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Marker-assisted selection for the rice blast resistance gene *Pi-ar* in a backcross population

Leila Garcês de Araújo[1*], Anne Sitarama Prabhu[2], Pedro Antonio Arraes Pereira[1], and Gisele Barata da Silva[3]

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ABSTRACT - *A doubled-haploid (DH) population, obtained by anther culture of F_1 plants from a cross between a highly susceptible rice cultivar* Lijiangxintuanheigu *and the resistant somaclone (SC09), of the cultivar Araguaia, was used to identify RAPD markers linked to the blast resistance gene Pi-ar. The 86 DH plants, inoculated with the race IB-9 of* Magnaporthe oryzae, *segregated in 1:1 ratio of resistant and susceptible plants. Of the 67 primers used 31 produced DNA profiles that differentiated resistant and susceptible bulks as well as the parental cultivars. The resistance gene was found linked to the primer $OPS16_{2072}$ ('AGGGGGTTCC') at a distance of 3.6 cM. The selection efficiency of this primer was assessed in a BC_3 F_1 population derived from another cross between a susceptible cultivar IAC 201 and SC09. The marker OPS16 showed efficiency of 86.9%, when six resistant and two susceptible plants were considered as negatives in RAPD analysis.*

Key words: Anther culture, double haploid, *Magnaporthe oryzae*, *Oryza sativa*, molecular markers.

INTRODUCTION

Rice blast caused by *Magnaporthe oryzae* B. Couch [anamorph-*Pyricularia oryzae* Cavra] is a major rice disease in different parts of the world, despite the continuous efforts of breeding for blast resistance. The disease causes significant grain yield losses under upland conditions mainly in west-central Brazil. An unprecedented rice blast outbreak was reported in the 2004/2005 rice growing season in a newly released cultivar Colosso. Some farmers could not even harvest a single bag of rice (Prabhu et al. 2009). Somaclones of the upland rice cultivar conferring vertical resistance to blast have been successfully obtained from susceptible rice cultivars Araguaia, utilizing immature panicles as an explant source. The dominant gene that conferred resistance to the IB-45 race of *M. oryzae* in these somaclones was designated as *Pi-ar* (Araújo et al. 1999). The Random amplified polymorphic DNA (RAPD) marker OPC 02 was found to be tightly linked (1.7cM) to the *Pi-ar* gene from the somaclone SC09 (Araújo et al. 2002). SC09 exhibited a resistance reaction to all 282 *M. oryzae* isolates of 15 different races (Araujo et al. 2004).

The availability of different molecular markers allows characterization of genes of interest. RAPD analysis was widely used to identify markers tightly linked to blast resistance genes (Araújo et al. 2002, Araújo et al. 2004). Bulked segregant analysis by Michelmore et al. (1991) was used for increasing the efficiency of RAPD markers linked to blast resistance gene to *M. oryzae* in rice whereas Chesquière et al. (1997) used DH populations for *Rhynchosporium*

[1] Departamento de Biologia Geral, Universidade Federal de Goias, 74001-970, Goiania, GO, Brazil. *E-mail: leilagarcesaraujo@gmail.com
[2] Embrapa Arroz e Feijao, C.P. 179, 75375-000, Santo Antonio de Goias, GO, Brazil
[3] Departamento de Fitopatologia, Universidade Federal de Vicosa, 36570-000, Vicosa, MG, Brazil

secalis, in oats. According to McCouch et al. (1994) DH populations derived from anther cultures provide the advantage of fixed lines after only one generation of meiosis, but can only be produced efficiently for some rice genotypes, whereas recombinant inbred (RI) populations obtained by single-seed descent requires seven or more generations of meiosis.

Marker-assisted selection (MAS), using Restriction fragment length polymorphism (RFLP) as well as RAPD has been utilized incorporating major genes through backcross (Chen et al. 2000), as well as pyramiding blast resistance genes (Hittalmani et al. 2000), and bacterial blight resistance in rice (Toenniessen et al. 2003). Due to its low reproducibility of RAPD markers SCAR is being utilized for identifying genes linked to rice blast resistance (Zhuang et al. 1998, Naqvi and Chatto 1996, Sandhu et al. 2003).

The objectives of the present investigation were to identify the RAPD markers linked to the known blast resistance gene *Pi-ar* in a doubled-haploid population of a somaclone SC09 of the rice cultivar Araguaia, and assess its selection efficiency in a backcross population between the susceptible cultivar IAC 201 and the resistant somaclone.

MATERIAL AND METHODS

Doubled-haploid plant material

A doubled-haploid population was obtained by anther culture of F_1 plants from a cross between susceptible rice cultivar *Lijiangxintuanheigu* (LTH) and the blast resistant somaclone SC09 of the cultivar Araguaia in Embrapa Rice and Bean Research Center, Santo Antonio Goias,GO, Brazil. Callus induction was performed using the immature panicles as an explant source. Rice culms were collected before complete emergence of the flag leaf when the distance between the flag leaf and penultimate leaf collar was 3.0 to 6.0 cm and incubated for eight days at 4 ºC. The panicles were sterilized for 15 minutes with 10% of sodium hypo chlorite solution and washed three times with sterilized distilled water. The spikelets aseptically dissected from the panicles were transferred to Petri dishes containing N6 culture medium for callus induction, supplemented with 1.0 mg L^{-1} of naphthalene acetic acid (NAA); 0.25 mg L^{-1} of picloran; 30.0 g L^{-1} of sucrose; 8.0 g L^{-1} of agar and pH adjusted to 5.8. The anthers were incubated in dark at 26 ºC for 4-8 weeks.

The induced calluses were transferred to plates containing an MS regeneration medium, supplemented with 0.5 mg L^{-1} NAA; 3.0 mg L^{-1} kinetin 40.0 g L^{-1} sucrose; 8.0 g L^{-1} agar and pH adjusted to 6. The cultures were maintained in growth chamber at a temperature of 26 ºC with 16 hours light intensity of 75 $\mu E\ m^{-2}\ s^{-1}$. The calluses with green islands were sub-cultured at 30-day intervals until the seedlings attained a 10 to 15 cm length. They were transplanted into pots containing 6.0 g of soil fertilized with 2.5 g of NPK (4-30-16) and 0.25 g of micro nutrients (FTE-BR12). The plants were maintained in greenhouse until maturation. Seeds were harvested from 97 individual DH plants with spontaneous chromosome duplications, identified based on the formation of fertile panicles and reduced tiller number.

Blast disease assessment

Disease evaluation was made by artificial inoculation tests in the greenhouse and under natural field conditions in the rice blast nursery. The progenies of 86 DH plants were sown in 10 plastic trays (30 x 15 x 10 cm) containing 3 kg soil fertilized with 5.0 g NPK (5-30-15), 1.0 g zinc sulfate and 2.0 g ammonium sulfate at planting. Ten rows with 12 seeds per row were sown per tray. An additional 2.0g ammonium sulfate was applied 20 days after planting. In another tray the parents and eight standard international differentials were sown for the identification of race.

Twenty one day-old plants were inoculated by spraying aqueous spore suspension ($3x10^5$ spores mL^{-1}) of the single spore isolate (race IB-9) of *M. oryzae* using De Vilbiss connected to a compressor maintaining uniform pressure Following inoculation, the trays were incubated for 24 hr in humid chambers and later they were maintained at high humidity (70-90%) with an average temperature ranging from 26-30 ºC. The leaf blast was assessed nine days after inoculation using the visual rating scale of 0, 1, 3, 4, 5, 7 and 9. The plants showing scores 0 to 3 were considered resistant (incompatible) and 4 to 9 as susceptible (compatible) according to the Standard Evaluation System for Rice developed by IRRI in 1988. The score 3 is a resistant lesion type characterized by small round to slightly elongated necrotic spots of 1 to 2 mm in diameter. Type 4 is a typical susceptible 3 mm long sporulating lesion.

In the inheritance study, the frequencies of the classes obtained were tested for significance by χ^2 (Araújo et al. 1999).

One plant from each one of the 86 progenies was transplanted to pots containing 6.0 kg of soil, for harvesting the seed. The disease reaction of these plants to the same isolate of *M. oryzae* was confirmed, in the next generation.

RAPD analysis

Leaves of parents and 86 DH plants, at four-leaf stages were collected and macerated in liquid nitrogen, a week after the disease assessment. Total DNA was extracted using the CTAB method as described by Doyle and Doyle (1987). DNA amplification was performed as described by Williams et al. (1990). The 25 µL reaction utilized contained 25 ng DNA, 2.5 µL 10X buffer reaction (200 mM Tris - HCl, pH 8.4 and 500 mM of KCl), 0.75 µL 50 mM $MgCl_2$; 0.5 µL dNTP (10 mM each dATP, dGTP, dCTP and dTTP); 1.0 µL 5.0 pmol primer (Operon Technologies, Boulevard, CA, USA); one unit Taq polymerase (Gibco), overlaid with 50 µL mineral oil to prevent evaporation. The enzymatic amplification was performed in a thermocycler (MJ Research, model PTC-100-60), programmed for 40 cycles. Each cycle was composed of: 15 sec. at 94 ºC; 30 sec. at 36 ºC and 1 min. at 72 ºC. After 40 cycles an extra extension step was performed for 7 minutes at 72 ºC. Amplification products were separated by gel electrophoresis on 1.4% agarose gel in a TBE buffer (90 mM Tris-borate and 2 mM EDTA). Five mL of 1% ethidium bromide was added to one hundred mL of agarose. DNA bands were photographed under ultraviolet light, utilizing a photo documentation system, Eagle Eye II (Stratagene).

For the detection of primers showing polymorphism in the parents, initially 577 primers were tested utilizing 29 kits of Operon Technologies: OPA, OPB, OPC, OPE, OPG, OPK, OPX, OPY, OPZ, OPAB, OPAD, OPS, OPP, OPD, OPF, OPI, OPL (except OPL04), OPT, OPJ (except OPJ06), OPM, OPO, OPQ (except OPQ04), OPR, OPU, OPAS, OPAX, OPAT, OPAP e OPAY. Some selected primers exhibiting polymorphism in parents were tested in two bulks, one resistant and the other susceptible, each one containing DNA from seven individual plants of doubled- haploids. The PCR reaction was performed one time.

The linkage between the marker and *Pi-ar* was confirmed, utilizing 86 double haploid plants. The χ^2 test was employed to compare the observed and expected genotypic proportions. The distance was estimated utilizing the Mapmaker III program (Lander et al. 1987). A log of the likelihood ratio (Lod) score minimum of 3.0 and the function of Kosambi were used.

Marker-assisted selection

A population composed of 66 plants of BC_3F_1 derived from a cross between a susceptible cultivar IAC 201 and a resistant somaclone SC09 was utilized, for marker-assisted selection. In each backcross, only the plants exhibiting resistant reaction to blast were utilized in crosses with the recurrent parent. The inoculation and evaluation procedure, with the single spore isolate (race IB-9) was the same as described in the item of disease assessment.

For DNA extraction, the leaves from 61 plants of BC_3 F_1, IAC 201 and SC09 were collected from 40-day old plants. The same DNA extraction and amplification procedures described earlier were utilized.

RESULTS AND DISCUSSION

Doubled-haploids

The frequencies of callus induction, regeneration and albinos were 75.5%, 12.6% e 7.2%, respectively, among 15,600 anthers transferred to Petri plates. The seed from 86 DH plants was harvested out of 193 plants regenerated. The chromosome duplication was natural, the frequency of spontaneous diploidization being 50.2%. The tillering of the haploid plants was high and did not produce seeds, whereas the doubled-haploid plants had few tillers and failed to produce seeds. A large number of resistant cultivars posses unknown multiple genes and other traits of interest for mapping and marker-assisted selection. It is difficult to evaluate resistance genes based on phenotypic reaction because of the epistatic and environmental effects in F_2 populations. For this reason complex resistance can be effectively analyzed utilizing fixed populations. One of these methods of obtaining fixed populations is anther culture, where the fixation of doubled-haploid occurs in meiosis. Each plant in a DH population is homozygous and can be multiplied by selfing (McCouch et al. 1994).

The 86 doubled-haploid plants, inoculated with the race IB-9 of *M. oryzae*, segregated in 1:1 ratio of resistant and susceptible plants. The resistant plants

showed reaction type 1 and the susceptible plants disease scores were 5, 7 and 9 as expected for monogenic rice blast resistance, in a doubled-haploid population (Table 1). In the next generation the progenies of 86 doubled-haploid plants maintained the proportion of one resistant and one susceptible (1:1) for the race IB-9 of *M. oryzae*, indicating the expected homozygosis in a doubled-haploid population. In the present study for blast resistant reaction using one isolate of *M. oryzae* the lines showed either resistant or susceptible reaction and no intermediate reactions were observed. The ratio of susceptible and resistant plants was as expected.

RAPD markers and linkage analysis

One hundred and seventy six primers differentiated parents LTH and the somaclone SC09. Of the 67 primers tested in susceptible and resistant bulks of DH plants, 31 showed polymorphism. DNA of seven different individual plants of each one of the bulks was tested using 31 primers. Of the 31 primers that amplified the RAPDs in a bulk specific manner five, OPS16 (Figure 1), OPC15, OPA17, OPG06 and OPAB02 produced one band that was present in the resistant parent, resistant bulk and in all seven individual plants that constituted the resistant bulk. The band was absent in susceptible parents, susceptible bulk and the seven individual plants that constituted susceptible bulk.

For the confirmation of association between five RAPD markers and the gene *Pi-ar*, the segregation of amplified products was monitored in 86 DH plants (Table 1). The primers OPS16, OPC15, OPG06 and OPAB02 confirmed the expected hypothesis of 1:1 (presence and absence of band), as well as phenotypic segregation of 1:1 (resistant:susceptible) for the gene *Pi-ar*. The marker OPS16 was closely linked to the gene at a distance of 3.6 cM and produced an amplified

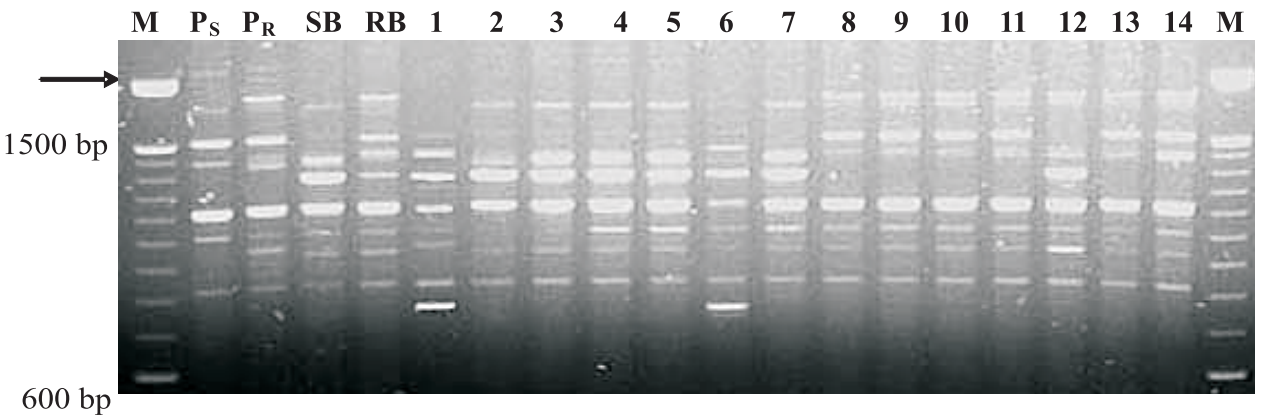

Figure 1. RAPD tagging of *Pi-ar* gene by bulked segregant analysis using primer $OPS16_{2072}$. Columns: Susceptible parental cultivar LTH (P_s), resistant somaclone SC09 (P_r), susceptible bulk (SB), resistant bulk (RB), followed by 14 doubled haploid plants of the cross composed of susceptible plants (columns 1 to 7) and resistant plants (columns 8 to 14). The arrow indicates the marker linked to the resistance gene *Pi-ar* of somaclone SC09 derived from Araguaia to race IB-9 of *Magnaporthe oryzae*. M = Marker (100 bp), Gibco BRL

Table 1. Segregation analysis of *Pi-ar* gene in the doubled-haploid population of cross LTH (susceptible)/SC09 (resistant) to race IB-9 of *Magnaporthe oryzae* and for RAPD markers

Locus	Observed data		Expected ratio	χ^2	Probability	Genetic distance (cM)
	Resistant	Susceptible				
Pi-ar	44	42	1:1[a]	0.04	0.84	-
	Presence of band	Absence of band				
OPS16	41	45	1:1[b]	0.18	0.67	3.6
OPC15	38	48	1:1[b]	1.16	0.28	9.8
OPA17	31	55	1:1[b]	6.69	0.09	-
OPG06	37	49	1:1[b]	1.67	0.19	35.0
OPAB02	46	40	1:1[b]	0.41	0.52	73.3

[a] Expected proportion for monogenic dominant inheritance in the doubled-haploid population (1 resistant: 1 suscetible)
[b] 1:1 (presence of band: absence of band)

fragment of approximately 2072bp. The selection efficiency of the resistant and susceptible plants with the marker OPS16 was 95.3%.

In the present study the homozygous nature of resistant plants was inferred by inoculation with the same race IB-9 of *M. oryzae*. The efficiency of DH was demonstrated for the construction of genetic maps for blast utilizing RFLPs (Chesquière et al. 1997). However, the use of RAPD markers is an alternative in reducing the time and effort in identifying the markers linked to the genes of interest. The success of the RAPD technique lies in the capacity of individual primers to detect multiple loci and a large number of primers can be tested at the same time. The results in the present investigation showed that the marker OPS16 linked to the gene *Pi-ar* at a distance of 3.6 cM can be used for pyramiding or incorporating this gene in other agronomical desirable varieties. The gene pyramiding strategy has been widely advocated for obtaining cultivars with durable resistance (Hittalmani et al. 2000).

Marker-assisted selection

In inoculation tests with the race IB-9 of *M. oryzae*, the BC_3F_1 population segregated into 27 resistant (score1) and 39 susceptible plants (scores 4 to 7) whereas the controls, cultivar IAC 201 and the somaclone SC09 exhibited susceptible (score 7) and resistant (score 1) reactions, respectively. Of 39 susceptible plants only 34 were evaluated with the OPS16 marker, including 27 plants with disease scores 5 and 7, and seven plants with score 4 (Table 2).

The selection efficiency of the marker OPS16 was 85.2% considering six resistant plants and two susceptible plants (scores 5 and 7) as negatives and

Table 2. Selection efficiency of the OPS16 marker to *Pi-ar* gene of the somaclone SC09 in the BC_3F_1 population of cross IAC201/SC09

BC_3F_1 plants	Blast disease score BC_3F_1	Presence (+)/Absence (-) of marker OPS16	Phenotype reaction BC_3F_1
1	1	+	Resistant
2	1	+	Resistant
3	1	+	Resistant
4	1	+	Resistant
5	1	+	Resistant
6	1	+	Resistant
7	1	+	Resistant
8	1	+	Resistant
9	1	+	Resistant
10	1	+	Resistant
11	1	+	Resistant
12	1	+	Resistant
13	1	+	Resistant
14	1	+	Resistant
15	1	+	Resistant
16	1	+	Resistant
17	1	-	Resistant
18	1	-	Resistant
19	1	-	Resistant
20	1	+	Resistant
21	1	+	Resistant
22	1	-	Resistant
23	1	+	Resistant
24	1	-	Resistant
25	1	-	Resistant
26	1	+	Resistant
27	1	+	Resistant

to be continued …

Table 2. Cont.

BC_3F_1 plants	Blast disease score BC_3F_1	Presence (+)/Absence (-) of marker OPS16	Phenotype reaction BC_3F_1
28	4	-	Susceptible
29	4	-	Susceptible
30	5	-	Susceptible
31	5	-	Susceptible
32	7	+	Susceptible
33	5	-	Susceptible
34	5	-	Susceptible
35	7	-	Susceptible
36	5	-	Susceptible
37	7	-	Susceptible
38	7	-	Susceptible
39	5	-	Susceptible
40	5	-	Susceptible
41	5	+	Susceptible
42	5	-	Susceptible
43	5	-	Susceptible
44	5	-	Susceptible
45	7	-	Susceptible
46	5	-	Susceptible
47	5	-	Susceptible
48	5	-	Susceptible
49	5	-	Susceptible
50	5	-	Susceptible
51	5	-	Susceptible
52	7	-	Susceptible
53	5	-	Susceptible
54	7	-	Susceptible
55	5	-	Susceptible
56	7	-	Susceptible
57	4	-	Susceptible
58	4	-	Susceptible
59	4	-	Susceptible
60	4	-	Susceptible
61	4	-	Susceptible
IAC201 [a]	7	-	Susceptible
SC09 [b]	1	+	Resistant

[a] Susceptible parent. [b] Resistant parent

increased to 86.9% when the reaction type 4 was also considered. These results demonstrated the utility of the marker OPS16 in the detection of a resistance gene. The same fragment was absent in the susceptible cultivar IAC 201 and present in the resistant somaclone SC09 (Table 2). The electrophoretic profiles of parents and BC_3 F_1 plants are presented in Figures 2 and 3.

The identification of RAPD markers for blast resistance depends on a cross between two genotypes with distinct differences in resistance and susceptible reaction. In leaf blast evaluation the phenotypic reaction types 3 and 4 are some times confounded. The results in this study showed the efficiency of marker OPS16 in identifying all plants exhibiting susceptible reaction type 4 by the absence of a marker band. On the other hand, the absence of markers in the plants considered resistant could be considered escapes in an artificial inoculation with spore suspension of *M. oryzae*. Similar results were obtained in studies conducted with RAPD marker ANT-$TO1_{830C}$ linked to

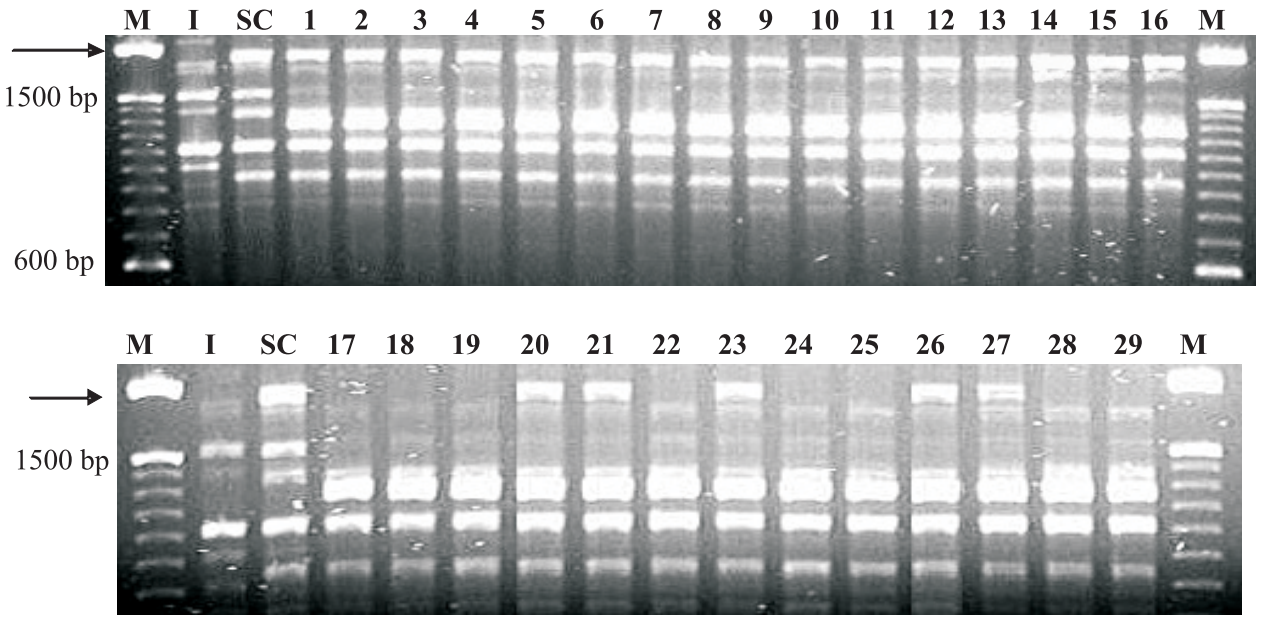

Figure 2. Eletrophoretic analysis of DNA amplification products with the primer $OPS16_{2072}$ of 27 BC_3F_1 resistant plants (1 to 27) and two susceptible plants with score 4 (28 and 29). The arrow indicates the marker linked to the resistance gene *Pi-ar* of somaclone SC09 from Araguaia to race IB-9 of *Magnaporthe oryzae*. M = Lambda Marker 100 pb, Gibco BRL; I = IAC 201; SC = Somaclone SC09)

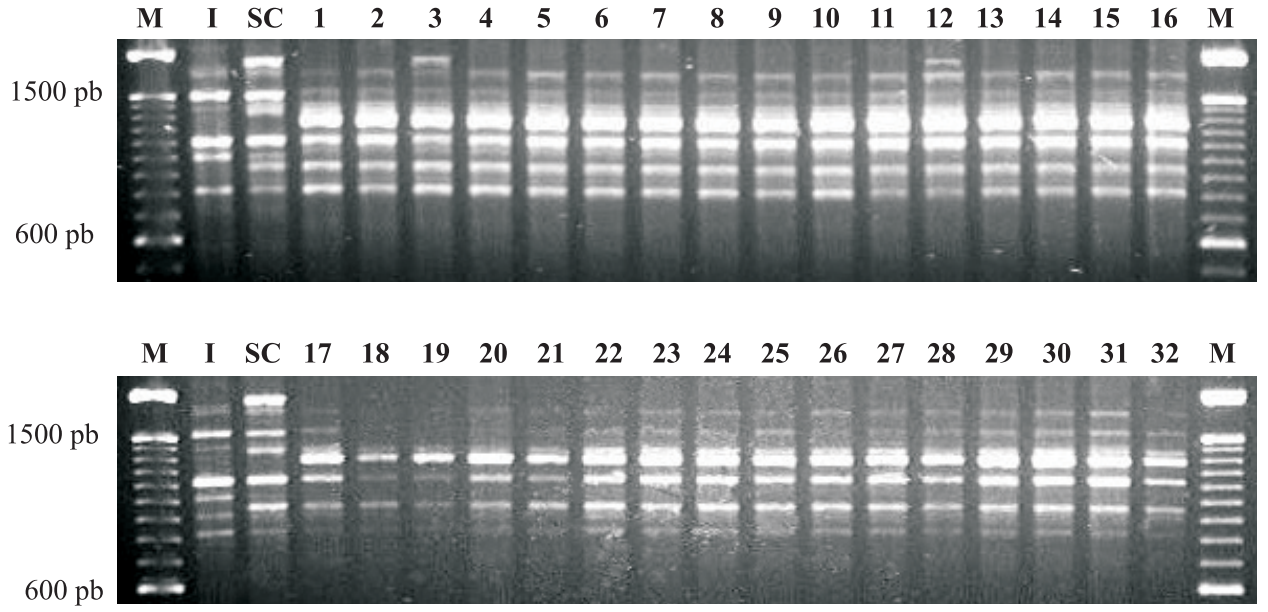

Figure 3. Eletrophoretic analysis of DNA amplification products with the primer $OPS16_{2072}$ of 32 BC_3F_1 susceptible plants, including 5 plants with score 4 (28 to 32) M = Lambda Marker 100 pb, Gibco BRL; I = IAC 201; SC = Somaclone SC09)

the resistance gene *Co-4* in identifying F_2 resistant and susceptible plants based on the disease scores 3 and 4, in relation to bean anthracnose caused by *Colletotrichum lindemuthianum* (Alzate-Marin et al. 1999).

The field selection for resistance is complicated by the presence of a large number of races. The marker-assisted selection can be utilized in the backcross program for identifying the gene of interest against a specific known race and for the rapid recovery of a

recurrent parent (Sharma et al. 2002). Another form of utilizing molecular markers in backcross programs is the individual selection that presents a genetic constitution closer to the recurrent parent. The backcross program with MAS can reduce two to four generations necessary for the recovery of the genetic constitution of the recurrent parent. Due to these advantages the breeders have been successfully utilizing MAS for qualitative traits such as blast resistance (Chen et al. 2000).

The incorporation of the blast resistance gene *Pi-ar* in backcross breeding with MAS utilizing the marker OPS16 has immense value because the gene exhibits a broad spectrum of resistance to a great number of isolates of the pathogen. However, the efficiency of MAS for the incorporation of gene *Pi-ar* can be further increased by the transformation of the marker OPS16 to SCAR in future studies.

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Seleção assistida por marcador para o gene de resistência *Pi-ar* à brusone do arroz em uma população de retrocruzamento

RESUMO - *Uma população de duplo haplóides (DH) obtida através da cultura de anteras de F_1 do cruzamento da cultivar altamente suscetível* Lijiangxintuanheigu *(LTH) e do somaclone resistente (SC09) da cultivar Araguaia foi usada para identificar marcadores RAPD ligados ao gene de resistência à brusone Pi-ar. A população de 86 DH inoculada com a raça IB-9 de* Magnaporthe oryzae*, segregou para uma razão de 1:1 de plantas resistentes e suscetíveis. Dos 67 primers usados, 31 produziram perfis que diferenciaram os grupos resistente e suscetível, bem como as cultivares parentais. O gene de resistência encontrou-se ligado do primer, $OPS16_{2072}$ ('AGGGGGTTCC') à distância de 3,6 cM. A eficiência de seleção para este primer foi avaliada em uma população F_1RC_3 derivada de outro cruzamento entre a cultivar suscetível IAC 201 e SC09. O marcador OPS16 mostrou eficiência de 86,9%, quando foram consideradas seis plantas resistentes e duas plantas suscetíveis como negativas na análise de RAPD.*

Palavras chave: Cultura de antera, duplo haplóide, *Magnaporthe oryzae*, *Oryza sativa*, marcadores moleculares.

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