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RAPD marker linked to a gene conferring resistance to race IB-9 of *Pyricularia grisea* in a somaclone of the rice cultivar Araguaia

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Abstract

The gene *Pi-ar* confers resistance to *Pyricularia grisea* in a somaclone of the upland rice cultivar Araguaia developed from callus culture of immature panicles. The somaclone SC09 exhibited resistant reaction to all of the 182 *P. grisea* test isolates belonging to 15 different races. The study on inheritance showed that the resistance to pathotype IB-9 of *P. grisea* is monogenic and dominant. In order to identify marker linked to this gene, the F_2 population from a cross between the highly susceptible cultivar Lijiangxintuanheigu (LTH) and the somaclone SC09 of rice cultivar Araguaia was screened using RAPD primers. Initially, the polymorphism between parents, the cultivar LTH and somaclone SC09 was analyzed using 577 random 10-bp primers. The susceptible and resistant bulks of the F_2 population, along with DNA of the two parents were tested with 176 primers that differentiated susceptible and resistant parents. Thirty-six primers differentiated the susceptible and resistant bulks, as well as the cultivar LTH of the somaclone SC09. However, one primer OPK17 was found to be closely linked (5.3 cM) to the resistance gene of somaclone and this can be used in the marker assisted selection.

Introduction

Rice blast caused by *Pyricularia grisea* (Cooke) Sacc. [*Magnaporthe grisea* (Hebert) Barr], is the most destructive rice disease under upland conditions in Brazil. Araguaia, the first upland rice cultivar developed for blast resistance was released in 1986. However, the resistance of this cultivar was overcome due to the increased frequency of the virulent race IB-9 of *P. grisea* (Filippi and Prabhu, 2001). The durability of resistant cultivars is known to be limited due to great variability of rice blast fungus (Correa-Victoria and Zeigler, 1993). Despite its short lived nature, genetic resistance is considered as the most economic and efficient disease control measure (Tabien et al., 2000). Breeding for blast resistance requires continuous search for new sources of resistance and their incorporation into commercial rice cultivars constitutes one of the major goals of research. Tissue culture has been widely utilized as one of the tools for the induction of genetic variability for disease resistance in many crops (Larkin and Scowcroft, 1981). A large number of plants resistant to rice diseases were obtained from the susceptible rice cultivars well adapted to local conditions (Araújo et al., 2001). Somaclones of Araguaia, resistant to rice blast were developed from the callus cultures of immature panicles (Araújo et al., 2000). The resistance of this somaclone to the race IB-45 of *P. grisea* was controlled by a dominant major gene and designated as *Pi-ar* (Araújo et al., 1999). The incorporation of this gene by conventional backcross method may be limited due to the presence of other resistance genes and possible epistatic effects (Haley et al., 1993).

Different types of molecular markers have been utilized for indirect selection of characters of interest including disease resistance (Kelly, 1995). The molecular markers linked to known resistance genes facilitate the incorporation and pyramiding of these genes in commercial cultivars (Yoshima et al., 1995). RAPD markers can provide an efficient assay for polymorphism and permit rapid identification and isolation of

specific DNA fragment. They are widely used for gene mapping, gene tagging and plant breeding application because of their simplicity and low cost as an alternative to RFLP analysis (Williams et al., 1990). RAPD markers have been widely utilized to identify the disease resistance loci in different species (Naqvi et al., 1995; Zhang et al., 1996; Zheng et al., 2000). In wheat the efficiency of SCAR derived from RAPD UBC521 linked to the resistance gene to *Septoria nodorum* was tested along with RAPD in accessions that have this gene. The efficiency of SCAR as well as RAPD was shown to be 100% (Cao et al., 2001). The bulked segregant analysis, originally developed by Michelmore et al. (1991), has been shown to be the most effective for marker identification in specific genomic regions. RAPD markers combined with bulked segregant analysis has been utilized for identifying specific markers linked to blast resistance genes in rice (Naqvi et al., 1995; Chen et al., 2000; Zheng et al., 2000). Araújo et al. (2002) in an earlier investigation, identified the primer OPC02 tightly linked (1.7 cM) to the major resistance gene to *P. grisea* race IB-45 in a somaclone SC09 of the cultivar Araguaia.

The present study reports the RAPD marker linked to the gene conferring resistance to pathotype IB-9 of *P. grisea* in a somaclone of upland rice cultivar Araguaia.

Materials and methods

Plant material

The somaclone SC09 developed from a susceptible rice cultivar Araguaia, in an earlier investigation (Araújo et al., 2000), was utilized as a source of blast resistance. The cultivar Lijiangxintuanheigu (LTH) is highly susceptible to all races of *P. grisea*, and supposedly posses no known resistance gene was used as a susceptible parent in crosses with somaclone SC09. The japonica rice cultivar LTH was shown to be susceptible to 1739 isolates of *P. grisea* collected from China (Ling, 1995). Part of the F_1 seed obtained was used for backcross with LTH (BC_1s) and with SC09 (BC_1r). The remaining F_1 plants were self fertilized in a greenhouse for obtaining F_2 population.

Isolates and inoculation procedure

Single spore isolates of *P. grisea* obtained from sporulating lesions of rice cultivars IAC-47 (ECG4F^{1}89), Rio Paranaíba (ECG5F^{3}87 e L1^{2}96), Mars (CLC20L2^{1}94), Bluebele (CLC13L3^{3}94), Carajás (L1^{4}96) and Guarani (L1^{2}96) in experimental plots of Embrapa Rice and Bean Research Center, were established with the objective of identifying isolates virulent to LTH and avirulent to SC09. The stock cultures were conserved in sterilized filter paper discs at (4 ± 1 °C) in the refrigerator.

For inoculation, the test material was planted in plastic trays (30 cm × 15 cm × 10 cm) containing 3 kg of soil fertilized with 5 g of NPK (5–30–15), 1 g of zinc sulfate and 3 g of ammonium sulfate. Top dressing was done 20 days after seeding with 2 g of ammonium sulfate. The plant material was sown (10 and 12 seeds/genotype) in eight 10 cm rows per tray. The standard eight international differentials (Dular, Kanto 51, NP125, Raminad Str 3, Usen, Zenith, Caloro and Sha-tio-tsao), were planted in another tray for race identification.

The inoculation and evaluation procedures utilized were as described by Filippi and Prabhu (2001). The isolates that induced typical sporulating lesions (5, 7 and 9) were considered virulent or compatible and nonsporulating lesions (0, 1 and 3) as incompatible or avirulent according to a scale of 0–9 (Leung et al., 1988). The race was identified based on the reaction type on differentials according to Atkins et al. (1967). The selection of virulent isolate to the cultivar LTH, was based on sporulating lesion number per cm^2 of leaf, 9 days after inoculation.

Resistance spectrum of the somaclone SC09

A greenhouse study was conducted to determine the resistance spectrum of somaclone SC09 along with 31 other rice genotypes such as Araguaia, Bonança, Caiapó, Canastra, Carajás, Carisma, Confiança, Guarani, IAC-47, IAC 201, Maravilha, Primavera, Progresso, Rio Paranaíba, Moroberekan, Dular, Kanto 51, NP125, Raminad Str 3, Usen, Zenith, Caloro and Sha-tio-tsão, C101 LAC, C101 A51, C104 PKT, C101 PKT, C101-TTP-4L-23, Aimoré, and two introduced lines of *Oryza glaberrima* (OG 217 and OG 218), utilizing 182 single spore isolates of *P. grisea*, collected from 22 rice genotypes, during a period of 5 years (1996–2000).

Inheritance studies

The F_1, F_2 and backcross progenies (BC_1s and BC_1r) were assessed for their reaction on individual seedling

basis and F_1 and parents on row basis. Twenty plants of F_1, 671 of F_2 progeny, 31 of BC_1s and 20 of BC_1r were inoculated with the race IB-9 (ECG4F[1]89) of *P. grisea*.

DNA extraction

For the identification of molecular markers linked with the resistance gene, the leaves of parents and 120 F_2 plants, at the fourth leaf stage were collected 1 week after the blast disease assessment. The leaves were macerated in liquid nitrogen and DNA extracted using the CTAB method and stored in the freezer at −80 °C (Doyle and Doyle, 1987).

RAPD analysis

DNA amplification reactions were performed based on the method described by Williams et al. (1990). Each 25 µl reaction contained: 25 ng DNA, 2.5 µl 10× 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, 0.2 µM); one unit Taq polymerase and 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 one denaturation step at 94 °C for 15 s; one annealing step at 35 °C for 30 s and one extension step at 72 °C for 1 min. After 40 cycles an extra extension step was performed for 7 min at 72 °C. The fragments of DNA were separated by gel electrophoresis on 1.4% agarose gel in TBE buffer (90 mM Tris-borate and 2 mM EDTA). One-hundred milliliters of agarose was added to 5 µl 1% ethidium bromide. After the DNA bands were photographed under ultraviolet light, utilizing an Eagle Eye II photo documentation system (Stratagene).

For the identification of RAPD markers in F_2 population, 577 *primers* were tested, including 29 kits: 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 and OPAY (Operon Technologies). These primers were initially utilized for the detection of polymorphism between the parents and later the method proposed by Michelmore et al. (1991). The selected primers based on the detection of polymorphism in the parents were tested in two bulks, one resistant and the other susceptible, each containing DNA of seven F_2 individual plants. The selection efficiency was calculated based on the total number of F_2 individual plants assessed for phenotypic reaction in relation to the presence or absence of marker according to Corrêa et al. (2001).

Linkage analysis

One-hundred-and-twenty F_2 individual plants were utilized to verify the linkage between marker and the resistance gene. Chi-square (χ^2) test for goodness of fit was used to analyze all F_2 and backcross progeny. The distance was estimated using the software MAPMAKER III, with one minimum *lod score* of 3.0, adopting the mapping function of Kosambi (Lander et al., 1987).

Results and discussion

Identification of virulent race of P. grisea

In inoculation tests, utilizing eight standard international differentials with seven isolates of *P. grisea*, five of them were identified as pertaining to the race IB-9 and two to the race IB-45. The cultivar LTH and the somaclone SC09 showed susceptible and resistant reactions, respectively for all tested isolates. The isolate Py 14 (race IB-9) was selected for the studies on inheritance of resistance, because it was more aggressive than other isolates.

Inheritance of resistance

The F_2 population of the cross between the cultivar LTH and the somaclone SC09 segregated at the 3:1 ratio of resistant and susceptible plants, indicating that the resistance to race IB-9 is monogenic and dominant. Backcrosses to the susceptible parent LTH segregated in a 1:1 ratio of resistant and susceptible plants reaction in the backcross with resistant parent SC09 (Table 1).

These results are in conformity with those obtained by Araújo et al. (1999) in earlier studies in relation to the inheritance of resistance of the somaclones SC09, SC10 and SC23 for the race IB-45 of *P. grisea*. Filippi and Prabhu (1996) observed that in the majority of crosses studied the resistance to the predominant races IB-1 e IB-9 of *P. grisea* was dominant and controlled by one or three genes.

Table 1. Segregation of F_1, F_2, BC_1s and BC_1r, populations of cross LTH (susceptible)/SC09 (resistant) to race IB-9 of *P. grisea* and for RAPDs markers

Locus	Population	Observed data		Expected ratio	χ^2	Probability	Genetic distance (cM)
		Resistant	Susceptible				
Pi-ar	F_1	20	0	Resistant	–	–	–
Pi-ar	F_2	508	163	3:1[a]	0.17	0.68	–
Pi-ar	RC_1s	16	15	1:1[b]	0.032	0.85	–
Pi-ar	RC_2r	20	0	Resistant	–	–	–
		Presence of band	Absence of band				
OPK17	F_2	90	30	3:1[c]	0	1.0	5.3
OPS16	F_2	64	56	3:1[c]	15.02	0.0001	–
		Absence of band	Presence of band				
OPK09	F_2	61	59	3:1[d]	37.37	0.0	–
OPAP08	F_2	46	74	3:1[d]	86.03	0.0	–
OPAY16	F_2	40	80	3:1[d]	111.0	0.0	–

[a] Expected proportion for monogenic dominant inheritance in the F_2 progeny (3 resistant:1 susceptible).
[b] Expected proportion for monogenic dominant inheritance in the BC_1s progeny (backcross to LTH).
[c] 3:1 (presence of band:absence of band).
[d] 3:1(absence of band:presence of band).

Resistance spectrum of the somaclone SC09

In inoculation tests, the somaclone SC09 exhibited resistant reaction to all of the 182 *P. grisea* isolates pertaining to 15 different races, indicating its broad resistance spectrum (Table 2). The somaclone developed based on greenhouse tests, with two distinct physiologic races of the fungus, utilizing 200 R_2 plants derived from 10 regenerated plants from the callus cultures of the cultivar Araguaia, referred to as R_1 plants, showed hypersensitive reaction, indicating vertical resistance (Araújo et al., 2000). These somaclones differed from the original parental cultivar in fan-shaped plant type, blast resistance and yield potential. In the advanced R_6 generation, among 20 somaclones highly resistant to blast, both in the greenhouse and field tests, the somaclone SC09 was selected, based on similar yield potential as Araguaia (Araújo et al., 1999, 2000). The expression of blast resistance among the regenerated plants may be attributed to the pre-existing variation present in the original explant resulting from residual heterozygosity of the genotype introduced *in vitro* (Morrish et al., 1990) or mitotic crossing over (Evans et al., 1984).

The race IB-9 was more frequently encountered (53.8%), confirming its reported predominance in the earlier race survey (Filippi and Prabhu, 1996). The somaclone also showed vertical resistance to leaf blast under natural field and in blast nursery tests conducted during two consecutive years (Araújo et al., 2000).

RAPD markers

Of 577 primers tested on resistant and susceptible parents, 523 produced clearly amplified DNA fragments. During this survey, 176 primers differentiated resistant and susceptible parents, of which 36 were specific for the resistant parent and resistant bulk, as well as for the susceptible parent and susceptible bulk. These primers were further used to test the DNA of seven different individual plants from each one of the bulks. Of 36 primers that amplified RAPDs in a bulk-specific manner, two primers OPK17 and OPS16 produced bands present in the resistant parent and in all plants of the resistant bulk. The same DNA fragment was absent in susceptible parent, susceptible bulk and in all seven individual plants that constituted the susceptible bulk. In contrast, from 36 primers, three (OPK09, OPAP08 and OPAY16) produced one DNA fragment that was present in susceptible parent, susceptible bulk and in all seven individual plants of the susceptible bulk. This fragment was absent in resistant parent, resistant bulk and in all seven individuals plants of the resistant bulk.

Table 2. Origin of isolates of *P. grisea* and physiologic races

Origin of isolates[a]	Number of isolates	Races[b]														
		IB-9	IB-41	IB-1	IC-25	IB-45	IC-17	IC-1	IB-11	IB-33	IB-37	IB-13	IC-9	IA-41	IG-1	IF-1
Primavera	53	28	2	–	10	–	7	5	–	–	–	–	1	–	–	–
Maravilha	37	19	14	1	–	–	–	–	2	–	–	1	–	1	–	–
Canastra	07	5	2	–	–	–	–	–	–	–	–	–	–	–	–	–
Caiapó	07	5	–	2	–	–	–	–	–	–	–	–	–	–	–	–
Carajás	07	2	–	1	–	3	–	–	–	–	1	–	–	–	–	–
Progresso	07	3	–	2	–	–	–	–	1	1	–	–	–	–	–	–
Guarani	07	2	1	4	–	–	–	–	–	–	–	–	–	–	–	–
Rio Paranaíba	06	6	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Araguaia	06	4	–	2	–	–	–	–	–	–	–	–	–	–	–	–
IAC 201	06	5	–	1	–	–	–	–	–	–	–	–	–	–	–	–
IAC 47	06	4	–	2	–	–	–	–	–	–	–	–	–	–	–	–
Confiança	05	2	1	–	–	1	–	–	–	–	–	–	–	–	1	–
Carisma	03	1	1	–	–	1	–	–	–	–	–	–	–	–	–	–
Veneza	05	4	–	–	–	–	–	–	–	1	–	–	–	–	–	–
Oryzica L-5	01	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–
CNA$_S$8540	04	2	–	–	–	2	–	–	–	–	–	–	–	–	–	–
CNA$_S$8711	02	–	1	1	–	–	–	–	–	–	–	–	–	–	–	–
CNA$_S$8713	01	–	–	1	–	–	–	–	–	–	–	–	–	–	–	–
CNA$_S$8812	04	2	1	–	–	1	–	–	–	–	–	–	–	–	–	–
CNA$_S$8983	03	–	–	–	–	–	–	–	–	–	–	–	–	–	–	3
CNA$_S$8934	05	3	1	–	–	1	–	–	–	–	–	–	–	–	–	–
Total	182	98	24	17	10	9	7	5	2	2	1	1	1	1	1	3

[a] The isolates were collected from sporulating leaf lesions of cultivars.
[b] The races were identified based on the reaction on standard international differentials.

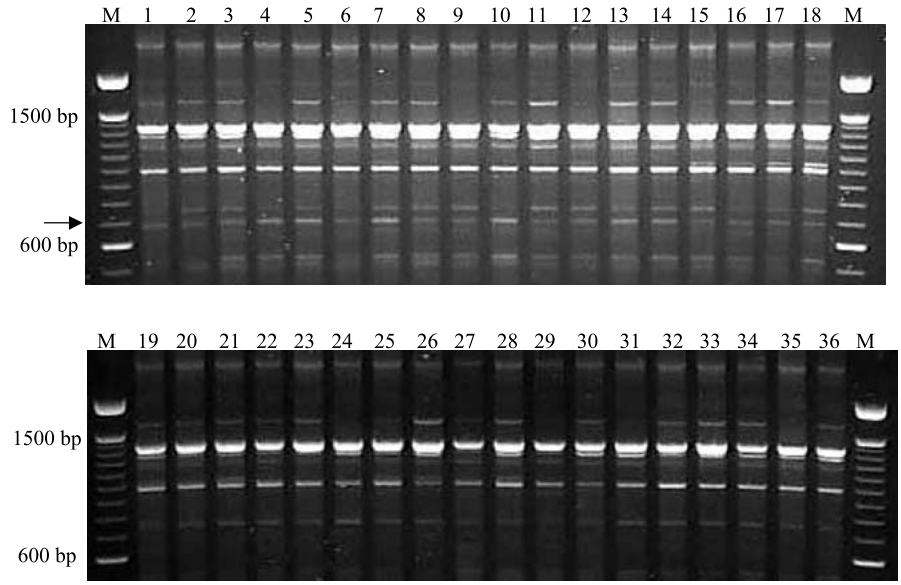

Figure 1. Eletrophoretic analysis of DNA amplification products with the primer $OPK17_{680}$ of 18 F_2 resistant (1–18) and 18 susceptible (19–36) plants. The arrow indicates the marker linked to the resistance gene *Pi-ar* of somaclone SC09 from upland rice cultivar Araguaia to race IB-9 of *P. grisea*. M = Lambda Marker 100 bp, Gibco BRL.

The phenotypic segregation of 3:1 (resistant: susceptible) or (presence:absence) of RAPD band in F_2 plants was consistent with the monogenic dominant inheritance for the gene only for the marker OPK17, indicating its utility as a marker (Table 1). The co-segregation analysis of 120 F_2 plants showed that the marker OPK17 was found linked in coupling phase, at the distance of 5.3 cM. The fragment produced by this primer possesses approximately 680 bp (Figure 1). The efficiency of selection of resistant and susceptible plants with the marker OPK17 was 95%. Two RAPD markers linked to the resistance gene to angular leaf spot of common bean exhibited the selection efficiency of 90.1 and 94.7% (Corrêa et al., 2001). It is interesting to note that the studies conducted with other cultivars with the marker $OPK17_{1400}$ was also linked to the resistance gene to leaf and panicle blast at 2.4 cM (Zheng et al., 2000).

RAPD markers and bulked segregant analysis were used to identify specific markers linked to resistance genes in various pathosystems (Naqvi et al., 1995; Naqvi and Chattoo, 1996; Zheng et al., 2000). However, markers with linkage distance over 10 cM are not used in plant breeding (Kelly, 1995). In rice, the distance of RAPD markers to the resistance genes to blast varied from 2.1 to 7.5 cM (Naqvi et al., 1995; Zhang et al., 1996; Zheng et al., 2000). Based on the co-segregation analysis of 126 F_2 plants of a cross between the resistant somaclone SC09 and the susceptible rice cultivar Araguaia, from which the somaclone was derived, the marker $OPC02_{1200}$ was mapped at 1.7 cM of the resistance gene *Pi-ar*, to the race IB-45 (Araújo et al., 2002). The studies on inheritance showed that the marker identified by primer OPC02 ($\chi^2 = 0.0105$, $p = 0.9183$) was monogenic and dominant. This was the first report of identification of a RAPD marker linked to resistance gene *Pi-ar* in a somaclone derived from a rice cultivar susceptible to blast.

The research was further extended to verify the presence of this gene in the somaclone SC09, in another cross with a variety LTH, which does not posses any known resistance gene and is highly susceptible to all known races. In the initial tests of the present investigation, with the cross SC09 × LTH, we utilized OPC02 to identify the gene in SC09, but failed to obtain positive results. For this reason, we decided to look for another marker in this population, besides the OPC02 with different race IB-9, predominant under upland conditions in Brazil. The relationship between the gene *Pi-ar* identified in the present work utilizing the pathotype IB-9 linked to the marker OPK17, and the other marker OPC02 in an earlier study utilizing the pathotype IB-45 was not determined. However, the same gene offers resistance to both pathotypes in inoculation tests.

In the present investigation, the marker OPK17 has shown linkage to the resistance gene at a greater distance of 5.3 cM in the population LTH × Sc09. The utility of RAPD markers in populations other than those where they were identified depends among other factors, the degree of linkage between the marker and the gene of interest and the genetic similarity between parents utilized in different populations. In general, in the identification of markers and the development of improved varieties, populations from crosses made with different parents are utilized, and this limits the expected impact of marker assisted selection (Tanksley and Nelson, 1996; Faleiro et al., 2003). Despite the limitations in breeding varieties by marker assisted selection involving different parents and populations, the new marker OPK17 may be utilized for the incorporation of this gene in the highly susceptible cultivar LTH, for basic studies on rice blast resistance. Further studies on the marker assisted selection for this gene, in the population derived from crosses with SC09 utilizing different susceptible parents may possibly be improved by transforming the RAPD marker to SCAR.

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