IDENTIFICATION OF RAPD MARKER LINKED TO BLAST RESISTANCE GENE IN A SOMACLONE OF RICE CULTIVAR ARAGUAIA*

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ABSTRACT

The gene Pi-ar confers resistance to *Pyricularia grisea* race IB-45 in a somaclone derived from immature panicles of the susceptible rice (*Oryza sativa*) cultivar Araguaia. RAPD technique was used to identify molecular markers linked to this gene utilizing bulked segregant analysis. Initially, the two parental DNAs from the resistant donor SC09 and 'Araguaia' were analyzed using random primers. Of the 240 primers tested, 203 produced amplification products. The two parental DNAs along with the resistant and susceptible bulks of F_2 population were screened using 48 primers that differentiated resistant and susceptible parents. Even though eight primers differentiated the resistant bulk from the susceptible bulk, as well as somaclone SC09 and 'Araguaia', only one primer, OPC02 ('GTGAGGCGTC'), was found to be tightly linked (1.7cM) to the resistance gene of somaclone SC09.

Additional keywords: molecular markers, disease resistance, somaclonal variation, *Pyricularia grisea*.

RESUMO

Identificação de marcador RAPD ligado ao gene de resistência à brusone no somaclone da cultivar de arroz Araguaia

O gene Pi-ar confere resistência para a raça IB-45 de *Pyricularia grisea* no somaclone derivado de panículas imaturas da cultivar de arroz (*Oryza sativa*) Araguaia. A técnica RAPD foi usada para identificar marcadores ligados a este gene utilizando a análise de misturas segregantes. Inicialmente o DNA do pai doador resistente SC09 e da cultivar Araguaia foram analisados usando primers arbitrários. Dos 240 primers, 203 produziram produtos amplificados. O DNA dos pais e dos bulks resistente e suscetível da população F_2 foram testados usando 48 primers que diferenciaram os pais resistente e suscetível. Enquanto oito primers diferenciaram o bulk resistente do suscetível bem como o somaclone SC09 e Araguaia, somente o primer OPC02 ('GTGAGGCGTC') estava fortemente ligado (1,7cM) ao gene de resistência do somaclone SC09.

INTRODUCTION

The development of blast [*Pyricularia grisea* (Cooke) Sacc.] resistant cultivars is one of the major goals of the Rice Research Institute breeding program, Embrapa Rice & Beans, in Brazil. Upland rice (*Oryza sativa* L.) cultivars with different degrees of blast resistance have been developed over the years. 'Araguaia', the first blast resistant rice cultivar, was developed from a cross between the susceptible cultivar IAC 47 and the resistant Nigerian line TOS 2578/7-4-2-3-B2. 'Araguaia' showed moderate resistance to leaf blast and a high degree of panicle blast resistance when it was released in 1986. However, the resistance of this cultivar was overcome within a period of two years after its release, resulting in considerable losses in grain yield.

The recovery of stable traits such as single gene mutation opens the door for use of cell culture as a tool to induce variation in all seed propagated crops (Evans *et al.* 1984). The artificial culture medium generates a high frequency of stable and heritable variants for different characters including disease resistance that can be utilized for breeding resistant cultivars (Larkin and Scowcroft, 1981). Somaclonal variation, both for qualitative and quantitative characteristics has been exploited in different plant species (Mandal *et al*., 2000). A great number of disease resistant plants have been obtained from susceptible cultivars that are well adapted to local conditions including rice (Xie *et al*., 1990; Araújo *et al*., 2001). Major and recessive gene mutations have been reported in rice somaclones for different characters (Fukui *et al*., 1983; Xie *et al*., 1990).

Somaclones highly resistant to blast were obtained from the susceptible rice cultivar 'Araguaia', and the resistance was shown to be controlled by a single dominant major gene to *P. grisea* race IB-45 (Araújo *et al*., 1999; Araújo *et al*., 2000). These somaclones differed from the original parental cultivar in fan-shaped plant type, blast resistance, and yield potential.

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Different types of molecular markers are available and they are being widely used for indirect selection of traits (Kelly, 1995). The construction of a detailed RFLP genetic map of rice genome facilitated the identification of markers linked to important disease and pest resistance genes. RFLP analysis has been used to map genes for blast resistance and bacterial blight in rice (McCouch *et al*., 1991).

Recently, the simple and cost effective RAPD technique developed by Williams *et al.* (1990) has been widely used as an alternative to RFLP analysis to identify markers linked to resistance genes against diseases caused by fungi, viruses, and nematodes as well as insect pests in different crops (Miklas *et al*., 1993; Penner *et al*., 1993; Carvalho *et al*., 1998). Near-isogenic lines have been utilized to detect RAPD markers linked to Pseudomonas resistance genes in tomato (*Lycopersicon esculentum* Mill) (Martin *et al*., 1991) and to a downy mildew resistance gene in lettuce (*Bremia lactucae* Regel) (Paran and Michelmore, 1993). The bulked segregant method developed by Michelmore *et al.* (1991) has been shown to be appropriate for identification of specific regions of genome and does not require availability of near-isogenic lines. This method is based on comparison of two DNA bulk samples, one homozygous for resistance and other homozygous for susceptibility of a segregating population obtained from a cross, utilizing RAPD markers or other molecular markers. Using a mixture of segregating F_2 population plants, Michelmore *et al.* (1991) identified three RAPD markers in lettuce linked to a resistance gene to downy mildew located at a distance of 25 cM of locus of interest. Miklas *et al.* (1993) used bulked DNA samples formed separately from three resistant and three susceptible individuals from a BC_6 F_2 population of common bean (*Phaseolus vulgaris* L.) in order to identify a RAPD marker tightly linked to the Up_2 locus that condition hypersensitive resistance to *Uromyces appendiculatus* (Reben) Wint. Furthermore, RAPD markers linked to a leaf blotch resistance locus in barley (*Hordeum vulgare* L.) (Barua *et al.* 1993), and a gene conditioning resistance to angular leaf spot of Andean bean line (Carvalho *et al.* 1998) have been identified using bulked segregant analysis. The utilization of molecular markers linked to resistance genes will enable marker assisted pyramiding of genes into susceptible rice cultivars and accelerate breeding program (Yoshima *et al.* 1995).

The present paper reports the identification of RAPD markers tightly linked to the blast resistance gene *Pi-ar* in a somaclone derived from the susceptible rice cultivar 'Araguaia', using bulked segregant analysis.

MATERIALS AND METHODS

Genetic material

The segregating population composed of 126 F_2 plants was derived from the cross between the susceptible commercial rice cultivar 'Araguaia' and its resistant somaclone SC09. In a previous study the monogenic dominant nature of resistance was shown and the gene controlling the hypersensitive reaction in the somaclone SC09 was designated *Pi-ar* (Araújo *et al.* 1999).

Greenhouse inoculation and disease assessment

Plants were grown in plastic trays (30 x 10 x 15 cm) containing 3 kg of soil fertilized with 5.0 g of NPK (4-30-16), 1.0 g of zinc sulfate and 2.0 g of ammonium sulfate at planting. Top dressing was done with 2 g of ammonium sulfate per tray. Inoculations were made on 21 day-old plants with 30 ml of spore suspension per tray (3.0 x 10^5 spores/ml). Spore suspension was prepared as described by Prabhu *et al.* (1992). Each tray had ten rows with about 80 plants of segregating populations. Following inoculation, the trays were incubated for 24 h in humid chambers and later were maintained at high humidity (70-90%) with an average temperature ranging 26-30 ºC. The reaction of parents and F_2 was determined on a row and individual plant basis, respectively. Disease reaction was assessed nine days after inoculation based on a modified 0 to 9 scale of Leung *et al.* (1988), where, 0= no visible symptoms; 1= hypersensitive reaction or brown necrotic specks; 3= few small spindle shaped lesions; 5= several typical sporulating lesions often coalescing; 7= many spindle shaped lesions coalescing, covering 50% or more leaf area; 9= many lesions coalescing, causing partial or total death of plants. (The disease severity ratings 0, 1 and 3 represented incompatible (resistant) reactions, and 5, 7 and 9, compatible (susceptible) reactions.

Genomic DNA extraction

For the identification of molecular markers linked with the resistance gene, leaves of F_2 plant population, at four-leaf stage were collected and macerated in liquid nitrogen using the CTAB method (Doyle and Doyle, 1987). This method consists of macerating 200 mg fresh leaf tissue with liquid nitrogen using a glass rod in 1.5 ml microfuge tubes, following incubation for one hour at 65 ºC with 700 µl of buffer (100 mM Tris-HCl, pH 8.0; 20 mM EDTA; 1.4 M NaCl; 2% CTAB; 1% polyvinyl and 2% of mercaptoethanol). The proteins were removed with a solvent composed of chloroform-isoamyl alcohol in a proportion of 24:1. For RNA digestion, the nucleic acids were precipitated in cold isopropanol, washed with 70% ethanol, dried under vacuum and re-suspended in TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA), containing 10 mg/ml of RNAase A, and incubated at 37 ºC for 30 min. The DNA concentration was estimated by fluorometer and adjusted to 10 ng/µl.

DNA Amplification

DNA amplification reactions were performed as described by Williams *et al.* (1990). Each 25 ml reaction contained: 25 ng of DNA, 2.5 µl of 10 X buffer reaction (200 mM Tris - HCl, pH 8.4 and 500 mM of KCl), 0.75 µl of 50 mM MgCl2; 0.5 µl of dNTP (10 mM of each dATP, dGTP, dCTP and dTTP); 1.0 µl of 5.0 pmol primer, Operon Technologies, Boulevard, CA, USA (0.2 mM); one unit of *Taq* polymerase, overlaid with 50 µl of mineral oil to prevent evaporation.

The enzymatic amplification was performed in a thermocycler (M.J. Research, Inc.), programmed for 40 cycles. Each cycle was composed of one denaturation step at 94 ºC for 15 sec; one annealing step at 35 ºC for 30 sec and one extension step at 72 ºC for 1 min. After 40 cycles an extra extension step was performed for 7 min at 72 ºC. Amplification products were separated by gel electrophoresis on 1.4% agarose gel in TBE buffer (90 mM Tris-borate and 2 mM EDTA) containing 10 mg/ml of ethidium bromide. DNA bands were photographed under ultra violet light, utilizing the photo documentation system, Eagle Eye II (Stratagene).

Construction of bulks and linkage analysis

For identification of RAPD markers in the F_2 population, 240 decamer primers from commercially available primer kits A, B, C, D, E, F, G, I, K, L, M and O (Operon Technologies Inc., USA) were initially utilized for the detection of polymorphism in the parents. The selected primers were tested in the resistant and susceptible bulks, each one containing DNA from seven F_2 individual plants. The linkage between the marker and *Pi-ar* was confirmed, utilizing a 126 F_2 plant population. The distance was estimated utilizing the MAP-MAKER 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. The genetic control of the resistance of rice to *P. grisea* and of RAPD marker was determined and the results were tested by $\chi 2$.

RESULTS AND DISCUSSION

Out of 395 plants of the F_2 population ('Araguaia' x SC09), 290 were scored as resistant and 105 as susceptible, showing segregation ratio of 3:1 (resistant/susceptible) in inoculation tests with *P. grisea* race IB-45 (Figure 1). These results indicate that only one dominant gene confers resistance to race IB-45 ($\chi 2$ = 0.42, P = 0.5169).

Monogenic race specific blast resistance genes are useful to incorporate in susceptible commercial rice cultivars well adapted to local conditions. Identification of specific genes for resistance to specific races is necessary to incorporate resistance in upland rice cultivars. Sequential release of blast resistant cultivars is a traditional recommended strategy in Brazil (Prabhu and Guimarães, 1990). The novel *Pi-ar* gene against the race IB-45, in the somaclone SC09 derived from the susceptible upland rice cultivar 'Araguaia', may be of great value for introgression in parental cultivar or in recurrent selection programs. The progenies from crosses of 'Araguaia' and 'IAC 201' with three resistant somaclones SC09, SC10 and SC23 exhibited resistance to race IB-45 of *P. grisea*. The dominant nature of resistance in the somaclones was determined in previous studies on inheritance (Araújo *et al.*, 1999). The durability of these genes has been widely tested in the blast nurseries located in different parts of the country (Araújo *et al.*, 2000). The conventional backcross method for the incorporation of this gene in the susceptible 'Araguaia'

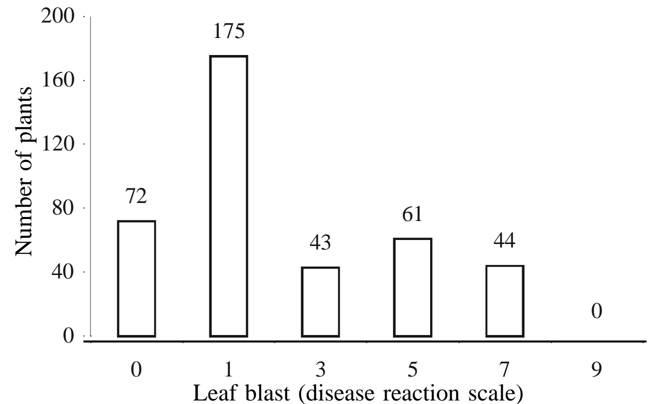

FIG. 1 - Frequency distribution of F_2 plants derived from a cross between the susceptible parent cultivar Araguaia and the resistant somaclone SC09, according to leaf blast ratings in artificial inoculation test with the race IB-45 of *Pyricularia grisea* ($\chi 2$ = 0.42, P = 0.5169). Leaf blast ratings 0 to 3 = incompatible reactions (resistant), 5 to 9 = compatible reactions (susceptible).

is complicated by the presence of other resistance genes. Additional problems in the artificial inoculation method with a specific race include contamination, disease escapes and intermediate reactions (Mackill and Bonman, 1992).

DNA from the resistant and susceptible parents was screened using 240 RAPD primers to identify RAPD fragments linked to the resistance gene in the somaclone SC09. Of the 240 primers, 203 produced amplification products. During this survey we identified 48 RAPD primers that differentiated resistant and susceptible parents, of which eight were specific for the resistant parent and resistant bulk, or for the susceptible parent and susceptible bulk. Such primers were further used to screen the DNA of seven different individual plants from each one of the bulks. Of the eight primers that amplified RAPDs in a bulk-specific manner, only one primer produced an amplification band present in the resistant parent and the resistant bulk, including all seven of the individuals that constituted the resistant bulk. This band was not observed in the susceptible parent or in the susceptible bulk, including all seven individuals constituting the susceptible bulk (Figure 2). This marker possessing approximately 1200 base pairs (bp), was generated by primer OPC02 ('GTGAGGC GTC'). Based on the co-segregation analysis of 126 F_2 plants, the marker $OPC02_{(1200)}$ was mapped at 1.7 cM of the resistance gene *Pi-ar*.

Random amplified polymorphic DNA (RAPD) markers have been widely used as effective tools for the indirect selection of disease resistance genes once the linkages between markers and the resistance genes have been established (Haley *et al.*, 1993; Miklas *et al.*, 1993; Carvalho *et al.*, 1998; Castanheira *et al.*, 1999). However, the linkage distance over 10 cM is not considered useful as a marker in

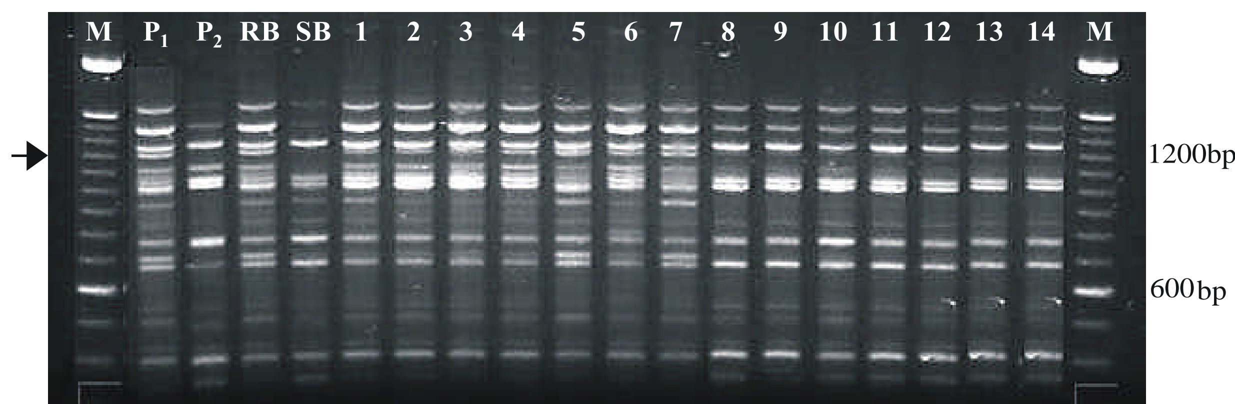

FIG. 2 - RAPD tagging of *Pi-ar* gene by bulked segregant analysis using primer $OPC02_{(1200)}$. Lanes: Resistant somaclone SC09 (P_1), susceptible parental cultivar Araguaia (P_2), resistant bulk (RB), susceptible bulk (SB) followed by 14 F_2 plants of the cross composed of resistant plants (lanes 1 to 7) and susceptible plants (lanes 8 to 14). The arrow indicates the marker linked to the resistance gene *Pi-ar* of somaclone SC09 derived from Araguaia to race IB-45 of *Pyricularia grisea*. M = Marker (100 bp)

breeding programs (Kelly, 1995). In this study the marker OPC02 was shown to be tightly linked to the gene *Pi-ar* (1.7 cM) that can be used for effective indirect selection of this gene. The results were identical when the experiment was repeated.

In general the nature of blast resistance is monogenic dominant (Mackill and Bonman, 1992). The inheritance of the marker identified by primer OPC02 ($\chi 2 = 0.0105$, P = 0.9183) was monogenic and dominant, indicating its viability as a genetic marker. This is 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. Indirect marker assisted selection of this gene may constitute a viable alternative to the conventional breeding procedure.

Breeding for blast resistance in Brazil is conducted for vertical or monogenic resistance to the prevailing races, the duration of which is short due to pathogenic variability of *P. grisea*. Gene pyramiding is another strategy, which is known to reduce crop vulnerability to changes in virulence of the pathogen. The use of traditional methods require differential races, and artificial inoculation of large numbers of progenies, which is both laborious and time consuming. The availability of RAPD markers linked to the known genes will greatly facilitate implementation of the gene pyramiding strategy. Further studies are imminent on the identification of genetic markers for other known resistance genes.

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