Tropical Agricultural Research Vol. 13: 373-379 (2001)

RAPD Markers Linked to Rice Blast (*Magnaporthe grisea* Cav.) Resistant Gene

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ABSTRACT. *A mapping population was constituted by crossing an indica rice cultivar (IR 5690) with durable resistance to blast disease with highly susceptible indica rice cultivar IR 50 and the F9 recombinant inbred lines (RILs) were produced by single seed descent method. The mapping population along with parents were evaluated for blast resistance in the glass house and in the field and categorized based on the reaction scores. In the parental survey, the resistant and susceptible parental lines (IR 5690 and IR 50) were analysed with 65 random amplified polymorphic DNA primers (RAPD). Subsequently resistant and susceptible bulks were constituted and Bulk Segregant Analysis (BSA) was done with 49 RAPD primers, which showed polymorphism in parental survey. Particular fragments of five primers OPAH 18, OPU 15 and OPC 14, OPK 17, OPK 11 were found to be co-segregated with resistant and susceptible phenotypes respectively in BSA. Results on individual RILs survey with the RAPD primer OPAH* 18_{710} *showed a perfect co-segregation pattern with resistant phenotype. This could be used as a molecular marker for identifying resistant phenotypes in a segregating population involving susceptible and resistant blast varieties.*

INTRODUCTION

Rice (*Oryza sativa* L.) is the most important food crop of people in Southeast Asia in general and India in particular. In India, it is cultivated in an area of 41.1 million ha, with an annual production of 84.74 million tonnes. Rice production has several constraints, among which, diseases caused by fungi, bacteria, viruses and mycoplasma-like organisms form a major limiting factor. Among the fungal diseases of rice, blast caused by *Magnaporthe grisea* Cav. is the most devastating disease (Babujee and Gnanamanickam, 2000). Rice growing nations are confronted with serious problem of this disease. In order to prevent this disease. the strategies employed are, breeding for resistant cultivars and chemical protection. Even though chemical protection has been the most widely adopted strategy, higher cost of fungicides, pollution to soil, water and air by the accumulation of obnoxious chemical residues and development of resistant races to the chemicals are reducing the usage of chemicals in the control of disease. Use of resistant cultivars is the most economical and effective way of controlling rice blast. Several dominant genes conferring complete resistance to blast have been identified (Mackill and Bonman, 1992). These objectives can be accomplished by pyramiding conventional blast resistant genes to generate cultivars with durable blast resistance. Phenotypic selection cannot be used to pyramid resistance because the presence of one major gene obscures the effect of other genes. Molecular markers linked to major blast resistance genes offer a powerful tool for marker aided indirect selection of resistance loci in gene pyramiding strategies. Molecular markers have been used to construct genetic maps and for molecular tagging of various agronomic traits in crop species. A number of major blast resistance genes have been mapped with tightly linked RFLP markers (Miyamoto *et al.*, 1996; Rybka *et al.*, 1997),

RAPD markers (Zhu *et al.*, 1993; Naqvi and Chattoo, 1995) and with AFLP markers (Chen *et al.*, 1999). As RAPD technique has the advantages of requiring only small amounts of DNA, and fast being suitable for experiments involving large number of genotypes. The present study was focussed to identify RAPD markers linked to the rice blast resistance gene.

MATERIALS AND METHODS

Mapping population

The Recombinant Inbred Lines (RILs) were developed by crossing, IR 5690 (IR 54745-2-10-17) an indica rice cultivar with durable blast resistance with a highly susceptible indica cultivar IR 50. The F1s were shelfed and forwarded till F9 by single seed descent method. Eighty five RILs chosen, based on phenotypic screening, constituted the mapping population. Plant materials required for RAPD analysis were obtained from the Paddy Breeding Station, Centre for Plant Breeding and Genetics, Tamil Nadu Agricultural University, Coimbatore.

Artificial screening of RILs in glass house

About 100 seeds of each RIL and the parents were sown in 15 × 20 cm mud pots and grown in the glass house. The plants were inoculated by spraying spore suspension from infected leaves and spores from the culture (local isolates) (10^6 conidia ml^{-1}). The pots were covered with polythene cover at night. Plants were scored 14 days after inoculation. Diseased leaf area was visually estimated for all plants from each line. The disease reaction of each line was scored according to the standard evaluation system for rice (SES for rice, 1996) following 0-9 score.

Natural screening of RILs in field condition

All the 85 RILs and the parents were sown in the nursery beds in sandwich method. Test entries were sown in single rows in raised beds bordered by susceptible cultivars TN 1, IR 50, MDU 5 on all sides to enhance the natural inoculum. The percentage of diseased leaf area was visually estimated 14 days after sowing and disease reaction was scored according to the standard evaluation system. Score was cross verified with previous years' data and 85 RILs were categorized as resistant for plants with score 2 and 3 (<30% of Percentage Disease Index (PDI)), susceptible for plants with score 9 (90-100% PDI), moderately resistant for score 5 (30-50% PDI) and moderately susceptible for score 7 (50-70% PDI).

RAPD analysis

Genomic DNA from parents was obtained from frozen leaf tissues following modified CTAB extraction procedure (Nagarajan and Kumar, 2001). The final concentration was adjusted to 25 ng/µl, and 65 random primers (Operon Technologies Inc., Alabama, USA) were used to identify polymorphism between the parents. All the primers

had a G-C content of 60-70%. PCR reaction was conducted in a volume of 18 µl containing genomic DNA (75 ng) 10 mM Tris buffer, 2 mM $MgCl_2$, 12 mM each of dATP, dCTP, dGTP and dTTP, 7.2 pm of primer and 1.8 units of Taq DNA polymerase. The PCR reaction was performed on a Gene Amp system 2400 (Perkin Elmer, Norwalk USA) with 35 cycles of 1 min at 92°C, 1 min at 37°C and 1 min at 72°C with initial denaturation for 2 min at 92°C and final extension at 72°C for 5 min. PCR amplified product was mixed with 1 µl of 6× loading buffer and was run on 1.5% agarose gel at 60 volts for 4-6 h. Gels were stained with ethidium bromide and documented using Alphaimager 1200 (Alpha Innotech Corporation, California, USA). The banding patterns were scored for each RAPD primer.

Bulked segregant analysis

Taking equal quantities of genomic DNA from each of 10 blast resistant RILs and 10 blast susceptible RILs, the resistant bulk and the susceptible bulks were constituted respectively. The two bulks and two parents were screened with the 49 RAPD primers which revealed polymorphism in parental survey to screen the segregation of bands with phenotypes in parents and the bulks. Individuals used for making bulks were screened with RAPD primers which gave phenotype specific bands in both parents and respective bulks using same temperature profile and same PCR ingredients. Products were run in 1.5% agarose gel to observe the co-segregration of particular bands with respective phenotypes, and each band was scored for its presence or absence.

RESULTS AND DISCUSSION

Screening for blast resistance

Eighty five RILs along with the resistant parent IR 5690 (IR 54745-2-10-17) and susceptible parent IR 50 were screened artificially in laboratory conditions and naturally, under field conditions. Scoring for blast was done 14 days after inoculation by standard evaluation system (SES for rice, 1996) based on the symptoms of reaction expressed by the RILs. Eighty five RILs were categorized as resistant, moderately resistant, moderately susceptible and susceptible. Of the 85 RILs screened, 16 RILs were found to be resistant (score 3); 40 RILs were found to be moderately resistant (score 5); 10 RILs were moderately susceptible (score 7) and 19 were susceptible (score 9). PDI was also calculated. Out of 85 RILs scored, 16 RILs had shown a PDI value between 20-40 (resistant); 40 RILs between 45-62 (moderately resistant); 10 RILs between 65-80 (moderately susceptible); 19 RILs between 80-100 (susceptible). A few resistant cultivars showed a moderately resistant reaction with score 5 (40-60% PDI) during this season. Increased susceptibility to blast observed in this study may be due to the breakdown of resistance to blast (Hittalmani., 1995) and also due to rapid adaptation of the pathogen population to resistant cultivar.

Parental survey

Of the 65 RAPD primers used in the parental survey, all the 65 primers amplified IR 5690 and IR 50. A total of 638 DNA fragments were amplified out of which 288 were

found to be present in IR 50 and 350 were found to be present in IR 5690. Eight primers produced less than 5 bands, 33 primers produced 6-10 bands, 16 primers produced 11-15 bands, 7 primars produced 16-20 bands and 4 produced more than 20 bands. Out of 5 kits of operon primers that were tested for parental survey OPU series showed maximum polymorphism (33.3%) followed by OPF (31%), OPC (30%), OPAH (23.4%) and lowest was exhibited by OPK series (23.3%). Total number of bands amplified was highest in OPU series (174) and the least being in OPK series (107). Out of 65 RAPD primers tested, in parental survey, 49 primers could detect 75.3% polymorphism. When individual primers were surveyed for percentage of polymorphism, it ranged from 4.76% (OPK 7) to 100% (OPF 9, OPU 2, OPU 9, OPC 17) other primers showed 15% to 50% polymorphism. This study revealed the advantage of GC rich primers in bringing about amplification. Fakuoka *et al.* (1992) found that in rice, increased GC content in the range of 40-60% tended to increase the number of amplification products. Relatively higher number of amplified products per primer in rice when compared to other plants like maize was reported by Welsh and Mc Clelland (1990). Apart from the fact that RAPD markers are capable of detecting high level of polymorphism, it also indicates that sufficient level of diversity exist between the parents used in this study. The success of any gene tagging experiment largely depends on the genetic divergence of the parents, especially in the target region. The mapping population (RILs) used in this study was developed from parents showing extreme reaction for blast, which is more effective and efficient method for finding a linked molecular marker. Forty-nine polymorphic primers produced a total of 762 fragments when tested on resistant and susceptible bulks, out of which 76 were polymorphic and the rest were monomorphic. The polymorphism detected by the primer series OPF was high (15.00%) followed by OPAH (11.20%), OPC series (10.60%), OPK series (8.60%) and lowest was detected by OPU series (2.94%). Out of 49 primers tested with the resistant and susceptible bulks along with the resistant and susceptible parents, 6 primers amplified phenotype specific fragments. Primers OPAH 12 (480 bp) OPAH 18 (710 bp) amplified fragments found to co-segregate with resistant bulk and the parent. Primers OPU 15, OPC 14, OPK 11, OPK 17 amplified fragments of sizes 850 bp, 490 bp, 1300 bp and 1420 bp respectively, which co-segregated with the susceptible bulk and the parent.

Several approaches have been suggested to identify molecular markers linked to disease resistance genes. One such approach is the use of bulk segregant analysis (BSA). BSA provides a rapid, technically sound method for identifying markers linked to gene of interest (Monna *et al.*, 1995; Chaque *et al.*, 1996 and Lawson *et al.*, 1998). In the present study, bulk segregant analysis was used to obtain phenotype specific markers using RAPD. One of the most time consuming requirements of DNA marker development is the need to screen large populations with every primer. The minimum size of the bulk is determined by the sequence with which linked loci might be detected as polymorphic between the bulked samples. This in turn will depend on the type of marker being screened (dominant or codominant) and the type of population used to generate the bulks (F_2, RILs, BC, *etc.*). For a dominant RAPD marker, the probability of a bulk of 'n' individuals having a band, a second bulk of equal size not having a band will be = 2-(1/4)(1/4) when a locus is unlinked to the target gene. Therefore, few individuals per bulk are required. For a sample, the probability of unlinked locus being polymorphic between bulks of 10 such individuals is 2×10^{-6}. As smaller bulks are utilized, the frequency of false positives will increase. However, as the linkage of all polymorphism is confirmed by analysis of RILs, bulk segregant analysis with only small numbers of individuals in bulks will provide great enrichment for markers linked to target loci (Michaelmore *et al.*, 1991; Shi *et al.*, 1997).

In the present study, bulks were constituted using the extreme class of resistance (1-3 score) and extreme class of susceptibility (9 score) and any marker/fragment that was present in either of the parent and their corresponding bulks was taken into consideration. 10 RILs from each phenotypic class were bulked to identify phenotype specific markers. When bulks are constructed from enough individuals, the BSA is sufficiently robust to cope with the low level of phenotypic misclassification. All polymorphic loci likely to be identified within 14 cM, of the target locus are likely to be identified by BSA. This strategy has the advantage of reducing identification of markers unlinked to the target residue (Chaque *et al.*, 1996). In the present study, we could identify phenotype specific markers present in the bulks. OPAH 18_{710} was present in the resistant bulk and OPK 11_{1300}, OPK 17_{1420}, OPC 14_{490} and OPU 15_{850} were present in the susceptible bulk. Use of extreme phenotypic classes to constitute the bulk mass was also reported by Xu and Mackill (1996) in an attempt to map a major locus for submergence tolerance in rice where co-segregation analysis with the resistant and susceptible RILs was used to constitute the Bulks.

Co-segregation analysis

RAPD primers producing phenotype specific bands were used to screen the twenty RILs individually, which contributed to the bulks (resistant and susceptible bulks). Each phenotype specific band segregated accordingly and was scored by giving (+) or (–) sign based on the presence or absence of particular band. The phenotype specific markers identified through bulk segregant analysis were further used with the 20 individual RILs and parents (10 resistant and 10 susceptible class). The RAPD marker OPAH 18_{710} showed a perfect co-segregation with all the 10 resistant RILs (Plate 1) and the RAPD marker OPK 11_{1300} showed a perfect co-segregation with all the 10 susceptible RILs used for co-segregation analysis. Other markers *viz.*, OPK 17_{1420}, OPC 15_{850} and OPC 14_{490} co-segregated with susceptible RILs respectively.

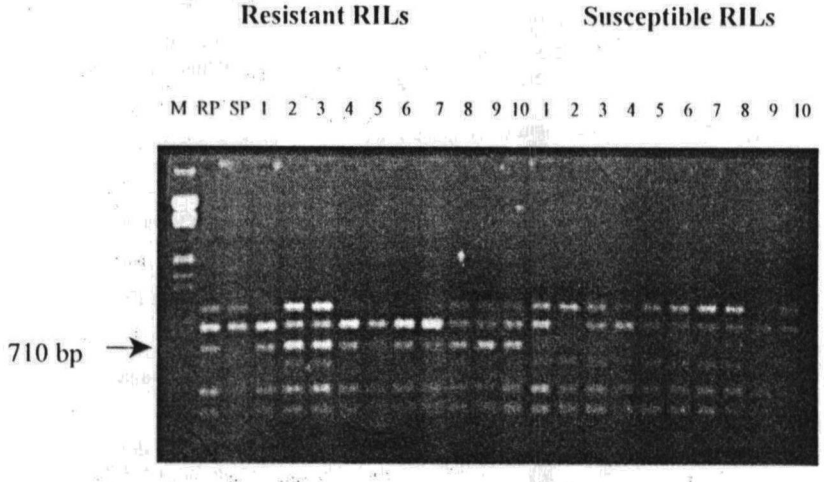

Plate 1. **Co-segregation banding pattern of the RAPD primer OPAH 18_{710}.**
[Blast Resistant Parent (RP); Susceptible Parent (SP); Resistant RILs (1-10) and Susceptible RILs (1-10); M - Marker]

CONCLUSIONS

The results suggested that though the linkage between the RAPD marker and the gene was not established statistically, it could be appropriate to say that the RAPD marker OPAH 18_{710} has been tagged with the gene confirming resistance to blast in rice. This close segregation pattern of this RAPD marker has practical value, particularly for selection within a large breeding population to obtain a manageable number of lines for multilocation yield trials.

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