The Bioscan 11(3):1861-1864,2016(Supplement on Genetics and Plant Breeding) www.thebioscan.com
AN INTERNATIONAL QUARTERLY JOURNAL OF LIFE SCIENCES

INTERACTION OF *AVR-R* GENE AND RICE BLAST RESISTANCE

***VARSHA GAYATONDE[1], P. MAHADEVU[2], PRASANNA KUMAR M. K.[3] AND PRUDHVI RAJ VENNELA[1]**
[1]Department of Genetics and Plant Breeding, BHU, Varanasi - 221 005 (UP), INDIA
[2]Department of Genetics and Plant Breeding,
[3]Dept. of Plant Pathology,
VC Farm AC College, Mandya, Karnataka - 560 065, UAS, Bangalore, INDIA
e-mail: varshapanchu@gmail.com

KEYWORDS
Avr
Blast
Isolates
Polymorphic
Resistance

Received on :
08.04.2016

Accepted on :
07.07.2016

***Corresponding author**

ABSTRACT
Magnaporthe oryzae is the causal agent of rice blast disease, a devastating problem all over the world. It is well known that disease management through cultivar resistance is a better remedy, identifying *Avr- R* gene interactions play a key role in host plant disease resistance which is abase of present investigation. Molecular validation for rice blast revealed out of 60 genotypes five gentotypes found with resistant response *i.e.*, MTU-1010, Vikas, Gangavati sona, Ratnachoodi and Alursanna. Maximum numbers of *R* genes were found in Alursanna which was moderately resistant in field conditions. Molecular interaction between *Avr-R* genes studied with polymorphic marker *Pik* revealed that out of 21 isolates studied, seven isolates i.e., Kempudoddi, MTU-1010, Jaya, Kagisaale, Gandasaale, Honasu and Kichadisamba were found with positive interaction among which Kempudoddi, Jaya, Kagisaale, Honasu and Kichadisamba showed resistance in field conditions also. *Avr-R* interaction was found in seven isolates (out of 21) indicates there is a predominance of blast disease in the study area which needs more interaction studies.The results confirmed both at the phenotypic and genotypic level confers blast resistance.

INTRODUCTION

Rice (*Oryza sativa* L.) is the second most important cereal crops of family Poeceae. About 90 per cent of world's rice is produced and consumed in Asia alone (Yang *et al*., 2013). Rice is one of the diverse crops grown in different agro climatic conditions. Asia is the home for more than half of world's poor and more than half of world's rice cultivators. Disease management through newer molecular techniques play a crucial role as some of the popular varieties are becoming susceptible to blast over the years. Rice blast disease, caused by the filamentous ascomycete fungus *Magnaporthe oryzae*(anamorph *Pyricularia oryzae*) (Couch and Kohn, 2002) is one of the most economically devastating diseases worldwide. Resistance (R) genes have been identified and incorporated into rice cultivars for managing rice blast disease throughout the world. Resistance conditioned by a single major *R* gene is typically effective in preventing infection by races of *M. oryzae* containing the corresponding avirulence (*Avr*) gene (Silue´ *et al*., 1992). One possible explanation for the molecular basis of gene for gene interaction is a ligand and receptor model where the *R* gene product acts as a receptor that recognizes a ligand, or elicitor, produced directly or indirectly by the pathogen's *Avr* genes. This response activates defense response (Jia *et al*., 2010) currently, 85 major rice blast genes have been genetically characterized and 19 of them have been cloned(Cesari *et al*., 2013). Frequent copy number variation and high levels of nucleotide diversity were observed at these *R*-gene loci,suggesting the rapid allelic diversification of these *R*-genes as an adaptive response to the rapidly changing spectrum of rice blast strains (Yang *et al*., 2008).

The present investigation deals with the study on the leading and local varieties as wll as landraces of rice from which the *R* genes and corresponding *Avr* genes have been identified in the blast isolates collected from blast hotspots of southern Karnataka.

MATERIALS AND METHODS

Field evaluation of rice genotypes for blast disease reaction has done by collecting rice genotypes including both standard resistant and susceptible checks, screened for leaf blast at ZARS, Mandya and at Agricultural Research Station (ARS) Ponnampet, represent hill zone of Karnataka and hot spot for blast disease under Uniform Blast Nursery (Chang *et al*., 1965). The screening was done both for phenotyping and genotyping Disease scoring and collection of isolates carried as per the standard IRRI procedures (Lebrun *et al*., 1990).

The marker validation of different rice varieties and blast isolates was carried out using linked SSR markers to confirm the presence of known resistant allele with tightly linked SSR primers and gene specific primers were used for blast fungal characterization. The material for this study consisted of 60 genotypes include the varieties, landraces advanced breeding lines and four check varieties (Tadukan, tetup-resistant and Co-39, HR-12 susceptible varieties) which were collected from various parts of Karnataka and maintained at AICRP on Rice, Zonal Agricultural Research Station (ZARS), Mandya.

The disease sample from young and healthy leaves of twenty five days old seedlings were used for DNA extraction following CTAB method (Cao and Oard, 1997). PCR amplification of the markers was performed into the 10 μl reaction volume consisting of 20 ng. of genomic DNA. 20ng primers, 0.1mM of dNTP's, 1X assay buffer (10 mM Tris pH-8, 50 mM KCl, 1.8mM MgCl2 and 0.01mg/ml gelatin) and 1U of *Taq* polymerase enzyme (Genei). DNA amplification was performed with Eppendorf thermo cyclers under the following PCR conditions: 95ºC for 1 minute for denaturation followed by 30 cycles of denaturation at 94ºC. One minute of annealing at 58ºC and final extension at 72°C for one minute. The amplified products were run on 3.5% agarose – ethidium bromide gel for studying polymorphism. The primers linked with specific genes are *viz*.,RM3825, RM5552, RM6838, RM1896, RM206, RM4862, RM224, RM1337, RM155 and RM7102. Agarose gel (3.5%) was prepared using electrophoresis grade agarose (Sigma) in a volume of electrophoresis buffer (1X TAE) sufficient for constructing a gel (300 ml for 18 X30 cm gel). Ethidium bromide added at concentration of 0.5 μg / ml of gel. The gel was allowed to set fully before removing the comb and loading the sample. 2μl of loading dye was added to 10 μl of PCR products and mixed well before loading into the wells. Care was taken to prevent mixing of the samples between the wells. A voltage of 1-5 v/cm was given for a time period of 3 hours for the separation of PCR fragments. After the run, the gel was viewed under UV light and the DNA banding pattern was recorded directly using Polaroid camera (Zolan and Pukkila, 1986).Scoring of SSR generated bandswas ascertained by comparing the amplicons of the test genotypes with those of standard resistant and susceptible checks.

The 21 rice isolates collected from two different locations (Mandya and Ponnampet) were subjected to single spore isolation, fungal mat preparation on liquid culture media and finally the DNA extracted from the fungal mycelial mat. DNA was confirmed by running the extracted fungal DNA with actin primers. Fungal collection and isolation has done by following the standard methods referred from Rout and Tewari (2012). Isolation of blast fungus DNA and scoring has done by grinding the mycelial mat in pestle and mortar was transferred to 1.5 ml sterilized Eppendorf tubes. Then 500 μl of extraction buffer (50mM Tris-HCl, 150mM NaCl and 100 mM EDTA) was added and vertex until evenly suspended and incubated at 37ºC for one hour after adding 50 μl of 10% SDS. Later, 75 μl of 5 M NaCl and 60 μl of CTAB / NaCl solution (10% CTAB in 0.7 M NaCl) were added and mixed thoroughly. The tubes were incubated at 65ºC for 15 min and equal volume of chloroform: isoamyl alcohol (24:1) added to extract DNA. The Eppendorf tubes were vigorously shaken and centrifuged at 10,000 rpm for 12 min. aqueous viscous supernatant was transferred to fresh Eppendorf tubes, two-thirds volume of ice-cold isopropanol was added and incubated at -20º for 8 hr. The tubes were centrifuged at 10,000 rpm for 15 min and the supernatant was discarded. Pellet was washed with 70% ethanol, air dried, dissolved in 100 μl of 1 $\times$ TE buffer and used in the PCR reaction (Huang *et al*., 2014).

The polymorphic DNA bands generated by each isolate were scored at each marker level.Five *Avr*- gene specific markers were used in the investigation. The presence of band was scored as 1 and its absence as 0. The genetic distance between the isolates was estimated using NTSYS software program to identify the number of clusters generated using qualitative similarity measures through SHAN clustering. Confirmation of *Magnaporthe* DNA was done by running the fungal DNA with Actin primer. Presence of 498 bp size bands confirms the blast fungus.In this investigation *Avr-Pizt, Avr-Pik, Avr-Pita, Avr-Co-39, Avr-Pia* primers were used.

The interaction was confirmed by looking the amplification pattern in genotypes for *R* genes as well as *Avr* genes. The defense responses are often activated by the action of a host resistance (*R*) gene and a pathogen avirulence (*Avr*) gene as proposed by the gene-for-gene hypothesis (Flor, 1971). One possible explanation for the molecular basis of gene-for-gene interactions is a ligand and receptor model where the *R* gene product acts as a receptor that recognizes a ligand, or elicitor, produced directly or indirectly by the pathogen's *Avr* gene.

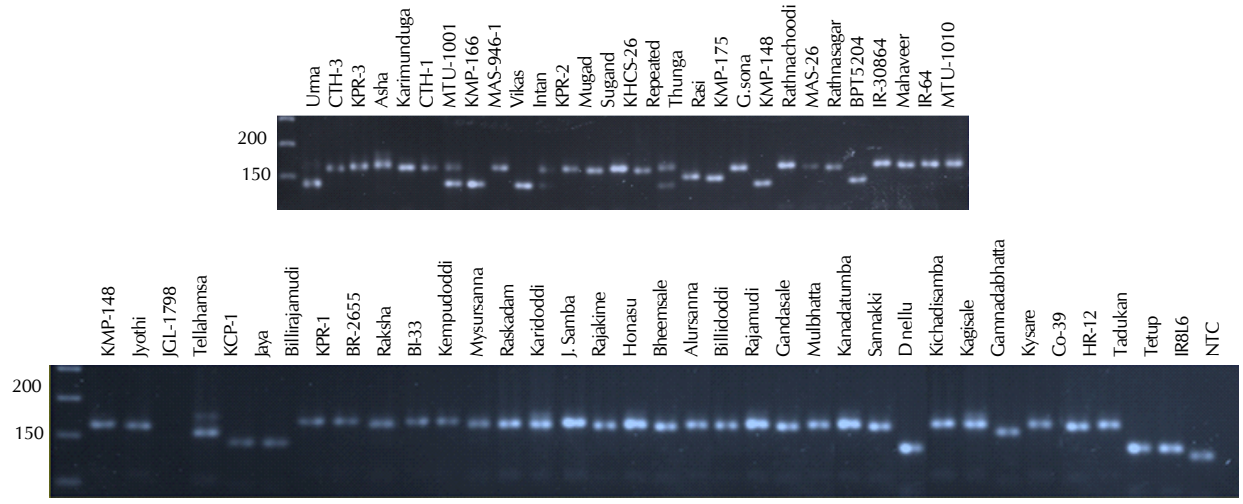
RESULTS AND DISCUSSION

For the efficient selection of varieties with resistance to leaf blast disease and their utilization in disease resistance breeding, molecular markers that are tightly linked to resistant genes that could serve as surrogates are gaining more importance. The use of DNA markers enabled several cycles of selection for blast resistance without depending on natural occurrence of pathogen (Mohan *et al*., 1997and Mc Couch *et al*., 1997).

In marker validation, out of 10 primers screened, three primers confirmed polymorphism whereas the rest 7 markers showed monomorphic pattern. The validated primers in the experiment were, RM 206 (*Pi38*), RM 1859 (*Pia*), and RM 224(*Pik*). The varieties were classified as resistant or susceptible to leaf blast disease based on the corresponding amplification pattern of Tetep, Tadukan, CO-39 and HR-12 and a positive control. Results of validated primers are given in Table 1. For genes with different allele types present among the genotypes and the number of genotypes found with particular phenotypic response (based on natural screening scores).Based on the amplification pattern, Marker RM 1896 specific to *Pii* (Fig.1) gene showed more number of *R*1 alleles (26). These alleles were found with resistance response in many genotypes, 16 *R*2 and 6 *R*3 alleles were also found in this gene. Among the 60 genotypes studied, *Pi 38* gene specific marker RM 206

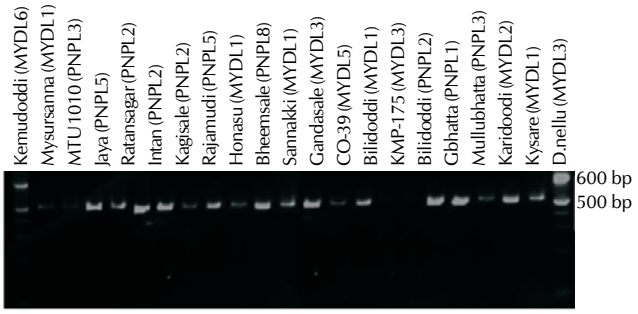
Table 1: List of genotypes with different types of alleles response to *R* gene specificmarkers

Markers	Gene	No. of genotypes with *R* 1 type of allele	No. of genotypes with R 2 type of allele	No. of genotypes with R 3 type of allele
RM1896	*Pii, Pi*3 *(t),*	26	16	6
RM 224	*Pik*	9	3	46
RM 206	*Pi*38	14	46	Nil.



[Expected product size - 157bp, Ladder - 50 bp, Varieties - 40., Landraces - 21, Checks (4) - Tadukan, Tetep (resistant to blast), HR - 12, CO - 39 (susceptible to blast), Positive control (6) - IRBL (6)]

Figure 1: Fingerprint profile of varieties and landraces for blast resistant gene *Pik* using linked marker RM-224



Expected product size - 493 bp, Total number of isolates - 21, Ladder size - 100 bp

Figure 2: Fingerprinting profile of blast Avirulence gene, *Avr-Pizt*.

showed 14 *R*1 and 46 *R*2 types of alleles.

Landraces *viz.,* Mysursanna and Kyasare found with RM-1896 showed susceptible response under the field conditions. Similar response was also found with Jaya variety when it was screened with Marker RM 206 (*Pi 38* gene specific marker).The SSR markers linked to blast resistance loci were used to screen varieties for leaf blast disease resistance. 40 released varieties and 20 landraces consisting 16 blast resistant varieties and 3 blast susceptible varieties were screened using known linked SSR markers. Out of 60 selected varieties, 16 harbored *Pik* gene which was sampled by RM 224 (Fig. 1); RM 1896 sampled resistance for *Pii* gene. *Pii* gene present with R1 type of alleles in 40 genotypes harbored R1 type alleles, among which 36 genotypes showed field resistance. The *Pi* 38 gene was available in all the 60 varieties. Whereas, resistant type of alleles found in only 16 genotypes out of which Kyasare and Rajamudi were showing susceptibility at field conditions. Variety -Vikas showed field level resistance harbored *Pik, Pia* and *Pii* genes. Similar results have been reported by Nandini (2013) in several landraces. Alursanna harbored *Pi 9, Pish* and*Pib* genes where MTU-1010 and Uma harbored 2 genes i.e., *Pik* and *Pi*38. Therefore Alur sanna could be utilized for simultaneous transfer of three blast resistant genes using marker assisted selection and could be preferentially used to develop cultivars with multiple blast resistant genes. The study indicates that, the land races are the good source of resistant alleles for both leaf and neck blast. Shivapriya (2002), also found the better response of local cultivars comparing to the popular varieties.

Interaction between *Avr* and *R* genes in disease expression

Presence of *Avr* gene in a particular variety is confirmed by the presence of the bands with specific product size. Maximum *Avr-R* gene combination, positive was observed for *Pita*/*Avr-Pita, Pi-a*/*Avr-Pia* and *Pik*. Out of 10 R gene specific primers screened, three primer pairs confirmed the presence of blast R genes in the released varieties and selected landraces. The validated primer was RM 224 (*Pik*). The varieties were classified as resistant or susceptible to leaf blast disease based on the correspondence of the bands with 4 check varieties *viz*., susceptible (CO39 and HR 12) and resistant (Tadukan and Tetep). Results of validated primers are given in the Table1

***Avr* genes characterization and *Avr-R* gene interaction studies**

The isolated 21 genotypes were screened with 5 *Avr* genes *viz*., *Avr-Pita, Avr-Pizt, Avr- CO-39, Avr-Pik* and *Avr-Pia*. The presence and absence of bands possibly reflected the differential action of different isolates on the host physiological system. Out of 21 isolates, 19 isolates confirmed the presence of *Avr-Pita gene* and 15 isolates for *Pizt* gene (Fig. 2). In *Avr-CO*39, among the 4 *Avr* genes amplified, 3 were showing field resistance response *viz.,* KMP-175, bilidoddi, Gandasale, Similarly 7 out of 8 *Avr- R* gene interactions showed the phenotypic resistance in *Avr-Pik* and 8 isolates were showing resistance out of 12 genotypes found with *Avr* gene *Pia.* In *R* gene studies, many genotypes did not amplify, whereas, 47 genotypes were in heterozygous condition (scored as either 2) and scored at different allelic level (R1, R2, and R3). The absence of band(s) / marker allele (s) in two cases (*Avr* and R) could be due to nucleotide sequence variation/alteration resulting from insertion, deletion or substitution in one or

both the primer binding sites which results in non-amplification (Shivapriya, 2002). Landraces like Kagisale, Bilidoddi and Gamnadabhatta were found with maximum number of *Avr* genes and there is a significant interaction found between the *Avr- R* genes. This indicates the aforesaid varieties resistant to blast in high yielding background could be utilized as potential donors for developing blast resistant cultivars. Some of the isolates found with *Avr* genes at molecular level but the resistance mechanism even having *Avr-R* interaction failed to show resistance phenotypically. The failure might be due to the damaged activation of systemic acquired resistance mechanism in plant system, which can be caused by an enzyme salicylate hydroxylase (Terrence, 1997). *Avr* proteins are diverse, and many have pathogenicity effector functions that play important roles in enhancing infection and breaking the *Avr-R* interaction by modifying nucleotide binding site (NBS) and leucine-rich repeat (LRR) domains (Zhou *et al*., 2007). Major *R* gene-mediated resistance can be robust and complete, but may not be long-lasting. Our findings also suggest that the development of a novel race carrying *R-Avr* interactions could allow the development of rice lines that have more effective, or durable, resistance to the rice blast pathogen.

ACKNOWLEDGEMENT

We thank Dr. Malali Gowda Director, NGS, C-CAMP, NCBS-Bangalore for sharing the valuable resources and MARS-Mandya for providing the facilities.

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