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Identification of a new major QTL associated with resistance to soybean cyst nematode (*Heterodera glycines*)

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Abstract Resistance of soybean [*Glycine max* (L.) Merr.] to cyst nematode (SCN) (*Heterodera glycines* Ichinohe), one of the most destructive pathogens affecting soybean, involves a complex genetic system. The identification of QTLs associated with SCN resistance may contribute to the understanding of such system. The objective of this work was to identify and map QTLs for resistance to SCN Race 14 with the aid of molecular markers. $BC_3F_{2:3}$ and $F_{2:3}$ populations, both derived from an original cross between resistant cv. Hartwig and the susceptible line BR-92–31983 were screened for resistance to SCN Race 14. Four microsatellite (Satt082, Sat_001, Satt574 and Satt301) and four RAPD markers (OPAA-11_{795}, OPAE-08_{837}, OPR-07_{548} and OPY-07_{2030}) were identified in the $BC_3F_{2:3}$ population using the bulked segregant analysis (BSA) technique. These markers were amplified in 183 $F_{2:3}$ families and mapped to a locus that accounts for more than 40% of the resistance to SCN Race 14. Selection efficiency based on these markers was similar to that obtained with the conventional method. In the case of the microsalellite markers, which identify homozygous resistant genotypes, the efficiency was even higher. This new QTL has been mapped to the soybean linkage group D2 and, in conjunction with other QTLs already identified for SCN resistance, will certainly contribute to our understanding of the genetic basis of resistance of this important disease in soybean.

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Introduction

The soybean cyst nematode (SCN) is economically the most damaging pathogen affecting soybean culture (Noel 1992). In a field infested with SCN, the nematode population only can be decreased, not eliminated (Vierling et al. 1996). The most economical and feasible solution for managing areas infested with SCN is the use of resistant cultivars. However, the genetic complexity and the heterogeneity of SCN populations have limited our understanding of the nature of the resistance and the development of resistant cultivars (Faghihi et al. 1986a, b).

Genetic studies have demonstrated that resistance to SCN is oligogenic (Caldwell et al. 1960; Matson and Williams 1965; Myers and Anand 1991; Rao-Arelli et al. 1992, Rao-Arelli 1994). Moreover, resistance to SCN can be conferred by linked genes, and these can have multiple alleles for each locus (Anand and Rao-Arelli 1989; Hancock et al. 1987).

Most of the heritability studies for resistance to SCN have considered it to be a qualitative trait. Mansur et al. (1993), using a strategy of quantitative analysis, obtained high heritability values for resistance to Race 3. These authors concluded that the additive model explained most of the variation in resistance to Race 3 in several crosses in which Peking and PI 88788 were used as resistant progenitors.

The use of molecular markers is an efficient alternative to the tedious work of genotype evaluation for SCN resistance and allows for an efficient selection of polygenic resistance to SCN (Vìerling et al. 1996). Molecular markers can be used in the indirect selection of traits that are difficult to evaluate and/or that are largely affected by the environment (Nienhuis et al. 1987; Paterson et al. 1988; Tanksley et al. 1989; Young and Tanksley 1989).

Although resistance to SCN is a complex trait, a quantitative trait locus (QTL) that explains more than 50% of resistance was mapped to linkage group G (Concibido et al. 1996). This QTL is present in several resistance sources, including PI 209332, PI 88788, PI 90763, PI 437654 and Peking, and confers partial resistance to several Races of SCN. Concibido et al. (1996) reported that in PI 209332 this QTL explains 35% of the resistance to Race 1, 50% of the resistance to Race 3 and 54% of the resistance to Race 6. These authors obtained 90% accuracy in selecting resistant individuals in an $F_{5:6}$ population using a molecular marker associated with this QTL.

Restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) markers linked to SCN resistance gene *Rhg4* were identified and mapped to linkage group A in crosses involving PI 290136 (Weisemann et al. 1992) and Peking (Mahalingan and Skorupska 1995). This gene is located near locus *i*, which conditions dark seed coat in soybeans. Vierling et al. (1996) identified four RFLP markers that explained 94% of the resistance of Hartwig to SCN Race 3. Also using RFLP markers, Webb et al. (1995) mapped three major QTLs to linkage groups A, G and M, respectively, that confer complete resistance to SCN Race 3 in PI 437654, the resistant ancestor of Hartwig. Although indirect selection of resistant plants with RFLP markers is less time-consuming than greenhouse evaluations, more efficient and simpler DNA markers are necessary to evaluate the great number of plants involved in marker assisted selection (MAS) (Mudge et al. 1997). Polymerase chain reaction (PCR)-based markers, such as RAPD and microsatellites, are currently being used because they are abundant, less expensive, more polymorphic and less laborious to manipulate than RFLP.

The objective of the investigation presented here was to identify RAPD and microsatellite markers linked to loci conferring the resistance to SCN Race 14 present in cultivar Hartwig.

Materials and Methods

Populations

A population of 126 $BC_3F_{2:3}$ families was used for molecular marker identification. This population was generated from a cross between the resistant source Hartwig and line BR 92–31983 (susceptible to all races of SCN) followed by three backcrosses with no selection to cv. Hartwig, which was used as the recurrent progenitor. An F_2 population of 183 plants was derived from a cross between a resistant [female index (FI)=0] and a susceptible plant (FI>100) from the $BC_3F_{2:3}$ population. The F_2 plants and the corresponding $F_{2:3}$ families were used to evaluate the correlation between the molecular markers and resistance to SCN.

Experiments with SCN

A SCN Race 14 population was collected from a soybean field located in the county of Chapadao do Ceu, state of Goias, Brazil. This population was increased and maintained for ten generations on the roots of cv. Centenial in a greenhouse at the Embrapa – Soja Center, Londrina, PR, Brazil.

Identification of SCN Race 14 was confirmed in each experiment using the differential soybean cultivars Pickett, Peking, PI 90763, and PI 88788, and cv. Lee as the susceptible control (Riggs and Schmitt 1988).

For the inoculation experiments, seeds were planted in 12-cm-diameter pots, one seed per pot, in a greenhouse maintained at 24°–28°C. Seven days after seedling emergence each pot was inoculated with 4000 eggs and juveniles of SCN Race 14 using an automatic pipette to apply two 1-ml aliquots, each containing crushed cysts in distilled water (2000 eggs and juveniles per milliliter) to each pot. Twenty-eight days after inoculation, the individual plants were uprooted, and the cysts were collected by washing the roots with pressurized water over wire mesh sieves. The total number of females and cysts were counted using a stereo microscope and transformed into a female index (FI), described by the following expression:

FI=(Number of females or cysts present on the evaluated genotype/average number of females or cysts present in Lee)×100.

Four plants from each 1 of the 126 $BC_3F_{2:3}$ families and the 183 F_2 plants were inoculated. After evaluation of the F_2 plants, the roots were washed and were replanted in new pots to obtain $F_{2:3}$ seeds. Six seeds from each $F_{2:3}$ family were germinated, and the corresponding plants were inoculated as described. All 6 individuals from each $F_{2:3}$ family were averaged and used for determining the SCN reaction of the corresponding F_2 line.

DNA analysis

Plant DNA extraction was according to Doyle and Doyle (1990). Bulked segregant analysis (BSA, Michelmore et al. 1991) was used to identify markers linked to SCN resistance loci in the $BC_3F_{2:3}$ population. The candidate markers were tested in all BC_3F_3 individuals, and those showing a high association with resistance were assayed using the $F_{2:3}$ families to estimate the proportion of phenotypic variation explained by each marker and to locate the QTL within the linkage groups.

DNA amplifications by the RAPD technique were based on Welsh and McClelland (1991). Each 25-µl reaction contained 10 m*M* TRIS-HCl pH 8.3, 50 m*M* KCl, 2 m*M* $MgCl_2$, 100 µ*M* of each deoxynucleotide (dATP, dTTP, dGTP and dCTP), 0.4 µ*M* of a decamer primer (Operon Technologies, Alameda, Calif.), 1 U *Taq* DNA polymerase and 30 ng of template DNA. Amplifications were performed in a thermocycler model 9600 (Perkin-Elmer, Norwalk, Conn.) using 40 cycles of the following program: denaturation at 94°C for 15 s, annealing at 35°C for 30 s and extension at 72°C for 1 min. After the 40th cycle, an additional extension of 7 min at 72°C was performed. The amplified fragments were separated on 1.2% agarose gels immersed in TBE (90 m*M* TRIS-borate buffer, 1 m*M* EDTA, pH 8.0), stained with ethidium bromide (10 mg/ ml), visualized under UV light and photographed by the Eagle Eye II photodocumentation system (Stratagene, La Jolla, Calif.).

Amplification of the microsatellite markers was based on Akkaya et al. (1995). Each 20-µl reaction contained 12.5 m*M* TRIS-HCl pH 8.3, 62.5 m*M* KCl, 2.5 m*M* $MgCl_2$, 125 µ*M* of each deoxynucleotide (dATP, dTTP, dGTP and dCTP), 0.7 µ*M* of primer, 1 U *Taq* polymerase and 30 ng of template DNA. The amplifications were performed in a thermocycler programmed for an initial step of 7 min at 72°C, followed by 30 cycles (1 min at 94°C, 1 min at 50°C and 2 min at 72°C). After the 30th cycle, an additional extension step of 7 min at 72°C was performed. The amplified fragments were resolved in 3% agarose gels immersed in TBE, stained with ethidium bromide and photographed as described above.

Data analysis

Segregation of the resistance gene and molecular markers was tested by the chi-square test.

The amount of phenotypic variation explained by the markers was determined by multiple regression analysis using the stepwise elimination method (Draper and Smith 1966). An association be-

tween a DNA marker and the SCN disease response was declared significant if the probability was equal to or less than 0.001 to minimize the detection of false positives (Lander and Botstein 1989).

The additive and dominance values were estimated as described by Edwards et al. (1987) using the alellic frequencies from the marker closer to the QTL. Phenotypic variance was calculated by the variance among the averages of the F_3 families. Additive and dominance variances were calculated using the allelic frequencies of the microsatellite markers and the additive and dominance values (Falconer 1981). The genetic variance was obtained by the sum of the additive and dominance variances. The heritabilities were estimated by dividing the genetic (broad-sense) and additive (narrow-sense) variances by the phenotypic variance.

The markers were mapped by using the MAPMAKER program (Lander et al. 1987), with a minimum LOD score of 3.0, a maximum recombination frequency of 50% and the mapping function of Kosambi (1944). QTL location was determined by interval mapping (Jansen 1993) using the QGENE software (Cornell University). The significance threshold for QTL interval mapping was determined by performing 1000 permutations (α=0.05) (Churchill and Doerge 1994).

Results and discussion

The public soybean genome linkage map from USDA/Iowa State University contains 1004 markers (486 microsatellite, 501 RFLP, ten RAPD, four isozyme and three classical markers) in 20 linkage groups (Cregan et al., 1999). The presence of a high number of microsatellite markers in this map allows for the prompt exchange of information among distinct research groups using different maps based on the assumption that microsatellites are highly conserved within a given species. In this way, microsatellite markers associated with a QTL of interest can be used as an anchor to locate such region within the soybean linkage map.

To identify markers linked to QTLs conferring resistance to SCN Race 14, we used a $BC_3F_{2:3}$ population derived from a cross between SCN-resistant cv. Hartwig and susceptible line BR 92–31983. DNA was prepared from two contrasting bulks of $BC_3F_{2:3}$ plants, one resistant and the other susceptible to SCN Race 14. A total of 1200 RAPD primers and 50 microsatellite primer pairs were tested. Eight molecular markers associated with resistance were identified: four RAPD markers ($OPAA11_{795}$, $OPAE\text{-}08_{837}$, $OPR\text{-}07_{548}$ and $OPY\text{-}07_{2030}$) and four microsatellites (Satt082, Sat_-001, Satt574 and Satt301). Marker $OPAA\text{-}11_{795}$ appeared only on resistant individuals (coupling phase), while markers $OPR\text{-}07_{548}$, $OPY\text{-}07_{2030}$, $OPAE\text{-}08_{837}$ appeared only on the susceptible ones (repulsion phase). All microsatellite markers were co-dominant (Fig. 1). The whole $BC_3F_{2:3}$ population was analyzed with these markers, and all of them showed a high correlation with FI (data not shown).

$F_{2:3}$ families were used to determine chromosomal locations of the putative SCN-linked markers. These families were derived from F_2 lines from a cross between 2 $BC_3F_{2:3}$ plants contrasting for resistance to SCN Race 14. The segregation of the markers in the F_2 population based on the analysis of the $F_{2:3}$ families fit a 3:1 ratio in the case of the RAPD markers, and a 1:2:1 ratio for the microsatellite markers (Table 1).

Using the strategy of interval mapping (Jansen 1993) and the permutation test (Churchill and Doerge 1994) we mapped the QTL for resistance to Race 14 with a peak of significance close to microsatellite Satt082 (Fig. 2). All markers showing a significant association to resistance mapped within an interval of 43.8 cM.

The order of the microsatellite markers differed from that obtained by Cregan et al. (1999) in linkage group D2. This could be explained by the fact that we used populations of different genetic backgrounds from those used by Cregan et al. (1999). In addition, the inversion we observed could have already been present in cv. Hartwig or it could have been generated by recombination events during the development of the populations. A total of eight recombination events were produced during the crosses, backcrosses and selfings between the genetic material derived from cv. Hartwig and that from line BR 92–31983.

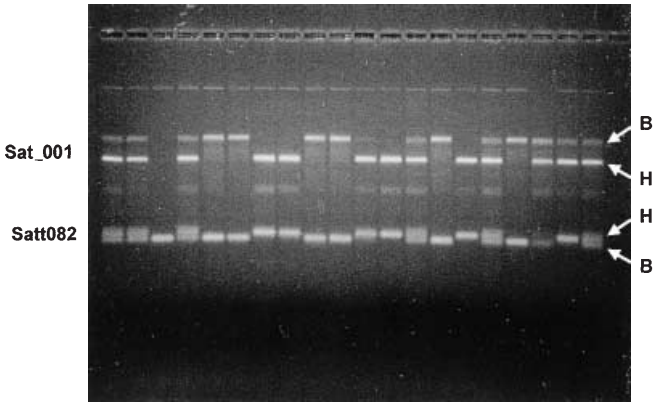

Fig. 1 Amplification of DNA samples from 20 individuals of a soybean population segregating for resistance to SCN Race 14 with microsatellite primers Sat_001 and Satt082. *B* and *H* the alleles of the resistant (*H*) and susceptible (*B*) parents

Table 1 Segregation of molecular markers linked to a QTL with a major effect on resistance to SCN Race 14 in the F_2 population

Locus	Hypothesis	Expected	Observed	χ^2	P
$OPAA\text{-}11_{795}$	3:1	137.25:45.75	144:39	1.33	24.88
$OPR\text{-}07_{548}$	3:1	135.75:45.25	126:55	2.80	9.43
$OPY\text{-}07_{2030}$	3:1	134.25:44.75	129:50	0.82	36.52
$OPAE\text{-}08_{837}$	3:1	137.25:45.75	132:51	0.80	37.11
Sat_-001	1:2:1	47.75:91.5:45.75	53:88:42	1.59	45.16
Satt082	1:2:1	47.75:91.5:45.75	55:86:42	2.52	28.36
Satt574	1:2:1	47.75:91.5:45.75	52:93:38	3.39	18.36
Satt301	1:2:1	47.75:91.5:45.75	48:91:44	0.30	86.07

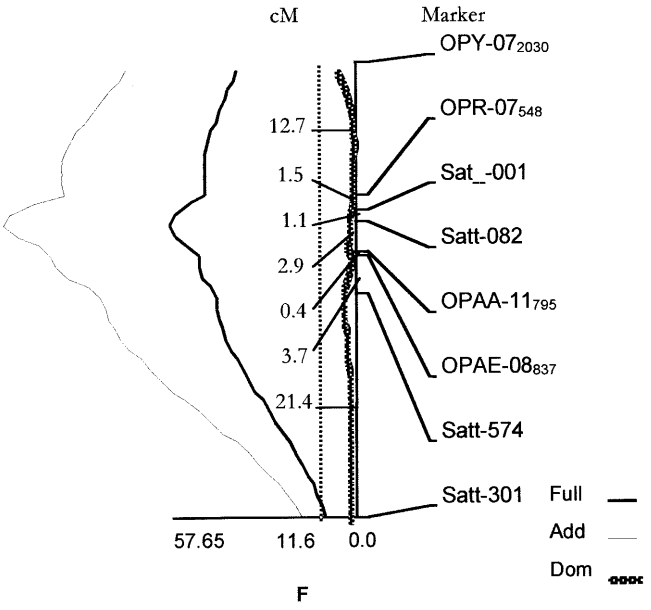

Fig. 2 Location of QTL for resistance to SCN Race 14 in MLG D2. The molecular markers are listed on the *right side* and the genetic distances (in centiMorgans) are on the *left side*. The threshold limit F=11.6 was established using permutation tests with 1000 permutations. The lines show the F values obtained for the QTL location in additive, dominance and full model effects

Table 2 Proportion of phenotypic resistance to SCN Race 14 explained by molecular markers linked to resistance QTL

Marker	Variation explained[a] (%)	*F*	Prob>*F*
OPAA-11$_{795}$	21.25	38.344	<0.0001
OPR-07$_{548}$	27.32	61.276	<0.0001
OPY-07$_{2030}$	20.28	38.992	<0.0001
OPAE-08$_{837}$	28.68	65.780	<0.0001
Satt082	41.13	114.923	<0.0001
Sat_-001	36.71	105.282	<0.0001
Satt574	32.08	83.115	<0.0001
Satt301	9.50	18.484	<0.0001

[a] Based on regression analysis between marker loci and the female index (FI)

Regression analysis demonstrated that microsatellite markers Satt082, Sat_-001, Satt574 and Satt301 explained 41.13%, 36.71%, 32.08% and 9.5% of the variation for resistance to Race 14, respectively; RAPD markers OPAA11$_{795}$, OPR-07$_{548}$, OPY-07$_{2030}$, OPAE-08$_{837}$ explained 21.25%, 27.32%, 20.28% and 28.68%, respectively (Table 2). Multiple regression analysis with stepwise elimination confirmed that all of these markers are associated within the same QTL as no model containing more than one marker was significant.

To date, two QTLs of major effect for resistance to SCN have been described and mapped in soybean, one dominant (*Rhg4*) and linked to the *i* locus (responsible for dark seed coat), located in linkage group A (Weiseman et al. 1992), and the other partially recessive (*rhg1*), located in group G, conferring resistance to several SCN Races (Webb et al. 1995, Concibido et al. 1997). QTLs of minor effects for resistance to Race 3 have been mapped to groups A, K, G and J and were reported by several authors.

Table 3 Additive and dominance components, average degree of dominance, variances and heritability of the resistance locus to SCN Race 14, based on the data analysis of molecular marker Satt082

a	d	d/a[a]	σ^2_A[b]	σ^2_D[c]	σ^2_G[d]	h^2_B[e]	h^2_N[f]
16.73	2.64	0.16	142.46	1.72	144.17	37.01	36.57

[a] Average degree of dominance: 1=complete dominance for susceptibility; 0=lack of dominance; –1=complete dominance for resistance; [b] Aditive variance; [c] Dominance variance; [d] Genetic variance; [e] Broad sense heritability; [f] Narrow sense heritability

In this paper we report the identification of a new major QTL for resistance to SCN Race 14 flanked by microsatellite markers Satt082, Sat_-001, Satt574 and Satt301 located in group D2 (Cregan et al. 1999). This finding was first communicated during the Plant and Animal Genome VII Meeting (Moreira et al. 1999). Kilo et al. (1999) also presented evidence implying the linkage of group D with SCN resistance based on their analysis of recombinant inbred lines from a cross between SCN resistant germplasm J87–233 and susceptible cultivar Hutcheson. By screening soybean bacterial artificial chromosome (BAC) libraries, Penuela et al. (1999) constructed contigs flanking the *rhg1* locus on linkage group G. In the process of chromossome walking, they identified additional contigs from an homeologous region located on linkage group D2 near the same microsatellite region reported in this work (N. Young, personnal communication). These findings strongly suggest the existence of duplicated regions in the soybean genome containing resistance genes to SCN. Duplicated chomosomal regions in the soybean genome containing QTL for seed protein and oil contents have been reported (Shoemaker et al. 1996).

The microsatellite markers identified a locus with partial dominance for susceptibility to Race 14, with an average degree of dominance (d/a) of 0.16 (Table 3). In this locus, the resistance is conferred by the recessive allele originating from Hartwig. Rao-Arelli et al. (1989), studying several F_1 populations derived from crosses between resistant and susceptible genotypes to Races 3, 4 and 5, reported that most crosses exhibit partial dominance (with dominance manifesting in susceptibility to SCN). Conversely, linkage group A contains the locus *Rhg4*, which appears to be dominant for resistance to SCN (Matson and Williams 1965).

The additive, dominance and genotypic variances and the broad- and narrow-sense heritabilities were estimated for the identified QTL (Table 3) by using the allelic frequencies of microsatellite marker Satt082, the recombination frequency between this marker and the QTL and the averages obtained within each genotypic class of this

marker. This analysis showed that the genotypic variance was composed almost exclusively of additive variance. The narrow-sense and broad-sense heritability values were low and quite similar to each other. The heritability estimated from the genetic variance was lower than R^2. This is because upon estimating R^2, part of the residual variance that could not be isolated was computed together with the genetic variance. The additive model by itself was sufficient enough to explain the variation of resistance to SCN Race 14 (Fig. 2).

The low heritability value found is the result of an analysis of an isolated single locus and not of a trait that is controlled by several QTL mapping at different loci. Mansur et al. (1993) found high heritability values for resistance to SCN Race 3 using an analysis of generation means. Webb et al. (1995), using variance components, also found high values of heritability for resistance to SCN. The heritability estimated in our work, using variance components, was also high (70.2%). However, these estimates consider all of the loci to be involved with resistance. To obtain these estimates, it is necessary to evaluate families in replicates (recombinant inbred lines, $F_{2:3}$, and so on).

Heritability values obtained by evaluating individual F_2 plants are generally of low magnitude. This was confirmed by the low value of the F_3-F_2 regression (15.59%) estimated in this work, which resulted in a narrow-sense heritability of 10.34%, after the appropriate correction factor had been applied (Smith and Kinmann 1965). This value is smaller than the one obtained based on the estimated variance by microsatellite marker Satt-082 and is the heritability value obtained when moving from the F_2 to the F_3 generation. This low heritability value indicates that phenotypic selection in the F_2 generation is not efficient because the phenotype does not precisely represent the genotype.

Application to plant breeding

Plants selected as resistant by RAPD markers OPAA-11_{795}, OPR-07_{548}, OPY-07_{2030} and OPAE-08_{837} showed, respectively, 43.18%, 59.27%, 54.01% and 63.11% fewer cysts than plants classified as susceptible (Table 4).

Plants classified as homozygous for resistance based on microsatellite markers Satt-082, Sat_-001 or Satt574 had, on average, 70% fewer cysts than plants homozygous for susceptibility and 60% fewer cysts than the heterozygous plants (Table 4).

The efficiency of selection based on the markers identified in this work was at least equivalent to that obtained with the conventional method and even superior as in the case of microsatellite markers Satt-082, Sat_-001 or Satt574. Table 4 shows the average FI values for F_3 families for each class of molecular markers and for the phenotypic selection in F_2. Selecting resistant F_2 plants based on their FI (FI<10) produced an F_3 generation with an average FI of 16.95. Selecting susceptible plants with FI≥10 resulted in an F_3 generation with an average FI of 32.47 (Table 4). Selecting homozygous F_2 resistant plants with the aid of Satt-082, Sat_-001 and Satt574 generated an F_3 offspring with an average FI lower than 13.5, while selecting homozygous F_2 susceptible plants generated an F_3 with an average FI higher than 46. These results indicate that selection assisted by microsatellite markers was more efficient in identifying resistant and susceptible plants than the conventional method.

Low heritability values indicate a high possibility of failure in the selection of plants by phenotype in the first breeding generations. On the other hand, the use of molecular markers allows for an efficient selection in early generations of the breeding process because they are not affected by the environment and because in the case of co-dominant markers selection is based on the genotype not the phenotype. Thereby, markers Satt-082, Sat_-001 and Satt574 are efficient tools for selecting resistant plants in early segregating populations, avoiding the tedious conventional evaluation procedure and expediting the development of cultivars resistant to SCN in breeding programs.

Table 4 Number of lines and female index (FI) observed in the F_3 families for each marker phenotype or genotype and phenotypic selection based on the FI of the F_2 generation

Marker	Phenotype/ genotype	Number of lines	FI
OPAA-11_{795}	+[a]	144	25.9
OPAA-11_{795}	–	39	45.6
OPR-07_{548}	+	126	36.6
OPR-07_{548}	–	55	14.9
OPY-07_{2030}	+	129	37.0
OPY-07_{2030}	–	50	17.0
OPAE-08_{837}	+	132	36.5
OPAE-08_{837}	–	51	13.5
Satt082	HH[b]	55	13.3
Satt082	HB	86	32.7
Satt082	BB	42	46.8
Sat_-001	HH	53	13.5
Sat_-001	HB	88	32.3
Sat_-001	BB	42	46.4
Satt574	HH	52	13.5
Satt574	HB	93	33.4
Satt574	BB	38	44.6
Satt301	HH	48	20.3
Satt301	HB	91	31.1
Satt301	BB	44	36.6
Phenotypic selection based on F_2 FI			
FI<10	Resistant	28	16.9
FI≥10	Susceptible	155	32.6

[a] +, Presence of band in marker loci; –, absence of band in these loci; [b] HH, Homozygosity for Hartwig genotype; BB, homozygosity for BR 92–31983 genotype; HB, heterozygosity

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