Genetics and Molecular Research

Comparative analysis of soybean genotype resistance to *Heterodera glycines* and *Meloidogyne* species via resistance gene analogs

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Genet. Mol. Res. 15 (3): gmr.15038562
Received February 17, 2016
Accepted March 28, 2016
Published September 2, 2016
DOI http://dx.doi.org/10.4238/gmr.15038562

ABSTRACT. Nematodes are important pests of soybean throughout the world and cause high yield losses. As a control strategy, the identification of resistance genes is an important aim of breeding studies. Plants possess resistance genes (R), which are responsible for the recognition of pathogens and activation of the defense system. R genes and resistance gene analogs (RGAs) possess conserved domains, from which nucleotide-binding site is the most common. Using degenerate primers originating from these domains, it is possible to identify and isolate sequences of R and RGA genes. In this study, soybean genotypes resistant to the nematodes *Heterodera glycines*, *Meloidogyne incognita*,

M. javanica, and *M. enterolobii* were compared by the use of RGAs and simple sequence repeat (SSR) markers. Forty-six soybean genotypes were studied, including plant introductions (PIs), commercial crops, and source of resistance genotypes. Thirteen combinations of RGA primers and different SSRs linked to QTLs were used to confirm resistance to soybean cyst nematodes (SCN). Fragments associated with resistance to the studied nematodes were amplified in the source of resistance and PI genotypes. RGA markers were efficient at distinguishing groups of genotypes that were resistant and susceptible to *Meloidogyne* spp and SCN. Combinations of specific primers were identified through their ability to amplify nucleotide sequences from possible resistance candidate genes. SSR markers contributed to the analysis of SCN race specificity, showing that the QTLs identified by these markers are distinct from those identified by RGA markers.

Key words: Soybean resistance; Nematode; Resistance gene analogs; Conserved domains; Molecular markers

INTRODUCTION

Soybean nematodes such as *Heterodera glycines*, Ichinohe, and *Meloidogyne* species are serious global soybean pests. Other than rotation with non-host crops, breeding cultivars with resistance to multiple nematode species is the most effective and environmentally friendly method to control these pests. Molecular strategies have been used to breed for nematode resistance for many years (Concibido et al., 2004; Jiao et al., 2015). The identification of molecular markers associated with resistance is helpful for developing resistant cultivars because phenotyping soybean for nematode resistance is time-consuming and costly (Kadam et al., 2016). Transgenic approaches have not been reported for soybean resistance to nematodes; however, marker assisted selection has been broadly studied and reported in soybean against other pests (Concibido et al., 2004; Xiao et al., 2014). In this context, strategies used to identify molecular markers for nematode resistance using those based on plant resistance genes (R genes) and the so-called resistance gene analogs (RGA) constitute one alternative that has not yet been employed in soybean.

R genes mediate a mechanism of resistance that is specific against pathogens, and which developed during the coevolution of plant-pathogen interactions (Kang et al., 2012; Sekhwal et al., 2015). Through this mechanism, products of the R genes recognize elicitors originating from pathogens and subsequently initiate the resistance response (Flor, 1971; Thakur and Sohal, 2013).

The proteins that recognize elicitor molecules share conserved domains that fulfill specific functions, such as recognition, protein-protein interaction, signaling, and activities that initiate the resistance response (Lei et al., 2014). Domains found in the majority of sequenced R genes include leucine-rich repetitions (LRR), nucleotide-binding sites (NBS), leucine zipper domains, coiled-coil (CC) domains, protein-protein interaction domains homologous to the interleukin-1 receptor (Toll/interleukin-1 receptor), and transmembrane (TM) domains, as well as other domains such as nuclear localization signal (NILS), tryptophan-arginine-lysine-tyrosine (WRKY), domain, and kinase domains (Collins et al., 1998; Gururani et al., 2012). In addition to R genes, a class of potential resistance genes, the RGAs, is characterized by the presence of conserved domains. RGAs are important in the study of resistance and in

the research and understanding of R gene evolution, and are found widely in the genomes of various species (Sekhwal et al., 2015).

Conserved domains are clustered in different arrangements, resulting in eight classes (Williamson and Kumar, 2006), which are all represented by products of the R genes and have been recognized and validated in monocot and dicot species (Gururani et al., 2012; Sanseverino and Ercolano, 2012). NBS-LRR represents the best known family of RGAs. The NBS domain can be identified in the genome by its motifs, namely P-loop, kinase 2, kinase 3, and GLPL, which enable the isolation and cloning of R genes and RGAs (Yu et al., 1996; Shen et al., 1998; Lei et al., 2014).

It is important to identify the genes in the soybean crop, *Glycine max* (L.) Merril, that are related to defense against potential pathogenic agents, including *Heterodera glycines*, the soybean cyst nematode (SCN) (Vuong et al., 2015), and nematodes of the genus *Meloidogyne*, both of which cause substantial yield losses. The first cloned R gene conferring resistance to a nematode was $Hs1^{pro-1}$ in sweet potato, for resistance to *Heterodera schachtii* (Cai et al., 1997). The conserved domains of this gene are of the LLR-TM type. The genes *rhg1* and *Rhg4* (Vuong et al., 2015), which contain LLR-TM-Kinase domains, are reported to confer resistance to *H. glycines* in soybean. The genes *Cre-1* and *Cre-2*, with NBS-LRR domains, confer resistance to the nematode *Heterodera avenae*. As for *Meloidogyne*, the genes *Mi-1* and *Mi-9* are reported to be responsible for resistance, and possess domains CC-NBS-LRR and *Me-3* in pepper (Gururani et al., 2012).

In addition to RGAs, resistance loci can be identified by microsatellite markers (Sekhwal et al., 2015), which are widely distributed in the genomes of eukaryotes and are frequently used to map resistance genes. Given the complexity of SCN resistance, which is controlled by quantitative trait loci (QTLs) (Concibido et al., 2004; Kim et al., 2010), simple sequence repeat (SSR) markers have often been used to identify QTLs associated with resistance (Vuong et al., 2015).

Molecular markers are useful for the identification of R genes in commercial cultivars and to identify possible sources of resistance. The use of resistant cultivars is one of the most efficient methods in the control of *H. glycines* and *Meloidogyne* spp; however, this strategy is limited, as those genotypes are generally derived from a common parent, such as PI 88788 or Peking. Therefore, it is important to identify new sources of resistance to these nematodes.

The molecular comparison of groups of genotypes that are resistant and susceptible to nematodes may contribute to the research of R genes and to the identification of genomic regions that are present in resistant genotypes. In the present study, polymorphisms were identified by RGA markers originating from the NBS domain, which is the most common domain found in resistance genes. In addition, the results were compared using microsatellite markers linked to QTLs, either for resistance, in the case of SCN, or in genotypes resistant and susceptible to nematodes in soybean. Hence, this study aimed to identify differential regions of the soybean genome that are related to resistance against nematodes of the genus *Meloidogyne* spp and the species *H. glycines* in soybean genotypes by means of RGA and SSR markers.

MATERIAL AND METHODS

Plant material

The 46 soybean genotypes used in this study were provided by Empresa Brasileira de Pesquisa Agropecuária (Embrapa) and the Institute of Biotechnology Applied to Agriculture (Bioagro). The genotypes used were selected based on the literature, with the aim of obtaining groups of genotypes that are resistant and susceptible to nematodes of the genus *Meloidogyne*

(*M. javanica*, *M. incognita*, and *M. enterolobii*) and to different races of *H. glycines*, and SCN (Table 1). Among the genotypes used, 32 were cultivars obtained by four different breeding programs in Brazil (Embrapa, Coodetec, Fundação Mato Grosso, and UFV).

Table 1. Nematode resistance of the soybean genotypes used in this study.

Genotype / BP - O	Resistance to SCN	*Mi*	*Mj*	*Me*	Parents
CD201 / CD	S	R	R	S	-
CD217 / CD	R 3	R	S	S	-
Conquista / E	S	R	R	S	-
BRS 211 / E	-	R	R	T	-
BRS Valiosa / E	S	MR	R	T	-
BRS 256RR / E	S	R	R	T	-
BRS Raimunda / E	S	R	R	T	-
BRSG Paraíso / E	-	R	R	T	-
Luziânia / E	S	MR	R	S	-
BRS Favorita / E	S	MR	R	T	-
BRSGO Iara / E	R 1, 3	S	S	-	Peking (S)
BRS262 / E	R 1, 3	S	S	-	Peking (S); PI 437654 (H)
BRSGO Raissa / E	R 1, 3	S	S	-	Peking (S)
BRS Invernada / E	R 1, 3	S	S	-	Peking(S); PI 88788; PI 209332
BRSGO Chapadões / E	R 1, 2, 3, 4, 5, 14	MR	S	-	PI 437654 (H)
BR Jiripoca / E	R 1, 3, 14 MR 5, 6, 9, 1	S	S	-	Peking (S); PI 437654 (H)
BRS263 D / E	R 1, 3 MR 14	S	S	-	Peking (S; F; L); PI 437654 (H)
TMG115 / FMT	R 1, 3 MR 14	S	S	-	Peking; PI 437654
TMG121RR / FMT	R 1, 3 MR 14	S	S	-	Peking; PI 437654
Tucunaré / FMT	R 1, 3 MR 14	S	S	-	Peking (S); PI 437654(H)
Tabarana / FMT	R 1, 3	S	S	-	Peking (Centennial)
TMG117 / FMT	R 3	S	S	-	-
Doko / FMT	S	S	S	S	-
M-Soy 8400 / MO	R 3	-	-	-	Peking (Coker)
M-Soy 8001 / MO	R 1, 3	S	S	S	Peking (Coker 6738)
M-Soy 6106 / MO	S	-	-	-	-
M-Soy 8914 / MO	S	-	-	-	-
Y23 / UFV	S	-	-	-	-
UFVS2001 / UFV	S	-	-	-	-
UFV16 / UFV	S	-	-	-	-
UFVTN104 / UFV	S	-	-	-	-
UFV18 / UFV	S	-	-	-	-
A7002	S	-	-	-	-
Bedford**	R	-	-	S	PI 88788
PI 595099	R	R	R	R	-
PI 594427	R	R	R	R	-
PI 209332 / Japan	R 3, 5, 14	-	-	-	-
PI 548316 / China	R 3 MR 14	-	-	-	-
PI 88788 / China	R 3, 14	-	-	-	-
PI 90763 / China	R 1, 2, 3, 5	-	-	-	-
PI 437654 / Russia	R*	-	-	-	-
PI 89772 / China	R 1, 2, 3, 5 MR 14	-	-	-	-
Hartwig / USA	R*E 4^{+}, 14^{+}	-	-	-	PI 437654
Pickett / USA	R 1, 3	-	-	-	-
Peking / USA	R 1, 3, 5	-	-	-	-
Lee	S	-	-	-	-

BP: breeding program; E: EMBRAPA; CD: COODETEC; FMT: Fundação Mato Grosso; O: origin; SCN: soybean cyst nematode; *Mi: Meloidogyne incognita*; *Mj: Meloidogyne javanica*; *Me: Meloidogyne enterolobii.* R: resistant; *R resistant to all races of SCN; MR: moderately resistant; T: tolerant; S: susceptible; resistance from Peking, S (Sharkey); F (Forrest); Centennial; Coker; L (Lancer, Lancer x BR80-6989). Resistance from PI437654, H (Hartwig). **Resistance from PI88788. Source: EMBRAPA (2011).

Molecular analyses

Genomic DNA was extracted from seeds using an adaptation of the method described by McDonald et al. (1994). The quality and concentration of the DNA were verified using a spectrophotometer Nanodrop™ 2000 (Thermo Scientific) and integrity was confirmed by 0.8% agarose gel electrophoresis. For the polymerase chain reaction (PCR), 13 combinations of degenerate primers were used, originating from the NBS domain of the *RPS2* gene of *Arabidopsis thaliana*, which confers resistance to *Pseudomonas syringae*; from the *N* gene of tobacco, which confers resistance to the tobacco mosaic virus (Leister et al., 1996; Shen et al., 1998), and from the *L6* gene of linen, which confers resistance to *Melampsora lini* (Table 2). Combinations of these RGA primers were amplified in a final volume of 20 µL containing 1X Master Mix (Fermentas), 30 ng DNA, 0.4 µM each primer, and 1 U Taq DNA polymerase. The reactions were subjected to initial denaturation for 5 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 40°C, and 2 min at 72°C, with a final extension of 10 min at 72°C. The PCR products were separated by gel electrophoresis on 1.4% agarose, stained with ethidium bromide, and visualized under ultraviolet light. Twenty-eight combinations of the RGA primers S2, As1, As2, As3, LM637, F1, and R1 were evaluated. To confirm the results, the reactions were repeated up to three times.

Table 2. Resistance gene analogue primers used in the present study that were obtained from the nucleotide-binding site (NBS) domain.

Primer	Motif	Gene (s)	Sequence 5'-3'	Reference
s1	P-loop	*N, L6,RPS2*	GGTGGGGTTGGGAAGACAACG	1
s2	P-loop	*N, L6,RPS2*	GGIGGIGTIGGIAAIACIAC	1
As1	GLPL	*N, L6,RPS2*	CAACGCTAGTGGCAATCC	1
As2	GLPL	*N, L6,RPS2*	IAAIGCIAGIGGIAAICC	1
As3	GLPL	*N, L6,RPS2*	IAGIGCIAGIGGIAGICC	1
Ploop1	P-loop	*N, L6,RPS2*	AAGAATTCGGNGTNGGNAAAACAAC	2
Ploop2	P-loop	*N, L6,RPS2*	AAGAATTCGGNGTNGGNAAAACTAC	2
Ploop5	P-loop	*N, L6,RPS2*	AAGAATTCGGNGTNGGNAAGACAAC	2
Ploop6	P-loop	*N, L6,RPS2*	AAGAATTCGGNGTNGGNAAGACTAC	2
GLPL1	GLPL	*N, L6,RPS2*	AACTCGAGAGNGCNAGNGGNAGGCC	2
GLPL3	GLPL	*N, L6,RPS2*	AACTCGAGAGNGCNAGNGGNAGTCC	2
GLPL4	GLPL	*N, L6,RPS2*	AACTCGAGAGNGCNAGNGGNAGCCC	2
F1	P-loop	*N, L6,RPS2*	GGAATGGGIGGIGTIGGIAARAC	3
LM637	P-loop	*RPS2*	ARIGCTARIGGIARICC	4

Genes *N*, *L6*, and *RPS2* are from *Nicotiana tabacum*, *Linum usitatissimum*, and *Arabidopsis thaliana*, respectively. Primers obtained from P-loop (or kinase 1a) domains = GGV/IGKTT; GLPL: GLPLAL. I = Inosina; R = G or A; Y = T or C; D = A, G, or T. 1: Leister et al. (1996); 2: Shen et al. (1998); 3: Pan et al. (2000); 4: Kanazin et al. (1996).

Amplifications were carried out in a total volume of 20 µL, containing 45 ng genomic DNA, 0.4 µM each primer, 1X Master Mix (Thermo Scientific), and 1.0 U Taq DNA polymerase (Fermentas). The reactions were carried out in a Veriti® 96-Well Thermal Cycler (Applied Biosystems) in accordance with the methods described by Leister et al. (1996) or Shen et al. (1998), depending on the combination of primers. The amplified products were separated by 1.5% agarose gel electrophoresis in 1X TBE buffer, stained with ethidium bromide, and visualized under UV light. To estimate the sizes of the amplified fragments, molecular weight markers of 100 bp were applied.

For comparative purposes, 25 genotypes with resistance to SCN were analyzed via RGA and SSR data. SSR data were obtained by Santana (2008) using SSRs linked to QTLs associated

with SCN resistance. The genotypes compared were as follows: BRS 262, BRS 263 Diferente, BRS Invernada, BRS Jiripoca, BRSGO Chapadões, BRSGO Iara, BRSGO Raíssa, CD 217, MSoy-8001, MSoy-8400, TMG 115, TMG 117, TMG 121RR, FMT Tabarana, FMT Tucunaré, PI 209332, PI 437654, PI 548316, PI 89772, PI 88788, PI 90763, Pickett, Peking, Hartwig, and Lee.

Data analyses

The polymorphisms generated by RGAs were converted into a matrix of binary data. The matrix was used to determine dissimilarity between genotypes by the method of simple coincidence, with later performance of grouping by the hierarchical method of mean linkage between groups (UPGMA). Based on the SSR data, the dissimilarity matrix was obtained by pondered index, and was later used for grouping with the UPGMA method.

The obtained data were presented as a heat map, which is a multidimensional way of representing groupings and amplifications by genotype. Thereby, amplification was represented by a light color, and the absence of amplification by the red color. All analyses were performed using the R version 3.2.1 software. Inferences about differences between genotype groups were made using the Bayesian method with the Structure 2.3.4 software. The data were generated by the method of simple coincidence.

RESULTS

RGA analysis

RGA markers based on conserved motifs of the NBS domain amplified 80 amplicons, most of which were polymorphic (88.75%), with a mean number of 7.72 amplicons per primer pair. Genotype diversity was estimated by the distance between individuals, which varied from 0.0 (CD201 and Doko) to 0.655 (TMG 115 and Peking). Clustering by the UPGMA method resulted in the formation of three groups (Figure 1, vertical clustering).

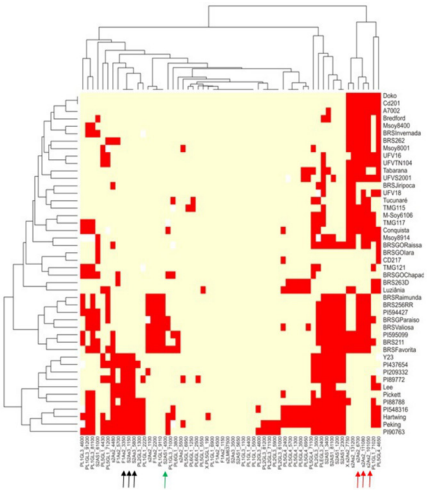

Figura 1. Amplification profile of RGA markers of 46 genotypes resistant and/or susceptible to *Heterodera glycines* or *Meloidogyne* nematode species. Grouping of genotypes (vertical); grouping of RGA amplicons (horizontal).

Light yellow bands indicate amplification and red bands represent ausence of amplification. Black arrows: combinations of primers that amplify in resistant genotypes to *Meloidogyne* species; green arrow: combination of genotypes resistant to primer that amplifies the SCN and commercial genotypes resistant to at least one race of this nematode; red arrows: primer combinations that amplified prominently in the SCN resistance sources.

One of the groups, composed of 11 SCN-differentiator genotypes, contained sources of resistance that are used widely in Brazil and in the United States against SCN, including PI 437654 and Peking. For these genotypes, there are no reports of resistance to nematodes of the genus *Meloidogyne* spp. Differentiator genotypes susceptible to SCN (Lee and Y23) were also placed in this group.

A second group was constituted by soybean genotypes resistant and tolerant to the three *Meloidogyne* species, including the genotypes Raimunda, Paraíso, Valiosa, BRS 211, and BRS 256RR with tolerance to *M. enterolobii* (originated from Embrapa), in addition to the genotypes PI 595099 and PI 594427, which are resistant to all gall nematodes and to SCN. The greatest genetic proximity found within this group was between BRS 256 and BRS Valiosa (dissimilarity of 0.016).

The third group was composed of 27 commercial cultivars, of which 11 were susceptible to SCN (Conquista, CD 201, Luziânia, M-Soy6101, M-Soy8914, UFVS2001, UFV16, UFVTN104, UFV18, A7002, and Doko), three were resistant to race 3 (CD217, TMG117, M-Soy8400), six were resistant to races 1 and 3 (BRSGO Iara, BRS262, BRSGO Raissa, BRS Invernada, Tabarana, M-Soy 8001), four were resistant to races 1 and 3 and moderately resistant to race 14 (BRS263 Diferente, TMG115, TMG121RR, and Tucunaré), and two were resistant to more than three races (BRSGO Chapadões and BRS Jiripoca). The cultivars Conquista and CD201 are resistant to *M. javanica* and *M. incognita.* Six cultivars with known susceptibility to *M. enterolobii* were allocated to this group, which is composed of genotypes from the different breeding programs in Brazil (Embrapa, Fundação MT, COODETEC, and Monsanto).

In the structure analyses, the population was attributed a value of K = 3. The results corroborate the grouping of genotypes obtained by the UPGMA method (Figure 2). The largest group was composed of cultivars originating from different breeding programs, and contained individuals that were resistant and susceptible to SCN and *Meloidogyne* spp. The second group was formed by differentiator genotypes and sources of resistance to SCN. The last group was allocated genotypes that were mostly resistant to *M. incognita* and *M. javanica*, besides PI 595099 and PI 594427.

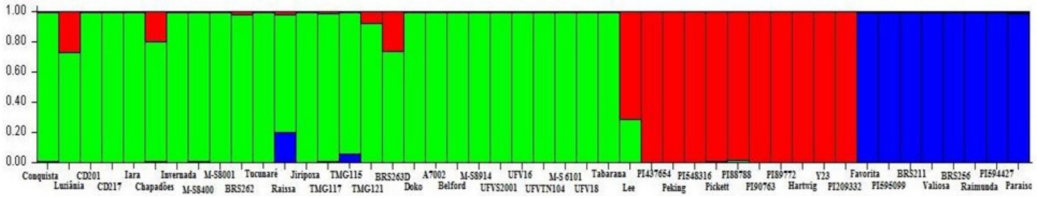

Figure 2. Structure analysis of 46 soybean genotypes by all RGA markers. Clusters were inferred based on Bayesian analyses considering the most probable number of groups (K = 3) estimated.

The primer combinations used to amplify motifs of the NBS domain, which were obtained from resistance genes of *A. thaliana* and *N. tabacum*, were analyzed independently in order to characterize the influence of the primers on the grouping of genotypes (Figure 3A and B).

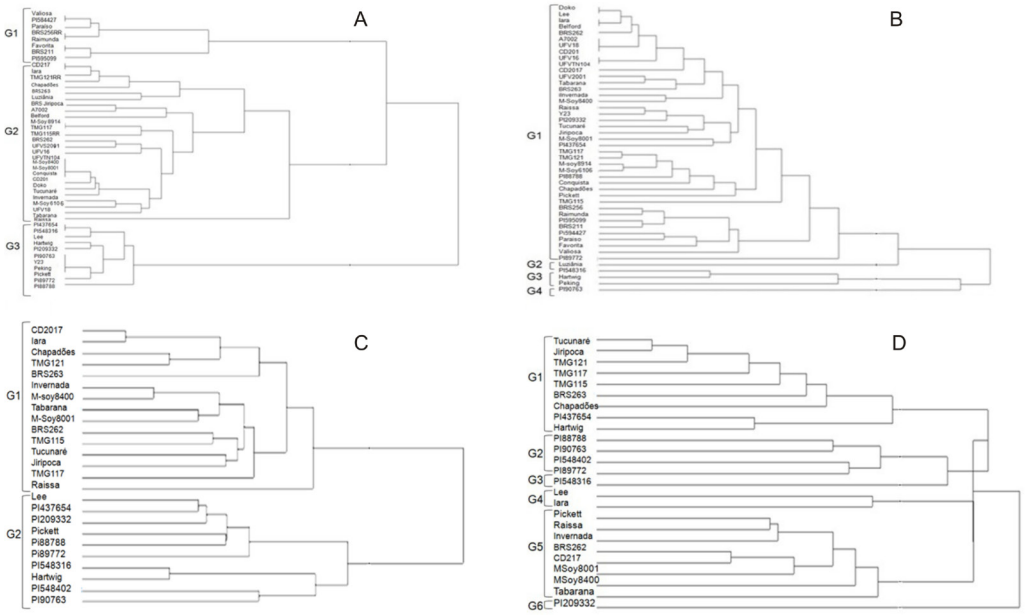

Figura 3. Clustering of 46 soybean genotypes resitant to nematodes via RGA markers obtained by Leister et al. (1996) (**A**) or Shen et al. (1998) (**B**). Clustering of 25 soybean genotypes resitant to SCN by RGAs markers (**C**) and SSR markers linked to QTL for resistance to SCN (**D**).

The initial grouping (Figure 1) was maintained with the primers s1, s2, As1, As2, and As3 (Figure 3A). Polymorphisms generated by combinations of the P-loop (P- loop 1, P-loop 2, P-loop 3, P-loop 4, P-loop 5, and P-loop 6) and GLPL (LM637, GLPL1, GLPL3, and GLPL4) primers did not maintain the subgroups observed in the clustering using all primers (Figure 3B). However, the group of genotypes with resistance to the genus *Meloidogyne* spp remained the same in all analyses, showing that the two primer sets assessed similar regions in the genome of these genotypes.

In addition to the grouping of genotypes, fragments amplified by RGA were also clustered in order to facilitate the identification of contrasting genomic regions between the groups of genotypes that were resistant and susceptible to the different nematodes (Figure 1, horizontal grouping).

Fragments common to all genotypes were obtained with the following combinations: F1/As2 (150 and 500 bp), S2/LM637 (500 bp), S2/As2 (500 bp), S2/As1 (850 bp), P-loop1/GLPL1 (400 and 500 bp), and P-loop1/GLPL3 (100 and 500 bp).

Differential amplicons were detected between the groups of commercial genotypes, those resistant to *Meloidogyne*, and sources of resistance to SCN. For instance, genotypes resistant to nematodes of the genus *Meloidogyne* (BRS Raimunda, BRS 256 RR, PI 594427, BRSGO Paraíso, BRS Valiosa, PI 595099, BRS 211, and BRS Favorita) presented amplicons of 350 bp (F1/As2), 150 bp (s2/As3), and 800 bp (s2/As3) in size (Figure 3, black arrows), which are also found in most of the commercial cultivars. The following fragments did not amplify as sources of resistance to SCN: PI 437654, PI 20332, PI 89772, Pickett, PI 88788, PI 548316, Hartwig, Peking, and PI 90736. Conversely, amplicons generated by the combinations S2/As2 (1050 bp), s2/As2 (1080 bp)

and s2/As2 (700 bp) only amplified in the PIs and in a few commercial genotypes, with most of these being resistant to more than two SCN races (Figure 1).

Cultivars from the group of commercial genotypes resistant or susceptible to the SCN races did not generate bands when amplified with the combinations s2/As2 (200, 1050, 1060 bp), P-loop1/GLPL1 (1020 bp), and P-loop 5/GLPL4 (450 bp), except for the genotypes Iara (R1,3), CD217 (R3 and R to *M. incognita*), TMG121 (R1,3 and MR 14) and Chapadões (R1, 2, 3, 4, 5 and 14). A 500-bp amplicon (s2/As1) was present in genotypes resistant to SCN and in commercial genotypes resistant to at least one race of this nematode (Figure 1, green arrow).

Based on these results, it was possible to observe that in most cases, genotypes resistant to nematodes of the genus *Meloidogyne* differed from those of the other groups by the absence of amplicons. In turn, in the commercial cultivars and PIs, groups of differential amplicons (absent in one group and present in another) could be verified, showing that the NBS domain is also important for SCN resistance. This will add to the literature, as genes for SCN resistance, *rhg1* and *Rhg4*, are described as belonging to the resistance gene class LLR-TM Kinase, and are therefore distinct from the NBS domain, which was the target of the present study.

To investigate the association between the fragments amplified by RGAs and QTLs of SCN resistance, RGA data from 25 genotypes with resistance and susceptibility to SCN were compared with data produced by SSRs associated with QTLs for resistance to SCN (Figure 3C and D). In this analysis, genotypes related to resistance to the genus *Meloidogyne* were excluded. On the basis of microsatellites, genotypes with resistance to races 1, 3, and 14 were distinguished from genotypes with resistance to races 1 and 3, and also from the sources of resistance to SCN (Figure 3D). Moreover, QTLs for race 14 permitted separation of the commercial genotypes.

The cultivar Iara (resistant to races 1 and 3) was the most divergent of the commercial varieties, and was grouped with the susceptibility pattern Lee. In comparison, in the analysis of RGAs, the sources of resistance were separated from the commercial cultivars, but without distinction of groupings per race (Figure 3C), indicating that genes specific for race 14 were not assessed in this analysis.

DISCUSSION

The conservation of domains present in resistance genes in various taxonomic groups enables R genes in the genomes of various species to be validated (Shen et al., 1998). Studies using RGA markers have successfully amplified sequences associated with resistance in various plant species (Soriano et al., 2005; Gururani et al., 2012). In the present study, primers based on the NBS domain of the resistance genes *RP2*, *L6*, and *N* were used (Leister et al., 1996). These oligonucleotides generated polymorphisms in nematode-resistant and nematode-susceptible genotypes, resulting in clear differentiation between those resistant to SCN and those resistant to *Meloidogyne* spp. Kang et al. (2012) reported approximately 314 genes with NBS domains within the soybean genome that are associated with pathogen resistance, demonstrating the variability of these regions and the need to differentiate genomic regions that are associated with specific pathogens.

Analysis of RGA revealed that there was close proximity between genotypes, such as the cultivars BRS Valiosa and BRS256RR, which have cultivar Conquista in common in their genealogy. Therefore, the application of these markers in genealogy and inheritance studies is demonstrated.

Group 1 was formed by PIs, which are important sources of resistance to SCN. This was also noted by Vuong et al. (2015), who placed PIs (PI 88788 and PI 437654) within the same subgroup. This suggests that the sources of resistance have common progenitors, which contribute to the narrowing of the genetic basis of pathogen resistance. This finding is concerning, because the use of a common set of genotypes as a source of resistance has resulted in genetic modifications within populations of cyst nematodes, leading to "resistance breakage" (Vuong et al., 2015). Therefore, research that explores new sources of resistance and identifies new R genes is necessary.

Studies on the genetics of resistance to *H. glycines* initially identified five genes in the soybean genome that are involved in the defense against SCN. The genes *rhg1*, *rhg2*, *rhg3*, *Rhg4*, and *Rhg5* are found in PIs and are sources of resistance, and these include Peking (*rhg1*, *rhg2* and *rhg3*) and PI 88788 (*Rhg4* and *Rhg5*). These genes are distributed in more than 18 QTLs associated with resistance and have been widely studied. Despite their importance in resistance to *H. glycines*, *rhg1* and *Rhg4* do not fit in the classes of R genes containing NBS domains, and instead possess LRR-TM and Kinase domains (Williamson and Kumar, 2006). The fragments generated in this study are not derived from these genes, but rather from other sequences that comprise the P-loop and GLPL motifs in the NBS domain. This observation highlights the interesting findings of the present analysis, including the cloning and characterization of these fragments in SCN resistance.

Most of the genotypes resistant to SCN are susceptible to nematodes of the genus *Meloidogyne* spp, just as those resistant to *Meloidogyne* are susceptible to SCN. The heat map analysis permitted the identification of fragments that are present in genotypes resistant to SCN (F1/As_200 bp; S2/As2_100 bp; S2/As1_500 bp), but absent in genotypes resistant to *Meloidogyne.* Furthermore, amplicons that are only present in genotypes resistant to *Meloidogyne* spp, but are absent in genotypes resistant to SCN (F1/As2_350 bp; S2/As3_150 bp and 800 bp) were identified. It is suggested that these fragments are associated with resistance to the nematodes studied here. Amplification of both sets of bands [those amplified only in genotypes resistant to SCN (F1/As_200 bp; S2/As2_100 bp; S2/As1_500 bp) or only in genotypes resistant to *Meloidogyne* spp (F1/As2_350 bp; S2/As3_150 bp and 800 bp)] in commercial genotypes may require further resistance sequences that contain such motifs, which are not related to these nematodes, to be studied.

The genotypes of the group composed by PIs presented differential fragments that were obtained by the primer combination s2/As2 (of 1050, 1080, and 700 bp). These sequences are present in most of the PIs and are rarely present in commercial genotypes resistant to more than one cyst nematode race, suggesting an association with resistance to SCN.

The R genes identified against *Meloidogyne* spp possess the NBS domain (Williamson and Kumar, 2006; Gururani et al., 2012), which was also amplified in the present study. For resistance to the nematodes of this genus, the RGAs were able to discriminate between resistant and susceptible genotypes, without differentiation for resistance between species of *M. incognita*, *M. javanica*, and *M. enterolobi.* These data indicate that, for resistance to *Meloidogyne* spp, some factor related to the NBS domain confers ample resistance to the nematode, which is supported by reports showing that a small number of genes of greater effect control such resistance (Barbosa-da-Silva et al., 2005).

The narrow genetic basis of resistance to *Meloidogyne* spp originates from the use of the cultivar Bragg as a source of resistance for commercial genotypes, which hinders their differentiation (Dias et al., 2010). Hence, the cultivar Bragg should be used as a control for

resistance to *Meloidogyne*, and for a source of resistance. Conversely, in *H. glycines*, many genes are involved in the resistance response in soybean, and inheritance of these genes is considered to be quantitative (Concibido et al., 2004). This way, the sources of resistance were differentiated based on the possession of regions distinct from those involved in SCN resistance.

The genotypes PI 595099 and PI 594427 are widely used in breeding programs (Beneventi et al., 2013), and are resistant to SCN and to *Meloidogyne* spp. Such genotypes are grouped together with the cultivars BRS Raimunda, BRS Valiosa, BRS Paraíso, BRS Favorita, BRS211, and BRS256RR, which are all resistant to *Meloidogyne* spp.

All primer classes used in this study (As, S2, P-loop, and GLPL) amplified in PI 595099 and PI 594427. The primers s1 and s2 amplified the P-loop motif, whereas As1, As2, and As3 amplified the GLPL motifs. The primers P-loop 1, P-loop 2, P-loop 3, P-loop 4, P-loop 5, and P-loop 6 (Kinase or P-loop motif), in association with LM637, GLPL1, GLPL2, GLPL3, GLPL4, and GLPL5 (GLPL motif), likewise amplified the P-loop and GLPL motifs, which are both from the NBS domain. Nevertheless, in the individual primer groupings (Figure 3), the GLPL/P-loop primer combinations were decisive in grouping the genotypes resistant to *Meloidogyne* spp. As observed following clustering with such primer sets (P-loop/GLPL), this was the only group that did not change when clustered using all primer classes (As, S2, P-loop, and GLPL). Therefore, the P-loop/GLPL combinations are important as they assess genome regions that are directly related to soybean resistance to the studied *Meloidogyne* species.

The relevance of primers containing P-loop motifs was confirmed by the heat map, from which we can infer the main primer combinations involved in the differentiation of genotypes. The set s1/As1 (500 bp) is given as the differential combination between the genotypes resistant to *Meloidogyne* spp and those resistant to SCN, since this band does not appear for genotypes resistant to *Meloidogyne* spp but is present in genotypes resistant to SCN, and in commercial cultivars that are resistant to at least one race.

The clustering based on SSR data grouped genotypes with resistance to races 1 and 3 with those resistant to more than two races; this grouping was not obtained with RGAs. This indicates that the latter did not amplify all the genes associated with resistance to this nematode, and thus did not permit race specificity to be identified. QTLs of greater effect have been described for resistance to SCN, including GL G from the *rhg1* gene, which confers most of the resistance to race 3 of SCN, and various QTLs of smaller effect may be associated with race specificity (Concibido et al., 2004, Kadam et al., 2016). Therefore, it is presumed that genes originating from loci of smaller effect were not amplified in this study, as it was not possible to identify resistance to the different races of SCN. Hence, the group of genotypes resistant to more than one nematode race and the group of genotypes acting as sources of resistance to SCN were clustered more closely due to possible amplification of QTLs of smaller effect. This increased the distance of this group of genotypes, which possess resistance to only two nematode races.

It can be inferred that the analyzed QTLs help in determining associations of races specific to the SCN. While the RGAs used here (based on the NBS domain) did not confer race-specific resistance to cyst nematodes, they are associated with QTLs of generally greater effect. Therefore, the results of the present study show that specific motifs of the NBS domain amplified well in cultivars resistant to *Meloidogyne* spp, allowing the discrimination of genotype groups with regard to resistance to the studied nematodes. In addition, analyses with SSRs were more informative than those with RGAs, as SSRs discriminated genotypes based on their resistance to the different SCN races. Conversely, the heat map data permitted the identification of fragments

associated with resistance to SCN, which are responsible for the distinction between resistant and susceptible genotypes. Finally, GLPL motifs, which are amplified by the primer set P-loop/GLPL, are directly associated with resistance to *Meloidogyne* spp.

The knowledge gained through these findings will help further subsidized breeding programs, thereby providing information on new candidate genes and increasing knowledge regarding R genes known to confer resistance to the nematodes studied.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

We would like to thank FAPES (Fundação de Apoio à Pesquisa do Espírito Santo), CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico), CAPES, and Federal University of Espírito Santo for financial support.

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