markers #1 and #20 or #20 and #1485. The lines 18, 32, and 86 had recombination events between #20 and #1. The SA resistance segregation among plants in the progeny tests were consistent with the segregation of #20 and #1485, but not #1, suggesting that *Rag2* is likely between #20 and #1485 (Table 2).

Direct re-sequencing in the interval between #20 and #1485 was then done to identify additional SNPs that could be used to refine the map position of *Rag2*. Although one target amplification primer pair was designed for each 10 kb within the interval, some regions did not have sufficient sequence information suitable to design target amplification primers as a result of the draft quality of the soybean genome at the time. A total of 27 primer pairs were designed with thirteen of the 27 primer pairs producing a single PCR product for both parents. Through sequencing of the PCR products from both parents, SNP markers were developed for seven primer pairs. In addition, the two primer pairs KS8 and KS10 produced a single PCR product from only LD02-4485 DNA and failed to amplify a product from PI 200538 and were used as a dominant marker in genotyping the recombinant lines. By testing all progeny plants from the greenhouse resistance evaluations with these dominant markers, lines derived from heterozygous and homozygous susceptible plants could be distinguished (Table 2).

Individual plants from the lines 162 and 181, which were identified as having recombination between SNP markers #20 and #1485, were genotyped with the nine new markers (Table 2). Line 162 was segregating for SNP markers from KS5 to #1 alleles and homozygous resistant from KS7 to #1485. No significant association between the segregation of aphid resistance and the SNP marker KS5 was observed for this line (Table 2) and all tested plants had a resistant phenotype. The results for line 162 show that *Rag2* must be to the left of KS5 in the genetic map. The left border of the position of *Rag2* was refined by analysis of line 181. This line was segregating for SNP markers from KS12 to #1 and homozygous resistant for markers from #1485 to KS14. There was a highly significant association ($P < 0.0001$) between aphid resistance and the segregation of the SNP marker KS12 in the line (Table 2), showing that *Rag2* was segregating and therefore to the right of KS14. A BLAST analysis indicated that the SNP detected by the marker KS5 is located at 29,266,469 bp and the SNP detected by the marker KS14 is located at 29,123,397 bp on chromosome 13. Therefore, the genomic region containing the gene was narrowed to an interval approximately 143 kb in length (Table 2; Fig. 1c) based on the Williams 82 sequence.

To further refine the position of *Rag2* within the 143-kb region, re-sequencing was done to identify additional DNA markers within the region. Nine target amplification primer pairs were designed for re-sequencing between KS7 and KS12. Of the nine primer pairs, three did not produce a PCR product from PI 200538 and two did not produce a PCR product from both parents. This poor success rate was likely the result of low sequence homology between the Williams 82 sequence, which was used to design the primers, and LD00-4485 and PI 200538, the parent used to test the primers. Four primer pairs produced products from both parents and these products were sequenced. The sequences of three products contained too many SNPs and INDELs which made it impossible to develop SNP assays. A SNP assay was successfully developed from only the remaining SNP marker (KS9-3).

A total of 2,632 BC_3F_2, F_2, and BC_4F_2 plants grown in the field at Urbana, IL in 2009 were tested with the markers KS7 and KS12 to find additional recombination events within the interval. Three plants were identified with recombinations in the interval and the lines K16, K31, and K37 were derived from these recombinant plants. Progeny plants from these lines were genotyped with all SNP markers and tested for SA resistance. The line K37 was segregating for SNP markers from KS9-3 to #1 and was fixed for markers #1485 to KS10. There was a highly significant ($P < 0.0001$) association between these segregating SNP markers and SA resistance, indicating that *Rag2* was to the right of KS10 (Table 2). Lines KS16 and

Table 4 Sequences of target amplification primers and MCA sensor probes used for SNP genotyping

Marker name	Type	Sequences
#1	Forward	CTCGAAAGGTGAACATGCACCA
	Reverse	AGAACATTAAGAGATATGGGAAGGAAGTAG
	Probe	Fluorescein-SPC-TTAATACACATATAAATTTTGAGAGC**A**TTTAAGT-Phosphate
#9	Forward	CAAACCAACCAAATGCTCAGAATACACG
	Reverse	AATGAATAATTCATATGATTAATAGG
	Probe	Fluorescein-SPC-AGTGCATGCATACCTTTAGTTGCTGGATTTGAGAT-Phosphate
#20	Forward	ACATTGCAATCAAAATCAAGATGTAGCTGG
	Reverse	GACGATTTTGGTTTCTGTGATCTTACGTG
	Probe	Fluorescein-SPC-TTCTGTAGCTTCTACCCAAGGGCTAGCCTTATCCA-Phosphate
#23	Forward	ATGCCAATCCATTCTAAAGT
	Reverse	GGATCATTGATGGCACGA
	Probe	Fluorescein-SPC-TTCTGCAAACATAAACGGATCAAAATATCA-Phosphate
#24	Forward	CCCCATGGAAATTAAGATTCCTGC
	Reverse	GCATGAGCACAAAGTTTTTCTTGGC
	Probe	Fluorescein-SPC-ATGCCCATGGTTAATTAAGTAAACACATTT-Phosphate
#25	Forward	GTGTGCATGTGTTTGAACTTTGAAGAGATT
	Reverse	ATCACAGAGACATGGAGGTTGCTAT
	Probe	Fluorescein-SPC-CTTGTCCTCCTGACTCTCTCCAGGTACTT-Phosphate
#34	Forward	AGAATATTATGAAGATCAAACATGAACAA
	Reverse	AATAATGTTTTGTTTAATACTTTTACTTGG
	Probe	Fluorescein-SPC-TTTCTCCTTTAAAAATAAGTAGAACCATTTTTTT-Phosphate
KS2	Forward	CTGCATCAGCTACTTCATGAGGAG
	Reverse	GGTCTGATTTGCTATTAAACCATCTTCCTT
	Probe	Fluorescein-SPC-ACCAGTCCTCTGAAAAAGTGAAGAGAAATCAACAA-Phosphate
KS4	Forward	ACCACAAAACAAGCAAATGAGTCACT
	Reverse	GTGCATGTTCGTTGTGATTTTCCCT
	Probe	Fluorescein-SPC-CAATGCACAAGTAGGAAAAATCATCCAAACGGGAA-Phosphate
KS5	Forward	CATGGAAGGCTGATAATACAGACATGTACC
	Reverse	CGTCGAGCTTAATGCGTGAAGGAAA
	Probe	Fluorescein-SPC-GGAAGAGGATGAGGACGCCATCATCGACATTCA-Phosphate
KS7	Forward	CAGGGCAAAGTGTGGAGACAAT
	Reverse	CAATCCATTATACGCTATACACTCCCCTTC
	Probe	Fluorescein-SPC-AGCTAGTTCGATTTTATCAACAATTAGGGTGATGA-Phosphate
KS12	Forward	ATCAAGCTCACTCCTTATTGAATAAACCT
	Reverse	ACATTGATCCATTATGTTTGCTTAACAAGT
	Probe	Fluorescein-SPC-AAAGGAATGCATTAGAAACTTATTGCCACTCCTCA-Phosphate
KS14	Forward	CTTCCGTCATCCATTAAGAGCAATTCATTT
	Reverse	TGGATGCAGAGGTTGTGTATGTGGTTTAG
	Probe	Fluorescein-SPC-AGTTGTTAACATGACAAGAGGTGAAAGAGACGAGT-Phosphate
KS16	Forward	CAGGTTCTTCACTCAAGTTGTTGCT
	Reverse	AATCAGAATCAGATTGAAAACAAGACACCA
	Probe	Fluorescein-SPC-GTCACATTTTGTTTTTGTTGTAATTTTGTTGGA-Phosphate

KS31 were segregating for SNP marker alleles from #1485 to KS9-3 and homozygous resistant from KS8 to #1. Because there was no significant association between aphid resistance and segregation for the SNP marker KS9-3 for these lines, *Rag2* must be to the right of KS9-3. A BLAST analysis revealed that the SNP marker KS9-3 was located at 29,212,318 bp on soybean chromosome 13 (LG F). These results indicated that *Rag2* maps within a 54-kb region defined by KS9-3 and KS5 on soybean chromosome 13 (Table 2; Fig. 1c).

Table 5 Sequences of target amplification primers and TaqMan probes for SNP genotyping

Marker name	Type	Sequence
#1	Forward	AGAACATTAAGAGATATGGGAAGGAAGTAGT
	Reverse	GGAACATTACTAAAAACGATATGTCAAAGTTAGAA
	Probe 1	ATTTTGAGAGCATTTAAG-VIC
	Probe 2	AATTTTGAGAGCTTTTAAG-FAM
#20	Forward	AAATCAAGATGTAGCTGGATGGATAAGG
	Reverse	GCTTTTGCACTTGAATTATTTGTTTTCTGT
	Probe 1	CCCTTGGGTAGAAGC-VIC
	Probe 2	CCCTTGGATAGAAGC-FAM
#1485	Forward	GCATAGAAATTTACACATCCATCAACCAT
	Reverse	CGTTTGGGAATAGCTTACAAGCTT
	Probe 1	ACTCTACCCTGACAATAG-VIC
	Probe 2	CTCTACCCTCACAATAG-FAM
KS9-3	Forward	ACGTCAAGTGATGACTTAACACTTGT
	Reverse	AGAAGTAGGAAGGACAAAACTTGAATATAAAGAAAAA
	Probe 1	ATCATTAGAAAACGAAATAA-VIC
	Probe 2	ATCATTAGAAAACAAAATAA-FAM

Discussion

The SA-resistance gene *Rag2* from PI 200538 was fine mapped in this study. Mian et al. (2008b) also mapped a single dominant gene named *Rag2* from PI 243540 to the same region on soybean chromosome 13 (LG F). Because these genes map to the same region and they both show resistance to SA biotype 2, it is likely that both sources have a resistance allele at the *Rag2* locus.

Our mapping efforts were greatly accelerated by the availability of the public sequence of the soybean genome (Schmutz et al. 2010). This sequence information was especially valuable in discovering SNPs through direct re-sequencing of target regions, determining the position of markers on the physical map, and identifying candidate genes in the region where the gene is located.

The current gene annotation of the 54-kb region containing the *Rag2* locus on the aphid susceptible Williams 82 8× assembly (Glyma1) predicts the presence of seven candidate genes. Of these genes, Glyma13g26000 is the only nucleotide-binding site (NBS)-leucine-rich repeat (LRR) candidate gene and is therefore the strongest *Rag2* candidate based on the Williams 82 sequence. Glyma13g26000 encodes a F-Box/LRR protein and shares significant homology with Arabidopsis and soybean sequences encoding disease resistance proteins such as the coiled-coil (CC)-NBS-LRR or toll-interleukin receptor-NBS-LRR classes of genes. Wang et al. (2005) demonstrated that the soybean F-box protein gene *GmCOI1* mediates Jasmonate (JA) that regulates plant defense and fertility in Arabidopsis. Li et al. (2008) suggested that JA-, ethylene-, and salicylic acid (SA)-regulated signaling pathways were at least partially activated by aphid feeding on soybean.

The majority of cloned resistance genes are members of the NBS-LRR gene family. Cloned NBS-LRR genes that confer aphid resistance include the *Mi* gene, which controls root-knot nematode (*Meloidogyne incognita*) and potato aphid (*Macrosiphum euphorbiae*) resistance in tomato (*Lycopersion esculentum* Mill.) (Milligan et al. 1998; Rossi et al. 1998), and the *Vat* gene, which confers resistance to *A. gossypii* in melon (*Cucumis melo* L.) (Brotman et al. 2002; Dogimont et al. 2009). In addition, an aphid-resistance gene in *Medicago truncatula* Gaertner was mapped to a NBS-LRR cluster region (Klingler et al. 2005).

A prominent aspect of the genome organization of the NBS-LRR gene family is that members tend to occur in localized clusters (Martin et al. 2003). Such clustering is seen both for resistance genes specific for different races of the same pathogen (Hulbert and Bennetzen 1991) and for resistance genes conferring resistance to unrelated pathogens (Witsenboer et al. 1995). For instance, three NBS-LRR genes were interspersed in a 213-kb region at the *Mla* locus in barley, which controls resistance to multiple strains of powdery mildew (Wei et al. 1999). In the 84-kb region between KS10 and KS5, where *Rag2* is located, three NBS-LRR genes are present. These include Glyma13g26000, our best candidate for *Rag2*, the *Rpg1-b* gene, which confers resistance to *Pseudomonas syringae* pv. *glycine* (Ashfield et al. 2003), and a third NBS-LRR candidate gene of unknown function. Both *Rpg1-b* and the third gene are outside the 54-kb region containing *Rag2*, so they are likely not *Rag2*. Our research on fine mapping *Rag1* also showed that the 115-kb region including *Rag1* contains two NBS-LRR genes (Kim et al. 2010). These gene predictions for the *Rag2* region are all based on the Williams 82 sequence and Williams 82 does not contain

the resistance allele at *Rag2*. The region containing *Rag2* will need to be cloned from PI 200538 and sequenced to determine whether these same predicted genes exist in PI 200538.

A major peanut root-knot nematode [*M. arenaria* (Neal) Chitwood]-resistant QTL was previously mapped to the same region as *Rag2* on soybean chromosome 13 using PI 200538 as a resistance source (Tamulonis et al. 1997). The mapping of both aphid and nematode resistance to the same region on chromosome 13 from PI 200538 suggests that *Rag2* could be analogous to the *Mi* gene in conferring resistance to both aphids and nematodes. Further work is needed to determine whether peanut root-knot nematode resistance maps to the same position as *Rag2*.

Tamulonis et al. (1997) also mapped a minor peanut root-knot nematode-resistance QTL to chromosome 15 (LG E) and there is evidence of homology between the *Rag2* region and the region on chromosome 15 that contains this minor QTL. This homology is shown by the STS GF097621, which was used to develop the SNP marker#1485. When the SNP was developed through re-sequencing, the STS was located on soybean chromosome 13 in SoyBase (http://soybase.org/) and the Williams 82 8× draft assembly (Glyma1) (verified Feb 2009), and its assembly sequence was perfectly matched to chromosome 13 and partly (85%) matched chromosome 15 (LG E). SNP marker #1485 also was mapped on soybean chromosome 13 through linkage analysis in our research when we used LOD score 5.0 or higher in JoinMap 3.0. At present, however, the location of the STS has been repositioned to between 4,130,5182–4,130,5716 bp onto chromosome 15 in SoyBase (http://soybase.org/) and Phytozome (http://www.phytozome.net), which is between the SSR markers Satt491 and Satt685 (1,966,3839–4,827,3769 bp). This is the same interval that Tamulonis et al. (1997) mapped the minor peanut root-knot nematode QTL from PI 200538 (GmComposite 2003, http://soybase.org/) indicating regions that include the STS on chromosome 13 and 15 have high homology or are duplicated. This homology is supported by Shoemaker et al. (1996) who reported that an extensive region of homology on soybean chromosomes 13 and 15 exist. Hayes et al. (2000) also reported that two NBS-LRR classes of disease-resistance genes identified by the same probe were mapped on soybean chromosome 13 and 15 and these genes have strong homology. These results suggest that the minor QTL for peanut root-knot nematode resistance on chromosome 15 could be a homolog of the major QTL on chromosome 13.

The high-resolution genetic and physical map of the *Rag2* locus will facilitate MAS for this gene from PI 200538 in soybean breeding programs as it has resulted in the identification of SNP markers closely linked to it. The introgression of this gene into new cultivars is important as it provides resistance to SA biotypes 1 and 2.

The SNP markers we developed will be especially useful in MAS and pyramiding of SA-resistance genes because of their very close proximity to the gene and the availability of efficient SNP marker detection assays. In addition, the identification of the physical location of *Rag2* on the soybean chromosome will greatly facilitate the cloning and functional characterization of the gene. The cloning of *Rag2* will improve our understanding of SA defense mechanisms in soybean plants. This information also can be applied to compare the function of this gene to other SA-resistance genes such as *Rag1* or cloned insect-resistance genes in other species. We are conducting ongoing studies to clone *Rag1* and *Rag2*.

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