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Molecular mapping of the genomic region conferring resistance to soybean stem canker in Hutcheson soybean

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Abstract Genetic resistance to soybean stem canker, caused by the fungus *Diaporthe phaseolorum* var. *meridionalis* (Dpm), is controlled by five major, dominant, nonallelic genes *Rdm1* to *Rdm5*. A genomic region containing the *Rdm4* and *Rdm5* genes was first described in Hutcheson soybean, where they were found to confer specific resistance to Argentinean physiological races of Dpm. Here, we report the genetic mapping of *Rdm4* and *Rdm5* loci using two pheno- and genotypically characterized $F_{2:3}$ populations derived from Hutcheson cultivar. The mapping populations were screened with amplified fragment length polymorphism (AFLP) markers using bulk segregant analysis, and with simple sequence repeat (SSR) markers. Linkage analysis indicated that the *Rdm4* and *Rdm5* resistance loci were located in a genomic region collinear with the molecular linkage group (MLG) A2 (chromosome 8) of the soybean genetic map. The linkage group contains two SSR markers, Sat_162 and Satt233, flanking the *Rdm4* and *Rdm5* loci. These SSR will be useful to increase the efficiency of selection in breeding programs aimed to incorporate *Rdm4* and *Rdm5* genes into soybean elite germplasm.

Keywords *Diaporthe phaseolorum* var. *meridionalis* · Disease resistance genes · Amplified fragment length polymorphism (AFLP) · Simple sequence repeat (SSR) · Bulk segregant analysis (BSA) · Linkage analysis · Marker-assisted breeding

Introduction

Soybean (*Glycine max* (L.) Merrill) is one of the leading oil crops as well as the main source of vegetable protein for animal feed produced and consumed worldwide. In the 2015/2016 growing season, world soybean production was 313 million metric tons, with the USA (34%), Brazil (31%) and Argentina (18%) as the leading producers (https://www.fas.usda.gov/data/world-agricultural-production).

Soybean annual losses due to diseases were estimated at 11% worldwide (Hartman et al. 2015). Particularly, soybean stem canker (SSC) is an economically important and potentially very destructive disease that causes plant death, usually from the middle of the growing season to crop maturity (Sinclair and Hartman 1999). This fungal disease is caused by the complex *Diaporthe phaseolorum*,

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anamorph *Phomopsis phaseoli*, which includes two varieties: *D. phaseolorum* var. *meridionalis* (Dpm) and *D. phaseolorum* var. *caulivora* (Dpc). SSC has caused significant losses in all major soybean-producing countries (Wrather et al. 1997, 2010). In Argentina, SSC caused by Dpm, was first detected during the 1992–1993 growing season (Pioli et al. 1993) and then an important outbreak was reported in 1996–1997 (Pioli et al. 1997; Ploper et al. 1999). Hence, resistance to SSC is an obligatory requirement for the release of new soybean cultivars in Argentina (www.inase.gov.ar).

The use of resistant cultivars provides an effective approach for disease control, which minimizes the use of fungicides, reduces crop losses and supports sustainable production management. Genetic resistance to SSC caused by Dpm is controlled by five major, dominant, nonallelic resistance genes, *Rdm1* to *Rdm5* (Kilen and Hartwig 1987; Bowers et al. 1993; Tyler 1996; Chiesa et al. 2009). None of the known *Rdm* genes of resistance to Dpm provide resistance against Dpc, and some of them displayed differential expression when interacting with different Dpm isolates (Pioli et al. 2003; Chiesa et al. 2013).

Particularly, the *Rdm4* gene was first identified in Dowling cultivar (Bowers et al. 1993), and an allele of this gene was later characterized in Hutcheson cultivar (Tyler 1996). More recently, it was found that the resistance to SSC in Hutcheson was conferred by at least two resistance genes, the previously reported *Rdm4* and a new gene, named *Rdm5* (Chiesa et al. 2009). The resistant cultivar Hutcheson has been successfully used as a source of resistance to SSC in Argentinean commercial varieties (Pioli et al. 2003).

The availability of molecular markers linked to the *Rdm* genes would improve the efficiency of SSC resistance incorporation in soybean-breeding programs. Recently, two single nucleotide polymorphisms (SNP) associated with resistance to Dpc and Dpm were reported in the chromosome 14 of soybean using genome-wide association studies (GWAS) (Chang et al. 2016). The authors indicated that the linkage disequilibrium region of the two SNPs overlapped each other, suggesting that the same resistance source underlies the SNPs associated with Dpc and Dpm resistance (Chang et al. 2016). However, it was previously demonstrated that the *Rdm1–5* major known genes that confer resistance to Dpm do not confer resistance to Dpc (Pioli et al. 2003) suggesting that the genomic region identified in chromosome 14 by Chang et al. (2016) would be a different source of resistance. In this work, we report the localization of the genomic region containing the *Rdm4* and *Rdm5* genes in the MLG-A2 (chromosome 8) of the soybean genetic map.

Materials and methods

Plant materials and population development

The Hutcheson cultivar (*Rdm4/Rdm4*; *Rdm5/Rdm5*) (Chiesa et al. 2009) was used as the resistant parent, and the experimental line J77-339 (*rdm/rdm*) (Kilen and Hartwig 1987) was used as the susceptible parent. The J77-339 × Hutcheson cross was made at the experimental field of the Facultad de Ciencias Agrarias, Universidad Nacional de Rosario, Zavalla, Santa Fe, Argentina. The F_1 plants were grown in the field and were self-fertilized to produce F_2 segregating seeds. The F_2 from populations J04c ($n = 51$) and J04d ($n = 108$) were self-pollinated, and the plants were harvested individually to obtain the $F_{2:3}$ families, as previously described by Chiesa et al. (2009). Thus, two separate and independent populations, derived from the same parents, were pheno- and genotypically assayed to map the *Rdm4* and *Rdm5* genes. Since *Rdm5* was differentiated from *Rdm4* by pheno-genotypic analysis of the J04d population interacting with physiological races of the pathogen, *Rdm4* resistance to isolate CE109 and *Rdm5* resistance to isolate CE112 was arbitrarily assigned (Chiesa et al. 2009).

Inoculation procedure and disease evaluation of the $F_{2:3}$ families

Seeds were sown and grown in 8-cm diameter plastic pots, filled with a sterilized mix of three parts humus-rich soil and one part perlite. The soil mix was maintained at field capacity by subirrigation. The inoculation assays were carried out in a greenhouse under natural sunlight during the springs of two different years. The mean photosynthetic active photon flux density was 500 μE m^{-2} s^{-1} (400–700 nm), measured with a LI-COR 185a radiometer and 190 s sensor (LI-COR, Lincoln, NE). The average day and night temperatures were 27 ± 3 and 18 ± 3 °C, respectively.

The pathogenic behaviours of the Dpm isolates CE109 and CE112, used for the phenotypic analysis, were previously characterized by Pioli et al. (2003). All inoculations were performed on seedlings in the fully expanded trifoliate leaf stage (12–15 days old) as described previously (Chiesa et al. 2009). Plant-pathogen

interactions were evaluated weekly from 7 days post-inoculation (dpi) to 54 and 41 dpi in the specific interaction with CE109 and CE112 isolate, respectively.

Phenotypic and genotypic analyses were performed by progeny tests in the J04c population by inoculating 10–11 seedlings from each $F_{2:3}$ family with CE109 isolate of Dpm. Also, progeny tests in the J04d population were performed, for each $F_{2:3}$ family, by inoculating independently 10–11 seedlings with CE109 isolate, and other group of 10–11 seedlings with CE112 isolate of Dpm (Chiesa et al. 2009).

To characterize the phenotypic response of the parental lines and the $F_{2:3}$ families, in order to infer the genotypes of the F_2 plants for the *Rdm* gene mapping, the following scale was used: (i) dead plants (DP) ≤ 19.9%, resistant phenotype and homozygous dominant genotype; (ii) DP = 20 to 69.9%, moderately resistant to moderately susceptible phenotype and heterozygous genotype; and (iii) DP = 70 to 100%, susceptible phenotype and homozygous recessive genotype (Chiesa et al. 2009).

DNA extraction

Young leaf tissue was collected at vegetative stages V1 (one trifoliate leaf) or V2 (two trifoliate leaves) from each field-grown F_2 plant in the J04c and J04d mapping populations and parental genotypes, and flash frozen in liquid nitrogen. The frozen leaf tissue was stored in a −80 °C freezer until DNA purification. DNA was extracted using CTAB method (Shagai-Maroof et al. 1984) and dissolved in 100 μL of distilled and sterile water. DNA samples were quantified using a Genesys 20 Spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA), and their quality was visualized under UV light after staining the 0.8% (*w*/*v*) agarose gel with ethidium bromide (0.5 μg/mL). The final concentration of the DNA samples was adjusted to 300 ng/μL and stored at −20 °C.

Molecular marker development and genotyping

Amplified fragment length polymorphism

Amplified fragment length polymorphism (AFLP) molecular markers were analysed according to Vos et al. (1995) with minor modifications. Briefly, 1 μg DNA from each sample was digested with the four-cutter restriction enzyme *Mse*I (New England Biolabs, Ipswich, MA) and the six-cutter restriction enzyme *Eco*RI (Promega). Restriction fragments were ligated with double-strand *Eco*RI- and *Mse*I-adapters. A pre-amplification was performed using the appropriate primer combinations with one added selective nucleotide, E + A/M + A, manufactured by AlphaDNA (Montreal, Canada). The reaction mix was diluted 1/20, and 5 μL were used as the template for the final amplification with two primers, each having three selective nucleotides (Table S1). The AFLP primer combinations that generated clear band patterns and detected polymorphisms among the DNA samples were used in this study. In both amplifications, a thermocycler PTC-100 (MJ Research, Inc., Waltham, MA) was used.

Simple sequence repeats

The simple sequence repeats (SSR) were selected based on their reported genomic locations and amplified using the primer sequences from SoyBase (http://soybase.org) and the BARCSOYSSR_1.0 database (Song et al. 2010). Primers were manufactured by AlphaDNA. First, SSR were selected after a systematic search to achieve the best possible genome coverage with a homogeneous distribution of possible markers linked to the *Rdm* genes in each of 20 molecular linkage groups (MLG) of the soybean genetic map (SoyBase). From each MLG, an average of 12.5 loci were selected, at an average genetic distance of 10 centi-Morgan (cM) between them. Markers known to be linked to soybean diseases resistance loci were also tested. A similar strategy was used previously to map other resistance loci in soybean (Silva et al. 2008). A total of 290 primer pairs were analysed. SSR molecular markers were analysed according to Cregan and Quigley (1997) with minor modifications. PCR amplifications were performed in a final volume of 20 μL with 50 ng template DNA, 1 × PCR buffer, 1.5 mM $MgCl_2$, 100 μM of each dNTPs (Promega, Madison, WI), 1 U of Taq DNA Polymerase (Promega) and 0.15 μM of each of the forward and reverse primer in E-pure sterile water to the final concentration. The thermal cycles were 95 °C for 120 s, followed by 33 cycles of denaturing at 94 °C for 30 s, annealing at 47–60 °C (according to the optimum temperature for the primer pair tested) for 30 s, extension at 68 °C for 30 s, then an additional extension of 180 s at 72 °C at the end of the last cycle. The products were maintained at 4 °C until detection.

The AFLP- and SSR-PCR products were mixed with 5 μL of loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol and 40% sucrose) and resolved by gel electrophoresis on a high-resolution denaturing polyacrylamide gel (Merck, Rahway NJ) (5.7% for SSR; 4.75% for

AFLP) containing 0.3% methylenebisacrylamide (Promega), urea (Promega) (5.6 M for SSR; 7.5 M for AFLP) and 30% formamide (Merck), in 0.5X TBE buffer (50 mM Tris-base, 50 mM boric acid and 1 mM EDTA, pH 8). To 65 mL of the polyacrylamide gel solution, 390 μL 10% ammonium persulfate (Promega) and 44 μL N, N, N_2, N_1-tetramethylethylenediamine (Sigma Aldrich, St Louis, MO) were added. Amplicons were visualized by staining the DNA using a silver staining system (Promega) following the manufacturer's instructions. A 100-bp ladder (Promega) was used to estimate the size of the fragments.

Only AFLP bands that showed a clear and reproducible polymorphism were scored as present (1) or absent (0), since all of them were dominant. Polymorphic fragments were named E*x*/M*x* (*x* = 1 to *n*), indicating the specific *Eco*RI and *Mse*I primers used in the selective amplifications, and a final letter was attached to differentiate fragments derived from the same amplification (Table S1).

The SSR markers were screened based on the DNA fragments that were polymorphic between the resistant and susceptible parents, and tested further in the entire $F_{2:3}$ mapping populations. All polymorphic SSR markers identified in this study were codominant in the $F_{2:3}$ families and were scored as AA (homozygous for the Hutcheson allele), BB (homozygous for the J77-339 allele) or AB (heterozygous).

DNA preparation and pooling for bulk segregation analysis

Putative genetic linkage between markers and resistance conferred by *Rdm4* and *Rdm5* was assessed by bulk segregant analysis (BSA) (Michelmore et al. 1991). The phenotypic characterization of resistance was used to score individual F_2 plants from the J04c population, and the ten most resistant and ten most susceptible plants were chosen for BSA. After quantification, equimolar amounts of DNA from each group of ten F_2 individuals were pooled to form the resistant and susceptible pools. The bulked samples were screened over different combinations of AFLP primer pairs, as described previously. Markers present in the resistant bulk (RB) and the resistant F_2 individual but absent in the susceptible bulk (SB) and the susceptible F_2 individual were considered to co-segregate with the resistance to CE109 isolate of Dpm, conferred by *Rdm4*. Likewise, AFLP markers present in the SB and the susceptible F_2 individual but absent in the RB and the resistant F_2 individual were considered to co-segregate with the susceptibility to CE109 isolate of Dpm. The markers polymorphic between the RB and SB were identified as candidates for association with resistance to CE109 isolate by *Rdm4* and were further screened using the entire J04c population to map the loci (de-bulk assay).

Statistical data analysis and genetic linkage map construction

The resistant phenotype was evaluated as a qualitative trait and scored as a codominant trait, according to the $F_{2:3}$ progeny tests. The genotype of the respective F_2 source plants (resistant *Rdm/Rdm*, segregant *Rdm/rdm* or susceptible *rdm/rdm*) was inferred from the $F_{2:3}$ progeny tests for the J04c and J04d populations derived from the cross between J77-339 and Hutcheson. This process allowed the heterozygotes in the F_2 generation to be identified and tests the segregation against the 1:2:1 expected ratio for a single gene (Tables 1 and 2). The segregation patterns of the phenotypes/genotypes, and the selected SSR and AFLP markers in the mapping populations were checked for goodness-of-fit of the observed-to-expected ratios using the Chi-squared (χ^2) test. The probabilities of deviations from expected values were calculated using the Fisher's exact test (Sokal and Rohlf 1995) in the InfoStat software (student version; Córdoba, Argentina). JoinMap 4.0 (Van Ooijen 2006) was used to evaluate the molecular linkage, determinate the linear order, estimate the recombination frequencies between molecular markers and create a linkage map of the *Rdm4*/*Rdm5* locus. The Kosambi mapping function was used to calculate distances between loci (cM), and linkage was determined at a logarithm of odd (LOD) threshold of 3.0 with a maximum map distance of 50 cM. Marker order within a linkage group (LG) was verified using MapMaker/Exp 3.0b (Lander et al. 1987). The order and putative distances of markers in the obtained LG were compared with the soybean genetic map in SoyBase and the BARCSOYSSR_1.0 database.

Results

Phenotypic response in the mapping populations

After interaction with isolate CE109 of Dpm, the resistant parent Hutcheson remained symptomless in all inoculation tests (incompatible interaction, 0% DP), while the susceptible parent J77-339 presented 70% DP (compatible interaction). This phenotypic response was indicative of the isolate virulence and the effectiveness of the inoculation technique. Additionally, five inoculated hybrid plants (*Rdm4/rdm4, Rdm5/rdm5*) remained symptomless (incompatible interaction, 0% DP).

The progeny test of the J04c population (51 $F_{2:3}$ families), inoculated with the isolate CE109 of Dpm, identified 14 resistant (*Rdm4/Rdm4*), 25 heterozygous (*Rdm4/rdm4*) and 12 susceptible (*rdm4/rdm4*) $F_{2:3}$ families ($\chi^2 = 0.19$; $p = 0.93$). The progeny test of an independent F_2 population (J04d, 108 $F_{2:3}$ families) from the same J77-339 × Hutcheson cross, inoculated with the isolate CE109 of Dpm, identified 29 resistant (*Rdm4/Rdm4*), 52 heterozygous (*Rdm4/rdm4*) and 27 susceptible (*rdm4/rdm4*) $F_{2:3}$ families ($\chi^2 = 0.22$; $p = 0.90$). Each family was classified according to the scale described by Chiesa et al. (2009) and used to infer their respective F_2 genotype (homozygous dominant, heterozygous or homozygous recessive) that were required to mapping *Rdm4* in the Hutcheson soybean. In total, 1636 F_3 plants from J04c and J04d populations were phenotyped and used to infer the genotype of each F_2 plant (homozygous dominant, heterozygous or homozygous recessive) required to mapping *Rdm4* in the Hutcheson soybean. The observed phenotypic and genotypic segregations of *Rdm4* in the specific interaction with isolate CE109 fitted with the expected segregation of a simple inherited and completely dominant gene (Chiesa et al. 2009).

On the other hand, when interacting with isolate CE112 of Dpm, the resistant parent Hutcheson showed 10% DP (incompatible interaction), and the susceptible parent J77-339 showed 80% DP (compatible interaction). The progeny test of the 105 $F_{2:3}$ families of the J04d population, inoculated with the isolate CE112 of Dpm, identified 26 resistant (*Rdm5/Rdm5*), 49 heterozygous (*Rdm5/rdm5*) and 30 susceptible (*rdm5/rdm5*) $F_{2:3}$ families ($\chi^2 = 1.84$; $p = 0.67$). In total, 1110 F_3 plants from J04d population were phenotyped and used to infer the genotype of each F_2 plant (homozygous dominant, heterozygous or homozygous recessive) required to mapping *Rdm5* in the Hutcheson soybean. The results showed that the observed pheno- and genotypic segregations of the *Rdm5* gene in the specific interaction with the isolate CE112 of Dpm matched the expected segregation of a simple inherited and completely dominant gene (Chiesa et al. 2009).

Segregation of AFLP markers in the J04c population

As first approach to identify the genomic regions associated with SSC resistance in Hutcheson, AFLP molecular markers were tested in RB and SB. The bulks were constituted by using equimolar amounts of DNA from resistant (*R*) and susceptible (*S*) F_2 individuals that were previously characterized genotypically, based on the progeny test described for the J04c population. Additionally, DNA from two independent segregant individuals, characterized as *R* (*Rdm4/Rdm4*) and *S* (*rdm4/rdm4*) were tested. In total, 45 E + 3/M + 3 combinations were screened between the four DNA samples, giving an average of 85 bands per combination (3825 loci scanned throughout the genome). Only 14 combinations (E35M33, E35M35, E35M37, E35M39, E35M41, E33M34, E33M37, E34M41, E36M35, E32M43, E37M40, E41M34, E42M41 and E43M39) produced more than two polymorphic bands (markers) between RB and SB that also segregated in the individual F_2 DNAs. From these combinations, 16 markers were obtained and presented Mendelian segregation characteristic of dominant markers (3:1 present: absent) in the J04c population (de-bulked analysis) (Table S2).

Segregation of SSR markers in the J04c population

In addition to AFLP, SSR markers were used to reveal polymorphism between Hutcheson (*R*) and J77-339 (*S*) parental genotypes. From the 250 pairs of SSR tested, 80 (32%) were found to be polymorphic between them. The segregation of 62 of these polymorphic SSR markers was analysed in the whole J04c population that was analysed pheno- and genotypically through the progeny test with isolate CE109, previously characterized. At least two polymorphic SSR were screened from each molecular linkage group. The results of the observed segregation are shown in Table 1.

Table 1 Segregation of the SSR markers in population J04c

SSR marker	Observed Segregation			χ^2 L[b]	P[c]	SSR marker	Observed Segregation			χ^2 L	P
	AA[a]	AB	BB				AA	AB	BB		
Satt431	12	29	10	1.11	0.57	**Sct_191**	15	21	15	1.40	0.45
Satt071	12	29	10	1.11	0.57	**Satt520**	17	23	11	1.90	0.39
Scaa03	16	22	13	1.30	0.52	**Sctt010**	15	24	12	0.60	0.77
Satt181	16	24	11	1.15	0.56	**Satt485**	16	20	15	2.65	0.30
Satt184	12	24	15	0.58	0.77	**Sat_180**	13	27	11	0.16	0.85
Satt001	17	25	8	2.80	0.20	**Satt079**	15	23	13	0.65	0.72
Satt292	17	23	11	1.70	0.39	**Sat_382**	11	23	17	1.70	0.39
Satt174	16	23	11	1.32	0.52	**Satt353**	16	25	10	1.40	0.49
Satt216	15	27	9	1.59	0.45	**Satt301**	11	33	7	4.60	0.10
Satt606	15	23	12	0.67	0.70	**Satt321**	17	18	16	4.70	0.11
Satt318	13	23	10	0.85	0.66	**Satt009**	16	24	11	1.12	0.56
AF1861	10	25	16	1.42	0.49	**Sat_186**	15	23	13	0.65	0.72
Satt398	15	27	9	1.59	0.45	**Satt152**	16	23	12	1.05	0.57
Satt041	16	24	11	1.30	0.56	**Satt453**	19	19	11	4.60	0.08
Satt197	10	32	9	3.35	0.19	**Satt371**	12	25	14	0.18	0.92
Satt066	15	20	16	2.73	0.30	**Satt444**	18	23	10	2.80	0.22
Satt002	15	25	11	0.65	0.72	**Satt549**	15	26	10	0.90	0.61
Satt175	8	30	13	2.58	0.28	**Satt180**	15	23	13	0.65	0.72
Sct065	16	22	13	1.53	0.52	**Satt595**	11	30	10	1.60	0.44
Satt503	14	26	11	0.36	0.83	**Sat_292**	20	20	11	5.53	0.06
Sat-418	13	26	12	0.04	0.97	**Satt358**	8	26	17	2.85	0.20
Satt306	11	24	15	0.63	0.70	**Satt083**	14	22	15	1.0	0.61
Sat_270	10	31	10	2.0	0.31	**Satt233**	15	26	10	0.90	0.61
Satt612	14	26	11	0.36	0.83	**Sat_199**	15	29	7	3.50	0.18
Staga01	14	25	12	0.17	0.92	**Sat_392**	14	30	7	3.50	0.17
Satt409	13	27	11	0.69	0.85	**Sat_250**	13	25	13	0.02	0.99
Satt385	16	23	12	1.05	0.57	**Satt437**	14	26	11	0.40	0.83
Sat_242	11	26	14	0.36	0.83	**Satt089**	13	27	11	0.34	0.85
Satt680	12	25	14	0.22	0.92	**Sat_115**	13	26	12	0.04	0.97
Satt562	11	26	14	0.36	0.83	**Sat_162**	11	26	14	0.36	0.83
Satt296	16	26	9	1.70	0.38	**GMES2791**	15	26	10	0.90	0.61
Satt610	16	24	11	1.10	0.56	**BARCSOYSSR08-0780**	12	25	14	0.18	0.92
Satt702	9	28	14	1.45	0.48	**BARCSOYSSR08-0820**	14	22	15	1.00	0.61
Satt624	17	19	15	3.52	0.18	**BARCSOYSSR08-0923**	15	23	12	0.67	0.70
Satt284	18	24	9	3.25	0.19	**BARCSOYSSR08-0941**	16	23	12	1.05	0.57
Satt373	15	27	9	1.60	0.45	**BARCSOYSSR08-1002**	16	24	11	1.10	0.56
Satt288	14	27	10	0.80	0.70						

The SSR markers highlighted in grey belong to MLG-A2 and are linked in the LG obtained

[a] *AA* band pattern (loci) from the resistant progenitor Hutcheson, *AB* band pattern (loci) from the resistant progenitor Hutcheson and the susceptible progenitor J77-339 (heterozygous individuals) and *BB* band pattern (loci) from the susceptible progenitor J77-339 of population J04c (51 $F_{2:3}$ families)

[b] Chi-square value for the observed segregation of each SSR marker and expected Mendelian segregation of a codominant marker (1:2:1)

[c] *P* probability ($P \geq 0.01$)

Identification of candidate genomic region containing the *Rdm4* resistance gene in J04c population using CE109 isolate

The linkage analysis using the genotypic data from the J04c population indicated that the 79 analysed loci (16 AFLP, 62 SSR markers and the *Rdm4* locus) grouped in 19 LGs associated with some mapped or anchored SSR to a specific MLG in the soybean genetic map. Particularly, the linkage analysis using all the previously obtained genotypic data from population J04c revealed that *Rdm4* in Hutcheson soybean was tightly linked to an AFLP marker (E42M41e) at a putative genetic distance of 3.1 cM (LOD > 3) and indicated that the marker E42M41e was also linked to the Satt233 marker at a genetic distance of 28 cM (LOD > 3). Hence, the *Rdm4* locus could be mapped linking it to Satt233 at a genetic distance of 35 cM (LOD > 3).

Fine mapping of *Rdm4* gene in J04c population using CE109 isolate

The Satt233 marker allows anchoring the region that putatively contains the *Rdm4* gene in the molecular linkage group A2 (MLG A2), or chromosome 8 (*Gm8*), of the soybean genetic map, where it is located (Song et al. 2004, 2010). Therefore, in order to fine map the region containing the *Rdm4* gene, an additional set of 40 SSR markers were selected from the Consensus linkage map in the SoyBase and from the BARCSOYSSR_1.0 database (Song et al. 2010), to saturate the genomic region containing the Satt233 marker. These markers covered a region of approximately 60 cM (from 40.5 to 100.1 cM) of the MLG-A2, with an average genetic distance of 1.5 cM (Table S3). The genomic positions of the analysed SSR markers, recently updated by Song et al. (2016), is detailed in Table S3. Thirteen of them (32.5%) showed polymorphisms between Hutcheson and J77-339 genotypes (Table S3). The Mendelian segregation of these 13 SSR polymorphic markers, analysed for population J04c, is shown in Table 1.

The data of these new markers was integrated with the previously obtained genotypic characterization of J04c population. A total of 92 loci (16 AFLP, 75 SSR and the *Rdm4* locus) were analysed with the JoinMap software. Based on the whole genotypic data from the mapping population J04c, the linkage analysis indicated that 84 of the 92 analysed loci were grouped in 19 LG. The mapped SSR loci had consistent order along the 19 LG, when compared to mapped or anchored SSR to a particular MLG in the soybean genetic map.

The linkage analysis further revealed that the *Rdm4* locus in Hutcheson was linked to the AFLP marker E42M41e and was located between Sat_162 and Satt233 at a genetic distance of 38 cM (LOD > 3). When all the other mapped SSR markers (Sat_115, GMES2791, Sat_199, Satt089, Sat_250, Sat_392, Satt437, BARCSOYSSR08-0780, BARCSOYSSR-08-0820 (CSSR461), BARCSOYSSR-08-0923 (CSSR420), BARCSOYSSR-08-0941 and BARCSOYSSR-08-1002) were compared to the SSR markers in the MLG-A2, they had consistent order along this linkage group. However, some differences were detected between the obtained LG and the MLG-A2, particularly in the position of Sat_250 and Satt233 markers.

Mapping of *Rdm4* and *Rdm5* genes in J04d population using CE109 and CE112 isolates

In order to map both genes, *Rdm4* and *Rdm5*, the F_2 population J04d was screened with the CE109 and CE112 isolates of Dpm. A total of 45 polymorphic SSR markers, belonging to all the molecular linkage groups, were assayed in the entire population. All the analysed SSR markers showed Mendelian segregation as expected for codominant markers (Table 2). Afterward, the segregation data of the 47 loci (45 SSR markers, *Rdm4* and *Rdm5* genes) analysed in the J04d population (108 $F_{2:3}$ families) was combined with the whole genotypic data from the J04c population (51 $F_{2:3}$ families and 92 loci). When the two mapping populations, J04c and J04d, were summed, a total of 159 plants were genotyped based on 93 loci (46 in common). The linkage analysis using the joint segregation data from the J04c and J04d mapping populations indicated that 76 of the 93 analysed loci (16 AFLP, 75 SSR, *Rdm4* and *Rdm5*) grouped in 19 LG containing three or more markers. Fifteen loci could not be linked to any other locus in the analysed populations. The mapping analysis indicated that most of the markers in a particular LG in one population were in the same LG in the other population. In the joint analysis, each of the 19 LG matched to a particular MLG of the reference soybean genetic map (SoyBase) through the mapped SSR markers. In addition, the mapped SSR loci had consistent order

Table 2 Segregation of the SSR markers in population J04d

SSR marker	Observed Segregation			$\chi 2^b$	P [c]
	AA[a]	AB	BB		
Satt431	33	55	20	3.16	0.21
Satt071	24	57	27	0.62	0.73
Scaa03	29	45	31	2.24	0.33
Sct191	22	58	27	1.35	0.54
Satt181	22	60	22	2.53	0.28
Satt184	22	45	33	2.93	0.15
Satt251	36	45	26	4.60	0.10
Satt292	22	58	27	1.35	0.54
Satt606	24	54	28	0.43	0.83
Satt174	20	64	21	4.70	0.08
Satt216	28	53	25	0.21	0.90
Satt641	24	54	28	0.43	0.83
Satt318	35	48	20	4.60	0.09
AF1861	22	54	29	1.10	0.58
Satt595	24	54	29	0.50	0.79
Satt398	29	54	23	0.75	0.69
Satt041	28	56	21	1.42	0.49
Satt197	31	49	24	1.40	0.50
Sat_418	25	57	26	0.40	0.84
Satt066	35	46	26	3.20	0.17
Satt002	21	56	25	1.60	0.46
Satt175	21	60	21	3.25	0.19
Satt001	24	54	29	0.50	0.79
Sct065	31	43	31	3.30	0.18
Sat_242	24	57	27	0.62	0.73
Satt503	24	54	29	0.50	0.79
Satt152	25	57	26	0.40	0.84
Satt612	22	52	31	1.60	0.45
Staga01	30	49	29	0.90	0.62
Satt385	20	56	24	2.25	0.33
Satt457	22	52	31	1.60	0.45
Satt680	22	51	25	1.30	0.54
Satt359	29	54	23	0.74	0.69
Satt233	26	50	25	0.50	0.79
Satt089	25	57	26	0.40	0.84
Sat_250	28	53	27	0.06	0.97
Satt437	28	50	30	0.67	0.72
Sat_115	25	48	30	0.99	0.61
Sat_162	23	52	21	0.75	0.69
GMES2791	27	54	31	0.43	0.81
BARCSOYSSR08-0780	16	53	27	3.60	0.17
BARCSOYSSR08-0820	22	48	26	0.34	0.85
BARCSOYSSR08-0923	37	39	28	8.05	0.02
BARCSOYSSR08-0941	32	42	30	3.93	0.14
BARCSOYSSR08-1002	22	44	30	2.00	0.37

The SSR markers highlighted in grey belong to MLG-A2 and are linked in the LG obtained

[a] *AA* band pattern (loci) from the resistant progenitor Hutcheson, *AB* band pattern (loci) from the resistant progenitor Hutcheson and the susceptible progenitor J77-339 (heterozygous individuals), *BB* band pattern (loci) from the susceptible progenitor J77-339 of population J04d (108 $F_{2:3}$ families)

[b] Chi-square value obtained for the observed segregation for each SSR marker and expected Mendelian segregation of a codominant marker (1:2:1)

[c] *P* probability ($P \geq 0.01$)

along the 19 LG, when compared to the soybean genetic map (data not shown).

As result, the linkage analysis identified the LG that most probably contained the *Rdm4* and *Rdm5* genes. In this LG, the order and estimated genetic distances between the mapped loci indicated that the genomic region containing the *Rdm4* and *Rdm5* genes in Hutcheson was collinear with the MLG-A2 (*Gm8*) of the soybean genetic map (Fig. 1). The linkage map was constructed covering a genetic distance of 115.6 cM, with 18 significantly linked loci (15 SSR belonging to MLG-A2, one AFLP marker and the *Rdm4* and *Rdm5* genes) (LOD >3). Further, the *Rdm4* and *Rdm5* genes were found to be linked in a genomic region of 44.3 cM long located between Sat_162 and Satt233 markers, with the AFLP marker E42M41e tightly linked to *Rdm4* at 1 cM, Sat_162 linked to *Rdm4* at 12.4 cM and Satt233 linked to *Rdm5* at 14.3 cM (Fig. 1b). Moreover, the distance between *Rdm4* and *Rdm5* genes (17.6 cM) in the map obtained in this work is coherent with the genetic distance previously determined (Chiesa et al. 2009). The SSR loci on the map obtained in this work have consistent order along the LG, when compared to the reference soybean genetic map from Song et al. 2010 (Fig. 1a) and the consensus map from Song et al. (2004) (Fig. 1c). However, some minor differences were observed in Sat_250 and Satt233 positions (Fig. 1).

It is interesting to note that three of the selected SSR (BARCSOYSSR-08-0780, BARCSOYSSR-08-1002 and BARCSOYSSR-08-1040) that amplified a single and polymorphic product in the current study had not been tested previously.

Discussion

In the present work, three different approaches were used to locate the *Rdm4* and *Rdm5* genes from Hutcheson in the soybean genetic linkage map: (i) potential regions associated with resistance to Dpm were

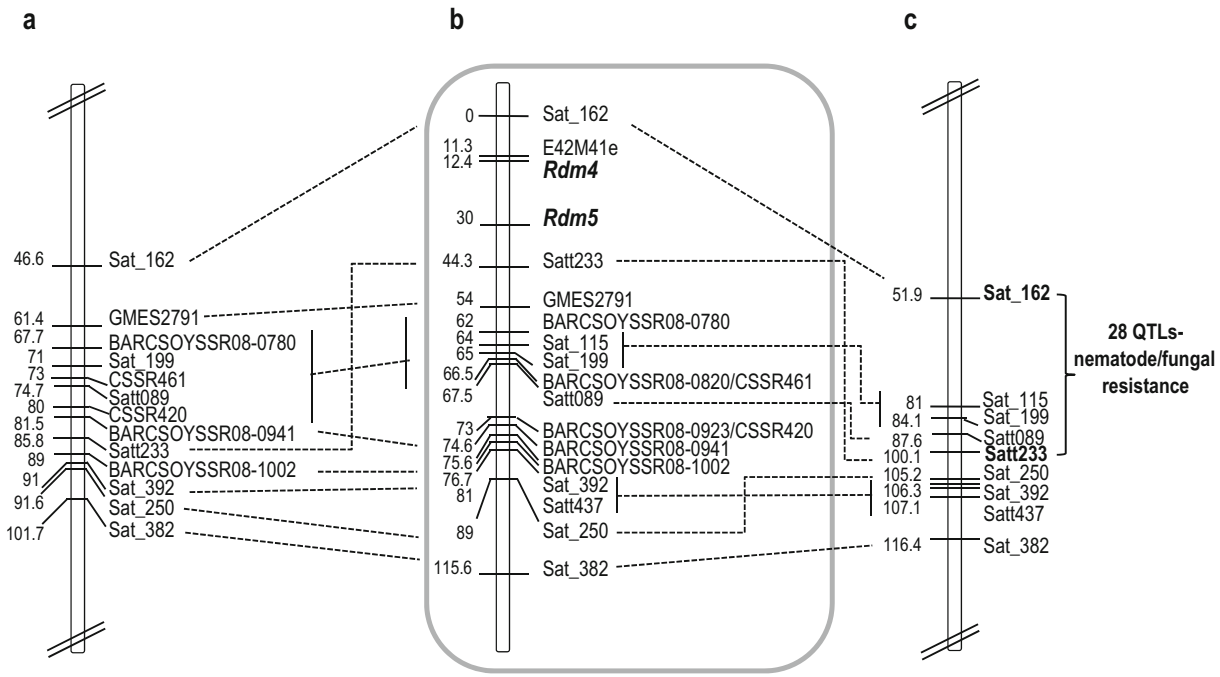

Fig. 1 Genetic linkage map of the genomic region containing the *Rdm4* and *Rdm5* resistance genes to *D. phaseolorum var. meridionalis*, in Hutcheson soybean, obtained from the pheno- and genotypic characterization and linkage analysis of 159 $F_{2:3}$ families, obtained by combining 51 individuals from J04c population and 108 individuals from J04d population, derived from the cross of the susceptible line J77-339 and the resistant cultivar Hutcheson. The genetic map was generated with SSR and AFLP markers genotypic data with JoinMap v. 4.0 (Van Ooijen 2006) using Kosambi's mapping function. **a** Reference map of MLG A2 (*Gm8*) (Song et al. 2010). **b** Map positions of the *Rdm4* and *Rdm5* loci on MLG A2. **c** Consensus map of the MLG–A2 of the Soybean Genetic Map (SoyBase) (based on Song et al. 2004). Common markers among maps are aligned. The distances are given in centimorgan (cM) at the left side of the maps

identified by combining the effectiveness of bulk segregant analysis, with a multi-loci marker technique (3825 AFLP loci) to initially scan the genome; (ii) systematic search of polymorphic regions between the parental genotypes was made by screening 250 anchored SSR loci, and then the specific genomic region detected was marker-saturated with 13 polymorphic markers; and (iii) linkage analysis was performed in two $F_{2:3}$ populations, first independently, and then by combining the whole genotypic data of 159 F_2 plants. These complementary approaches reinforce the validity of the results obtained. Also, the quality of the obtained linkage map is supported by the consistent order of most of the common markers compared to the reference soybean genetic linkage maps (Fig. 1).

The results obtained indicate that the *Rdm4* and *Rdm5* genes, which control race-specific resistance to Dpm in Hutcheson cultivar, are located in a genomic region that was found to be collinear with the MLG-A2 of the soybean genetic map. The linkage analysis confirmed that *Rdm4* and *Rdm5* are linked, as it was previously determined by classical genetic analysis (Chiesa et al. 2009). The *Rdm4* and *Rdm5* genes were found to be linked at a genetic distance of 17.6 cM, in a genomic region located between Sat_162 and Satt233 markers, with the AFLP marker E42M41e tightly linked to *Rdm4* at 1 cM, Sat_162 linked to *Rdm4* at 12.4 cM and Satt233 linked to *Rdm5* at 14.3 cM (Fig. 1b).

Of the 15 mapped SSR markers, 13 have consistent order along the LG when compared to the soybean genetic maps from Song et al. (2004) and Song et al. (2010). The exceptions were that Sat_250 is below Sat_392 and Satt437 in the map presented here; meanwhile, it is above Sat_392 and Satt437 in the Consensus 4.0 map (based on Song et al. 2004) and also in the Composite Gm2003 genetic map (SoyBase). However, Satt437 is in the map presented here and in the genetic maps of the SoyBase, but it is not present in the linkage genetic maps obtained by Song et al. (2010). The other difference observed in the linkage map obtained in this work is the position of Satt233 marker. This marker was located between Satt089 and Sat_250 (Song et al. 2004); meanwhile, in this work, it was located between Sat_162 and GMES2791 markers (Fig. 1). The observed discrepancy in the Satt233 marker order maybe attributed to an introgression region or to a translocation (i.e. interchange mechanism) in genomic regions specific to mapping populations with different genetic backgrounds (Choi et al. 2007; Lee et al. 2013). In the present study, the mapping populations were derived from a cross between the J77-339 line and Hutcheson cultivar, while the consensus integrated linkage map was constructed with three mapping populations: A81-356022 (US breeding line) × *G. soja* (wild soybean); Minsoy × Noir 1 (both collected in France) and a near isogenic line of US cultivar Clark × near isogenic line of US cultivar Harosoy (Cregan et al. 1999). While, Song et al. (2004) used five mapping populations: the three used by Cregan et al. (1999) as well as Minsoy × Archer (US cultivar) and Archer × Noir 1.

In soybean and other species of agronomic interest, *R* genes appears to be located in several irregularly distributed genomic regions, most in clusters or even single, and preferentially concentrated in some chromosomes (Kanazin et al. 1996; Michelmore and Meyers 1998; Bachman et al. 2001; Graham et al. 2002; Ashfield et al. 2003; Meyers et al. 2005; Sandhu et al. 2005; Marone et al. 2013). In the MLG-A2 (chromosome 8, *Gm8*) several major resistance loci like the ribosomal protein encoding gene (*Rps8*) against most isolates of *Phytophthora sojae* were reported by Burnham et al. (2003), as well as other quantitative trait loci (QTLs) that confer resistance to fungal pathogens such as *Sclerotinia sclerotiorum* and different *Fusarium* spp. (SoyBase). In addition, the MLG-A2 contains the receptor-like kinase encoding gene *Rhg4* against race 3 of *Heterodera glycines* (Matthews et al. 1998; Schuster et al. 2001). Also, 15 nucleotide-binding site leucine-rich repeat (NBS-LRR) genes were located in this MLG, representing a resistance-rich region of the soybean genome (Kang et al. 2012). Particularly, in the region between Sat_162 and Satt233 markers in the MLG-A2, which contains the *Rdm4* and *Rdm5* genes, 21 QTLs conferring resistance to different races of *H. glycines*, four conferring resistance against *Sclerotinia*, two for *Fusarium* spp. and one for *Phytophtora*, have been mapped (Soybase, Fig. 1c).

Interestingly, when the genomic region in the *Gm8* flanked by Sat_162 (genomic position 8283735) and the Satt233 (genomic position 17232172) was analysed in detail, four LRR-RLK genes (a probable LRR receptor-like serine/threonine-protein kinase At1g67720-like, Glyma.08 g107700, the receptor-like kinase *Rgh4* and a probable receptor-like protein kinase At5g56460-like) and one LRR-domain gene (FJ014736.1) were found (*Glycine max* genome assembly version Glyma. Wm82.a2, Gmax2.0, Soybase).

Recently, using GWAS, two SNP located in the chromosome 14 (MLG-B2) have been reported as significantly associated to Dpc and Dpm resistance (Chang et al. 2016). The authors suggested that the same resistance source to Dpc and Dpm underlies these two SNPs. However, it was previously demonstrated that the *Rdm1–5* major genes that confer resistance to Dpm do not confer resistance to Dpc (Pioli et al. 2003). Moreover, the genomic region in Hutcheson, which contains the *Rdm4* and *Rdm5* genes reported in this work, is located on chromosome 8 (*Gm8*), meanwhile, the SNPs reported by Chang et al. (2016) are located in chromosome 14 (*Gm14*), indicating clearly different resistance sources. Also, as the authors point out, further biological studies need to be conducted to understand how the new candidate resistance loci would be implicated in SSC resistance. In this context, it is worth to note that Hutcheson cultivar has been successfully used to incorporate resistance to SSC. Additionally, the availability of two flanking SSR markers linked to the *Rdm4* and *Rdm5* loci would facilitate the use of marker-assisted selection in soybean-breeding programs aimed to the introgression of these genes in elite germplasm. Future mapping of the other major *Rdm* genes, as well as the identification of novel ones, may give the possibility to pyramid them to obtain broader and more durable resistance against SSC (Pedersen and Leath 1988; Mundt 2014). Also, the results obtained in this work would provide the basis for the future genomic and molecular characterization of *Rdm4* and *Rdm5* genes and the defence pathways involved in the resistance observed in Hutcheson cultivar.

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Compliance with ethical standards

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

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