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*ARTICLE*

# Development and validation of SNP assays for the selection of resistance to *Meloidogyne incognita* in soybean

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**Abstract:** *This work aimed to develop and validate individual SNP molecular markers previously identified in a genetic association study of resistance to Meloidogyne incognita in soybean using a microarray panel. The markers identified in the array were converted in single TaqMan® markers. The single markers were used to create an SNP genotyping protocol and establish a marker-assisted selection (MAS) routine associated with resistance to M. incognita in soybean. Out of the eight TaqMan® assays tested, three were validated for use in MAS. The MAS protocol developed in this study uses sequential selection. Initially, molecular markers are used to identify susceptible plants; subsequently, the phenotypic evaluation of plants expressing resistance genotype for the markers is carried out, resulting in the accurate identification of resistant plants. The accuracy of this approach for MAS sequential for M. incognita varied from 94 to 96%.*

**Keywords:** *MAS, SNP markers, taqMan® assay, soybean, nematode, end-point PCR.*

## INTRODUCTION

Root-knot nematodes (*Meloidogyne* spp.) occur in most Brazilian soybean production regions. With the expansion of planting areas, the species has increasingly caused damage to crops (Dias et al. 2000, Castro et al. 2003, Embrapa 2011), resulting in yield losses ranging from 18 to 56% (Dias et al. 2006).

Association studies to identify genes or molecular markers related to resistance to nematodes in soybean are crucial to improve the selection of resistant varieties in the early stages of breeding programs. Resistant varieties, combined with crop rotation using non-host species, play an important role in reducing production losses caused by root-knot nematodes in soybeans (Xu et al. 2013).

Marker-assisted selection (MAS) for resistance to nematode in plants may accelerate the breeding work. Molecular markers of various types have been reported as being associated with *Meloidogyne* spp. resistance genes (Luzzi et al. 1994, Tamulonis et al. 1997, Fourie et al. 2008). Molecular markers provide an early and large-scale selection of plants and are advantageous due to the maintenance of small rearing populations when compared with traditional breeding. Moreover, lower inoculation levels of the target species are required to evaluate plants once the phenotypic selection is made (Silva 2001).

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SNP markers have been recently used in MAS due to their abundance in the genome of all species and the possibility of automation and large-scale use. Several methods can be applied to SNP genotyping, and TaqMan® assays are one of the most frequently used. This methodology is based on allelic discrimination and allows analyzing two variant alleles of SNP in a given DNA segment. The assay is relatively simple, fast, robust, easy to implement, leading to highly-specific and reliable results. It has been used in several successful gene identification studies (De La Vega et al. 2005) and allows evaluating few markers in a large number of samples. Single markers are more suitable for MAS in breeding projects than microarrays since the gene or QTL region of interest requires a few markers for selection in a large number of samples (Kumpatla et al. 2012).

We developed previously a genomic association study (data not shown) and identified SNP markers associated with resistance to nematode *Meloidogyne incognita* in soybean. The identified markers are part of the *Infinium iSelect HD Custom Genotyping BeadChips* 6k panel (Illumina, Inc., San Diego, CA, USA), customized for soybeans, containing six thousand SNP. For individual SNP to be used in MAS, these markers need to be validated in singleplex assays.

The validation of a marker includes the design of polymorphism-based assays and the genotyping of several germplasms, including segregant populations, to verify if the SNP under study is a Mendelian locus (Mammadov et al. 2010).

In the present study, some markers identified in a previous genetic association study for resistance to the nematode *M. incognita* in soybean were converted into individual TaqMan® markers and evaluated for Mendelian segregation in segregating populations. The efficiency of MAS was tested in an independent breeding population. This work aimed to validate an end-point protocol to genotype the SNP associated with resistance to root-knot nematode (*M. incognita*) in soybean and design a MAS protocol to be used in the routine selection of soybean plants resistant to this nematode in soybean breeding programs.

## MATERIAL AND METHODS

### Design of oligonucleotides and probes

Eight TaqMan® assays were designed using the SNP markers associated with resistance to *M. incognita*, using sequence information from *Infinium iSelect HD Custom Genotyping BeadChips* 6k panel (Illumina, Inc., San Diego, CA, USA). All markers are located on chromosome 10 of the soybean genome (Table 1).

Using the marker's names as marker ID in the NCBI (National Center for Biotechnology Information) database, a sequence of at least 100 nucleotides in the region containing the SNP was identified and used for primers and probes design. Primers and probes were designed using the Primer Express 3.0® software (Applied Biosystems, Foster City, CA). The parameters used to design the primers and probes were: primer lengths between 18 and 22 base pairs; primers average annealing temperature of 58 - 60 °C and probes average annealing temperature of 5 - 10 °C higher than that of the primers; absence of dimer and secondary structures; and G/C content between 40 and 50%.

***Table 1.*** List of markers used for the design of TaqMan® assays, with the accession number of the SNP marker in the NCBI, which corresponds directly to the position in the soybean genome, and the oligonucleotide and probe sequences, respectively

| Marker | Forward Primer (5' - 3') | Reverse Primer (3' - 5') | FAM Probe | VIC Probe |
|---|---|---|---|---|
| Gm10_1586434 | TCACTAATCCACCTCAACTATTG-TATCA | CATATAAAATTGCTGTTCACGTC-GAT | CTCACGTAACAAGACGA-MGB | CTCACGTAACAAGATGA-MGB |
| Gm10_831916 | GGCAAACTCAAAGAAGTATAAT-GAAAAGT | ACACGCTGCCTAGCTTCTATGTT | CAAACATTGGGATTCT-MGB | CAAACATTGAGATTCT-MGB |
| Gm10_981062 | ACACGCTTCATCATCACTGCTT | CAGATCCGACCCCCTTTCA | CTTCGAATTCCGACCGTA-MGB | CGAATTCCGACCATAG-MGB |
| Gm10_925972 | GTTTTAGAATGCTTGTTC | GTGAAATTCCAAATTAGG | TTTGTATTCCCCAATTCG-GAAT-MGB | TTTGTATTCCCTAATTCG-GAAT-MGB |
| Gm10_1232205 | GTAACCAATTAGTCTCACT | TGTCTCTAACTTGTTATCC | GGGCAAAATTGT-TACGTGGG-MGB | GGGCAAAATTAT-TACGTGGG-MGB |
| Gm10_711291 | AGGTATAAAAGAAATGGC | TAAAAGAGTATCCAAACG | AAATTCAAGTGCAA-GAATAGCG-MGB | AAATTCAAGTACAA-GAATAGCG-MGB |
| Gm10_1341309 | CTGATTCCAATAAATAAATTTC | TGTGAATATCGTTCGTTA | TTCAGGCAATAGCATTGAT-CA-MGB | TTCAGGCAATAACATTGAT-CA-MGB |
| Gm10_1051336 | GTCATTGTCATTATCACT | CATTCCAATAGGTAGAGA | GCAGTCCATGGTTTGAA-GTC-MGB | GCAGTCCATGATTTGAAGTC-MGB |

Oligonucleotide synthesis was performed by Life Technologies do Brasil. Each assay included two oligonucleotides primers, forward and reverse, to amplify the regions of interest, and two probes to detect the alleles.

### DNA extraction

DNA was extracted following the protocol described by Schuster et al. (2004). DNA concentration of each sample was estimated by absorbance at 260 nm in a Nanodrop1000 spectrophotometer, each absorbance unit corresponding to the concentration of 50 µg $mL^{-1}$ of double-stranded DNA. DNA integrity was evaluated using 0.8% agarose gel electrophoresis.

### Genotyping (TaqMan® Assay)

Genotype analyses were performed by the allelic discrimination method (end-point), performed in the Real-Time Polymerase Chain Reaction device (ABI 7500 SDS®), using TaqMan® SNP genotyping assays (Applied Biosystems, Foster City, CA).

Three resistant varieties (CD202, CD237RR, CD245RR), three susceptible varieties (CD205, CD206, CD221), and two heterozygous controls were used at the initial evaluation of the TaqMan® assays, in each reaction. Initial TaqMan® reactions aimed to optimize the reaction conditions. The following parameters were tested: concentration of oligonucleotides and probes, annealing temperature, number of PCR cycles, total reaction volume per well, and volume of genomic DNA (uL/PCR reaction). Two different types of TaqMan® Master Mix were tested: TaqMan® Universal PCR Master Mix and TaqMan® GTXpress™ Master Mix.

Afterward, PCR reactions were performed in duplicate with a total volume of 10 µL, containing 5 µL TaqMan® GTXpress™ Master Mix (2X) (AmpliTaq® Fast DNA Polymerase, UP, dNTPs, Tracking Dye, and ROX™ dye); 0.6 µL of $H_2O$ (DNAse-free); 1 µM of each oligonucleotide (Forward and Reverse); 0.2 µM of each probe (FAM and VIC); and 30 ng of DNA. The optimized cycling program consisted of an initial denaturation of 95 °C for 20 seconds, followed by 40 cycles with denaturation at 95 °C for 15 seconds; annealing at 60 °C for 1 minute; elongation at 60 °C for 1 minute; and a pre-read run and post-read run at 25 °C for 1 minute. The pre-read run records the sample's background fluorescence on the reaction well before PCR. The pre-read fluorescence is subtracted from the post-read fluorescence to obtain the pre-amplification of the background fluorescence, ensuring accurate results.

At least two negative controls and one positive control were included in each assay. Genotypes discrimination and plotting were performed by the TaqMan® Genotyper Software® (Applied Biosystems, Foster City, CA). Each reaction well was triplicated.

#### Genetic material

Three soybean populations were used to validate the TaqMan® assays. Initially, the same set of 167 varieties in which the markers were obtained in our previous work was used to evaluate the amplification of the correct region of the genome. This procedure allowed comparing the genotypes of the individual markers with those of the markers present in the *Infinium iSelect* panel.

An $F_2$ population containing 257 plants from the cross between the varieties CD201 x CD204 was also used to evaluate the Mendelian segregation and the distances between the markers. These parameters were assessed using the GQMol software (Cruz and Schuster 2008). The base pair (bp) ratio per centimorgan (cM) was estimated in the region of the markers, using the information of the markers' position on the sequence map in soybase (www.soybase.org) and the distances in cM.

Finally, a population of 81 soybean lines from the Coodetec breeding program, whose phenotypic data were available, was used to evaluate the efficiency of markers to select resistance to *M. incognita* in an independent soybean population. Genotypic information on the varieties was related to the phenotypic information and evaluated by the Chi-square test, using contingency tables in the Genes Software (Cruz 2006). Selection efficiency analyses of MAS were performed by comparing the genotypes of molecular data and phenotypes.

## RESULTS AND DISCUSSION

Eight TaqMan® assays were designed using the SNP markers for resistance to the nematode *M. incognita* in soybean (Table 1). To optimize the reaction, different amplification conditions were evaluated using DNA samples from six soybean varieties and two heterozygous samples. Three of the eight markers evaluated in this study presented reliable and reproducible results, with amplification curves in the expected format: Gm10_1586434_T_C, Gm10_831916_A_G, and Gm10_981062_A_G (Figure 1). The alleles of these markers formed three clusters, as expected in a good-quality reaction for the TaqMan® GTXpress™ Master Mix.

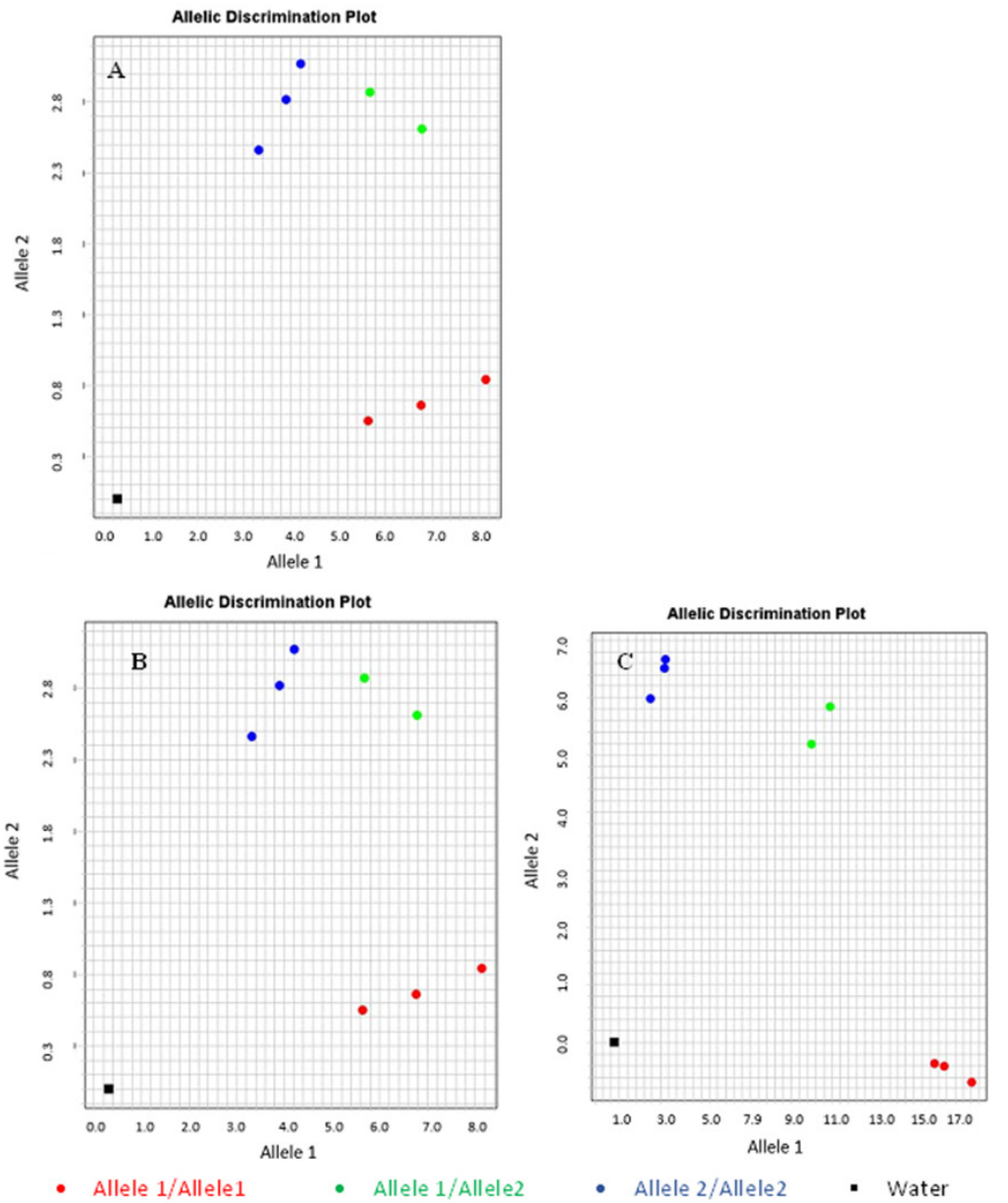


***Figure 1.*** Allelic discrimination plots with the result of the genotyping for each marker, forming three clusters that represent the genotypes AA (allele 1 - blue), AB (allele 1 and 2 - green), and BB (allele 2 - red), classified as resistant (R), heterozygote (H), and susceptible (S), respectively. A. marker Gm10_1586434; B. marker Gm10_831916; C. marker Gm10_981062.

TaqMan® reactions for the three validated markers were repeated in triplicate, *i.e*., three wells of each reaction were made, which faithfully reproduced the results and confirmed the efficacy.

The other five assays did not form clusters. Several reasons may lead to incorrect clustering of the alleles of a TaqMan® marker, such as incorrect assay design, the presence of more than one copy in the targeted region, the existence of a second SNP in the probe sequence, or the occurrence of multi-allelic SNP (De La Vega et al. 2005). Since all markers are in the same region of the soybean genome, the most likely cause for their non-amplification is the TaqMan® assay design.

An $F_2$ population from the cross CD201 x CD204 was genotyped with the three markers that successfully used in the first amplification test, to evaluate the Mendelian segregation. The three markers segregated according to the expected Mendelian proportions (Table 2). Genotypic data of the $F_2$ population were also used to evaluate the genetic linkage between these three markers (Figure 2). The markers Gm10_831916_A_G and Gm10_981062_A_G are very close, being 0.3 cM apart. The marker Gm10_1586434_T_C is located at 4.8 cM from marker Gm10_981062_A_G.

The distance in cM obtained from markers co-segregation in the $F_2$ population and the data of the marker position in the genetic sequence of chromosome 10 of soybean were used to estimate the ratio of bp/cM (Table 3). The number of bp/cM ranged from 126,119 bp to 497,153 bp, with a mean of 257,739 bp/cM in this interval.

Yu et al. (2001) compared genetic and physical maps in humans and identified that 1 Mb contains, on average, 1.3 cM, which also means that 1cM contains 769,000 bp. In this work, the evaluated region showed less bp/cM, indicating a region with more frequent recombination.

To verify whether the optimized assays corresponded to the same SNP previously identified by the analysis of association with resistance to *M. incognita* in soybean, these three TaqMan® assays were used in the genotyping of

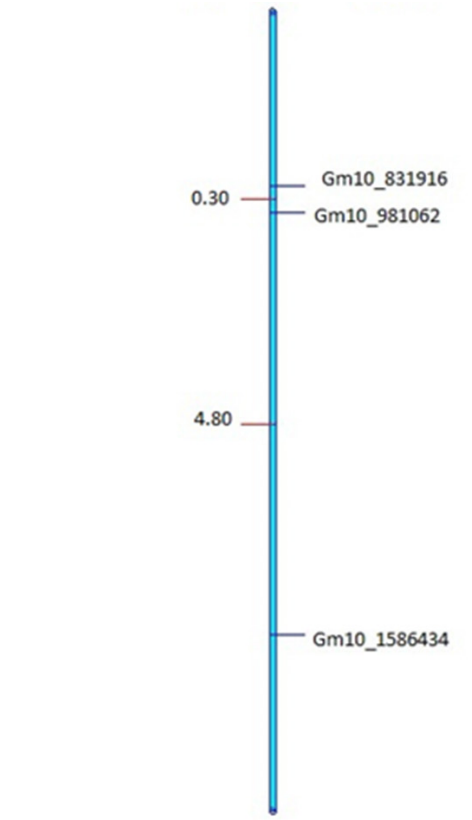


***Figure 2.*** Genetic linkage map of markers and distances (in cM) on chromosome 10 of the soybean genome.

***Table 2.*** Segregation of the three markers in the $F_2$ population (CD201 x CD 204). Marker 1: Gm10_1586434_T_C; marker 2: Gm10_831916_A_G; and marker 3: Gm10_981062_A_G

| Marker | **Homozygous Allele 1** | **Heterozygote** | **Homozygous Allele 2** | **Hypothesis** | $X^2$ | **Probability** (%) |
|---|---|---|---|---|---|---|
| 1 | 75 | 120 | 62 | 1: 2: 1 | 2.44 | 29.52 |
| 2 | 56 | 84 | 43 | 1: 2: 1 | 3.07 | 21.47 |
| 3 | 56 | 83 | 44 | 1: 2: 1 | 3.15 | 20.67 |

***Table 3.*** Relationship between physical distance and genetic distance between SNP markers associated with resistance to the root-knot nematode *M. incognita* in soybean

| **Interval** | **Dist** (cM) | **Dist** (Pb) | **Pb/cM** |
|---|---|---|---|
| Gm10_831916 - Gm10_981062 | 0.3 | 149.146 | 497.153 |
| Gm10_831916 - Gm10_1586434 | 5.1 | 754.518 | 149.945 |
| Gm10_981062 - Gm10_1586434 | 4.8 | 605.372 | 126.119 |
| Mean | | | 257.739 |

the same set of 167 soybean varieties in which the markers were identified in our previous work. When comparing the genotypes obtained by the individual TaqMan® markers with those obtained by the *Infinium iSelect* panel (Illumina), they coincided at 100% for all varieties, which means that the designed assays faithfully evaluated the same SNP previously identified in the *Infinium iSelect* panel.

To validate the use of these markers in an independent population, a set of 87 lines from the Coodetec soybean breeding program (whose phenotypic evaluation was available) was also genotyped with these three markers. Markers GM10-831916 and GM10-981062 presented complete linkage disequilibrium. Hence, no recombination between these markers was observed in this set of lines. Between these two markers and GM10-1586434, 24 of the 87 varieties showed resistance alleles for one marker and a susceptible allele for another, demonstrating that the first two markers are in equilibrium with the latter.

In the set of 87 lines used in the independent marker association validation, seven presented heterozygous genotypes when evaluated with marker GM10-1586434 and were discarded in the association analysis of this marker. For markers GM10-831916 and GM10-981062, six lines showed heterozygote genotypes and were also discarded in the association analysis. Among the 80 homozygous lines observed with marker GM10-1586434, 41 were susceptible, and 39 were resistant. Among the 81 homozygous lines identified with markers GM10-831916 and GM10-981062, 42 were susceptible, and 39 were resistant.

The use of marker GM10-1586434 to select resistant lines and eliminate susceptible lines showed an efficiency of 71%. Twenty-three of the 80 lines had a phenotype different from the marker's genotype (Table 4). For markers GM10-831916 and GM10-981062, efficiency was 88%, and ten of the 81 lines exhibited a different phenotype from that of the genotype.

Resistance to *M. incognita* in soybean is conferred by more than one gene/QTL. Oliveira et al. (2015) stated that resistance to *M. incognita* is conferred by three genes of complementary action. Thus, the lines must have the three genes to be resistant. The single-locus molecular markers are not expected to correctly identify the resistant or susceptible phenotypes.

Out of the 23 lines with different phenotypes from that of the genotype in marker GM10-1586434, 20 are susceptible and have resistant genotypes, which is expected when the resistance is controlled by more than one gene/QTL. The lines having this gene/QTL (or marker) need to have the other gene(s) to be resistant. In lines that do not have the other gene(s), the phenotype will be susceptible, even though they have this target marker. These 20 lines probably do not have at least one of the other complementary genes/QTL required for the plant to be resistant. The same occurs with the five susceptible lines that presented resistance genotypes in the markers GM10-831916 and GM10-981062.

Considering the complementary action of the genes/QTL of resistance to *M. incognita*, the absence of one of the genes/QTLs results in susceptibility. When a marker for only one of the gene/QTL is available, the best strategy for using

***Table 4.*** Efficiency of SNP markers in the selection of resistance to *M. incognita* in an independent soybean population and the strategy for the use of markers

| Marker | Maker genotype | Phenotype | Number | Strategy | Result |
|---|---|---|---|---|---|
| GM10-1586434 | R | R | 36 | Phenotype | R |
| | S | S | 21 | Not Phenotype | S |
| | S | R | 3 | Not Phenotype | S* |
| | R | S | 20 | Phenotype | S |
| Efficiency (%) | | | 71** | | 96*** |
| GM10-831916 or GM10-981062 | R | R | 34 | Phenotype | R |
| | S | S | 37 | Not Phenotype | S |
| | S | R | 5 | Not Phenotype | S* |
| | R | S | 5 | Phenotype | S |
| Efficiency (%) | | | 88** | | 94*** |

R: Resistant. S: Susceptible. * This line was excluded by the marker as susceptible, and has the resistant phenotype. ** Efficiency of the selection using only the marker, calculated as the percentage of phenotype classified correctly by the markers. *** Efficiency of the selection using the marker and phenotyping the lines classified as resistant by the markers. The efficiency is calculated as the percentage of final classification after sequential selection. In this sequential selection approach, the lines classified as susceptible by the markers are not phenotyped.

this single marker is the identification of susceptible lines, *i.e*., those that do not have the resistance genotype in the available marker. For a line that does not have this QTL, the probability of being resistant is small. Conversely, lines with the resistance allele for the marker may be resistant if they have the other QTL of resistance and may be susceptible if they do not have these other QTLs.

Since resistance to *M. incognita* is controlled by more than one gene/QTL, and only markers associated with one of these genes/QTL are available, a sequential selection strategy can be applied. Such a target marker will only identify susceptible plants. Afterward, plants that were not identified as susceptible should be phenotypically evaluated for the identification of resistant plants. Plants identified as susceptible by the marker, therefore, do not need to be phenotypically evaluated. This fact will reduce the number of plants that need to be verified for the resistance trait.

Considering this strategy of sequential selection in the set of lines evaluated in this study, the use of marker GM10-1586434 would eliminate 24 of the 80 lines (30%) from the phenotypic analysis. Out of these 24 lines that would not be phenotypically evaluated for being considered as susceptible by the marker, three are resistant. The other 56 lines, selected as resistant by the marker, can be phenotypically evaluated so that the selection of these lines can be validated for accuracy by the phenotype. As a result, the phenotype of 96% of the lines was accurately accessed (77 out of 80 phenotypes were correctly identified), with only 70% of phenotyping that equaled 96% of selection efficiency. Besides that, the 92% of resistant lines remained in the group of plants selected as resistant (36 of 39).

The markers GM10-831916 and GM10-981062 did not show recombination in the set of lines evaluated. The results obtained with markers GM10-831916 and GM10-981062 are similar, suggesting the use of only one of them. Using the same strategy with each one of these markers, 42 out of 81 lines (52%) would not be phenotyped for being identified as susceptible by the markers. The marker selected 87% of the resistant lines and also five susceptible lines evaluated in this study. Using sequential selection, the efficacy of selected resistant lines was 94% when only 52% of the lines were phenotyped. Ultimately, using these two markers resulted in the selection of 87% of the resistant lines and all susceptible plants were eliminated.

The use of marker GM10-1586434 resulted in some higher level of efficacy, and more resistant lines were selected. Therefore, more lines will be phenotyped. The use of markers GM10-831916 or GM10-981062 resulted in fewer plants to be phenotyped; however, it had lower efficacy. The application of the GM10-831916 or GM10-981062 further eliminated some resistant lines, *viz*. five compared to three for marker GM10-1586434). In summary, using the markers GM10-1586434 and GM10-831916 or GM10-1586434 and GM10-981062 did not improve the accuracy in terms of identifying resistant lines.

The TaqMan assays developed for these three SNP markers can be used with high efficiency in a sequential selection program for resistance to *M. incognita* in soybeans. The sequential selection program consists of the initial selection by the markers, eliminating plants with the susceptible allele and phenotypically evaluating only those with the resistance allele.

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