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Research Article

# MOLECULAR ANALYSIS OF BLAST RESISTANCE GENES IN RICE USING GENE LINKED AND GENE BASED MARKERS

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**Abstract-** Rice blast caused by *Magnaporthe oryzae*, spread in more than 85 countries and has caused great yield loss. Development and growing of rice varieties would be the most effective way to control blast disease. Molecular analysis and major rice blast resistance genes for genetic diversities were determined and molecular characterization (or) screening of major rice blast resistance genes was determined with molecular markers, which showed close set linkage to 10 major rice blast resistance genes (*Pi-54, Pi-1, Pi-2, Pi-9, Pik, Pikm, Pitp, Pi-38, Pizt* and *Pi 7t*), in a collection of 15 accession (including some varieties). Out of the 15, the *Pikm, Pizt* and *Pi7t* appeared to be widely present in all the varieties with respect their resistant allele size and gave positive express. For the gene, *Pi-54, Pi-1, Pi-2, Pi-9, Pik, Pitp* and *Pi-38* gene frequencies were 33.33 %, 40.01%, 86.67%,6.67%, 60.07%,33.33% and 33.33% respectively. Among the 15 accessions, 5 were positive for *Pi-54*, *Pitp* and *Pi-38* genes and six accessions were positive for *Pi-1* gene, thirteen for *Pi-2*, nine for *Pikm* and one for *Pi-9* gene. Four accessions *viz.,* BR 2655, JAYA, RAKSHA and BASUMATHI-370 were positive for two major and broad spectrum genes *i.e Pi-54* and *Pi-2*. Out of 15, only three accessions namely BR-2655, JAYA and BASUMATHI-370 were detected with maximum number (> 7) of genes. Less number of genes (<4) harboured in KMP-200, KMP-201 and IR-64. These results are useful in identification and incorporation of functional resistance genes from these evaluated varieties into elite cultivars through marker-assisted selection for improved blast resistance in India and worldwide.

**Keywords**- Rice, marker validation and blast resistant genes.

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## Introduction

Rice (*Oryza sativa* L.) is the staple food crop for more than 3 billion people around the world. The blast disease *Magnaporthe oryzae* is geographically widespread plant pathogen and it has various host-limited forms collectively infesting more than 50 different grass species [1]. The disease occurs on leaves, stems and seeds of the cultivated crop. Major Rice blast epidemics covering vast area occurs on a regular basis resulting 11 to 30% crop losses annually which represent a yield loss of about 157 million tonnes worldwide [2]. In South East Asia including India, it causes significant crop losses, sometimes even up to 50% damage under epiphytotic conditions [3]. Annual rice harvest loss ranging from 10% to 30% due to blast disease, neck blast may cause complete loss in grain yield of susceptible varieties in case the control is not yet on time [4]. The fungus colonizes leaves (leaf blast), node (nodal blast), panicle (neck blast) other parts culm, glume and leaf sheath of the rice plant.

Commonly fungicides can be used to control rice blast but they generate additional costs in rice production and chemical contamination of environment and foods. Therefore, the use of resistant varieties is thought to be one of the most economically and environmentally efficient ways of crop protection from the disease. Genomes sequencing of Asian cultivated two subspecies of rice i.e. *indica* (Basmati)and *japonica* has revealed that *indica* and *japonica* varieties have diverged significantly in resistance to *M. oryzae* strains. So far, 10 rice blast R genes (*Pib*,*Pita*, *Pi9*, *Pid2*, *Pi2*, *Piz-t*, *Pi36*, *Pi37*, *Pikm*, and *Pi5*) have been identified *via* map based cloning methods [5]. During the last three decades, a large number of genes governing disease resistance have been identified by both conventional methods and molecular approaches, and also utilized them for varietal improvement particularly to defend the attack of pathogens [6]. Recent progress in rice genomics has facilitated using the resistance genes in breeding by DNA marker assisted selection (MAS). So far, about 100 major rice blast resistance genes have been identified, and few of them have been characterized [7].

Recent advances in rice genomics research and completion of the rice genome sequence have made it possible to identify and map precisely a number of genes through linkage to molecular markers. Some examples of the genes tightly linked to markers are resistance to or tolerance to blast, bacterial blight, virus diseases, brown plant hopper (*Nilaparvata lugens*), drought, submergence, salinity, and low temperature and improved agronomic and grain quality traits. Mapping blast resistance genes and locating closely linked markers has made it possible to confirm the presence of given gene in a variety with multiple genes [8]. With this view, the following objective has been decided to validate blast resistant genotypes using gene linked/gene based markers.

## Material and Methods

The plant material selected, the procedural details of laboratory techniques employed, the method followed for the selection are described below:

### Isolation of genomic DNA

DNA was extracted from the frozen leaf sample (at –80° C) using CTAB protocol [9].

### Sample preparation

Already collected and stored tender, fully expanded leaves (20-25 days old) were surface sterilized with ethanol (75%) before extraction.

**DNA extraction and SSR analysis**

Twenty one days rice leaves were used to extract the DNA from rice leaves. Extraction buffer (2% CTAB, 100 mM Tris, 20 Mm EDTA, 1.4 M NaCl) preheated at 60°C for 5 min. DNA quantification and purity was checked by measuring the O.D at 260/280 nm using a Nano spectrophotometer [Table-1a].

**Polymerase Chain Reaction (PCR)**

The polymerase chain reaction was carried out in applied bio-system thermal cycler using three gene targeted primers.

- ✓ The PCR reaction mix includes the following: DNA 2µl of 10 ng/µl; 5µl of 2X Takara Mix, 2µl of DDW and 1µl of 10 µM each of forward and reverse primers.
- ✓ The PCR profile starts with Initial Denaturation 94°C for 5 min, Denaturation, 94°C for 30 sec, Primer Annealing 55°C for 30 sec, Extension 72°C for 1 min, Final Extension 72°C for 10 min, and cooling 4°C for infinite time was included. These steps were repeated for 34 cycles for amplification of DNA. After completion of amplification, PCR products were stored at –20°C.
- ✓ The amplified products were analyzed by electrophoresis using 3% agarose gels. Ethidium bromide was added while pouring the gel, so that the DNA fluoresces when gel was exposed to UV light. The DNA fragments was then visualized under UV Transilluminator and the banding pattern was observed and recorded using gel documentation unit (Gene flash) which was stored for further scoring and permanent records.

**Table-1** *Details of Gene based markers and tightly linked markers to major rice blast resistance genes*

| Sl. no | Gene | Marker | Forward Primer | Reverse Primer | Chr. no | Allele Size |
|---|---|---|---|---|---|---|
| 1 | *Pi-1* | RM224 | ATCGATCGATCTTCACGAGG | TGCTATAAAAGGCATTCGGG | 11 | 157 |
| 2 | *Pi54/Pikh* | Pikh MAS | CAATCTCCAAAGTTTTCAGG | GCTTCAATCACTGCTAGACC | 11 | 216 |
| 3 | *Pi-2* | AP56595 | CTCCTTCAGCTGCTCCTC | TGATGACTTCCAAACGGTAG | 6 | 288 |
| 4 | *Pi-9* | NMSMPi9-1 | CGAGAAGGACATCTGGTACG | GAGATGCTTGGATTTAGAAGAC | 6 | 168 |
| 5 | *Pizt* | Zt56591 | TTGCTGAGCCATTGTTAAACA | ATCTCTTCATATATATGAAGGCCAC | 6 | 257 |
| 6 | *Pik* | K-2167 | CGTGCTGTCGCCTGAATCTG | CACGAACAAGAGTGTGTCGG | 11 | 619/300 |
| 7 | *Pitp* | RM246 | GAGCTCCATCAGCCATTCAG | CTGAGTGCTGCTGCGACT | 1 | 116 |
| 8 | *Pi-38* | RM21 | ACAGTATTCCGTAGGCACGG | GCTCCATGAGGGTGGTAGAG | 11 | 157 |
| 9 | *Pikm* | Ckm-1 | TGAGCTCAAGGCAAGAGTTGAGGA | TGTTCCAGCAACTCGATGAG | 11 | 174/213 |
| 10 | *Pi-7(t)* | RM229 | CACTCACACGAACGACTGAC | CGCAGGTTCTTGTGAAATGT | 11 | 116 |

**Table-1a** *DNA quality and yield of selected rice accessions*

| Sl. no | Names of the DNA sample | DNA Quality (A260/A280) | DNA Yield (ng µl-1) |
|---|---|---|---|
| 1. | IR 64 | 1.86 | 1468.9 |
| 2. | JYOTHI | 1.81 | 1654.3 |
| 3. | BR 2655 | 1.65 | 956.4 |
| 4. | JAYA | 1.73 | 834.0 |
| 5. | THANU | 1.82 | 986.2 |
| 6. | MTU 1001 | 1.45 | 458.6 |
| 7. | KMP 201 | 1.56 | 754.6 |
| 8. | KMP 200 | 1.82 | 638.2 |
| 9. | KMP 128 | 1.69 | 369.4 |
| 10. | KMP 153 | 1.98 | 489.3 |
| 11. | KMP 175 | 1.45 | 569.7 |
| 12 | RAKSHA | 1.64 | 456.9 |
| 13. | RASHI | 1.61 | 569.3 |
| 14. | BASAMATHI 370 | 1.57 | 687.4 |
| 15. | MANDYA VIJAYA | 1.74 | 584.9 |

**PCR assay and Genotyping**

An allele-specific PCR marker for the ten rice blast R genes assays the genotype by examining the presence or absence of a PCR amplification product and Standard DNA ladder was loaded to determine the size of amplified product and scored to prepare genotypic matrix. The amplified fragments were scored as absence (0) or presence (1) of amplicon linked to each blast resistance gene DNA fragment.

**Data analysis**

Using Jaccard's coefficient, genetic similarities were analyzed from the binary data matrix [10]. The similarity coefficient was used for cluster analysis of rice germplasm utilizing UPGMA (unweighted pair group method with arithmetic averages).

**Results and Discussion**

Among the most devastating diseases that constrain rice production, Rice Blast ranks first because of its wide distribution and high incidence under favourable conditions. Although many resistant varieties have been developed, due to genetic plasticity in the pathogen genome, there is a continuous threat to the effectiveness of the developed cultivars [11]. To breed rice varieties with more durable blast resistance, multiple resistance utilizing both qualitative and quantitative genes must be incorporated into individual varieties [12]. Identification of new donors is very important in development of resistant cultivars.

**Table-2** *List of markers linked to blast resistant genes used for validation*

| Sl. No | Varieties/Gene | *Pi-54* | *Pi-1* | *Pi-2* | *Pi-9* | *Pik* | *Pikm* | *Pitp* | *Pi 38* | *Pizt* | *Pi7t* |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | IR 64 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 1 |
| 2 | JYOTHI | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 |
| 3 | BR 2655 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 1 |
| 4 | JAYA | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 |
| 5 | THANU | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 1 |
| 6 | MTU 1001 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 1 |
| 7 | KMP 201 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 1 |
| 8 | KMP 200 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 1 |
| 9 | KMP 128 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 1 |
| 10 | KMP 153 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 |
| 11 | KMP 175 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 1 |
| 12 | RAKSHA | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 |
| 13 | RASHI | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 |
| 14 | BASAMATHI 370 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 |
| 15 | MANDYA VIJAYA | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 1 |

**Allelic diversity of rice blast genes**

Ten R gene markers were used to detect the presence or absence of the related R genes in the selected collections. All the 15 accessions possessed three or more blast resistance genes as revealed by the positive bands for different markers. Specially, *Pik, Pizt* and *Pi-7t* genes were positive banding pattern for all 15 accessions. Two of the accessions, Jaya and Basumathi-370, showed positive bands for different markers associated with eight major rice blast resistance genes. Out of eight genes seven genes *viz*., *Pi-54, Pi-2, Pik, Pitp, Pi-38, Pizt* and *Pi 7t* were commonly present in Jaya and Basumathi-370 accessions but *Pi-1* and *Pikm* were individually present in Jaya and Basumathi-370 respectively.

While Raksha and Rasi have seven number of blast resistant genes with respect to their positive banding pattern for resistant allele. Of these seven, five genes like*Pi-2, Pik, Pikm, Pizt* and *Pi-7 t*were commonly present in Raksha and Rasi. *Pi-54* and *Pitp* absent in Rasi but they were present in Raksha and *Pi-1* and *Pi-38* which were absent in Raksha but they were present in Rasi. Five accessions, Jyothi, BR-2655, Thanu, KMP-153 and KMP-175, were positive for six markers while IR-64 and MTU-1001 contained five R genes *Pi-d2*, *Pi-z* and *Pi-ta2*. KMP-200 and KMP-201 were positive for four R genes [Table-2]. Out of the R genes detected in this study, maximum numbers of genes were located on the chromosome-11. *Pi-2*,*Pi-9* and *Piz-t* were located on chromosome 6 and only one gene i.e, *Pitp* located on chromosome number-1.

R gene frequency 100.0% for *Pik, Pizt* and *Pi-7t* genes. *Pi-2* was detected in 13 accessions and *Pikm* and *Pi-1* were present in nine and six accessions respectively. *Pi*-9 only present in one accession. 33.33% of gene frequency was observed in *Pi-54, Pitp* and *Pi-38.*

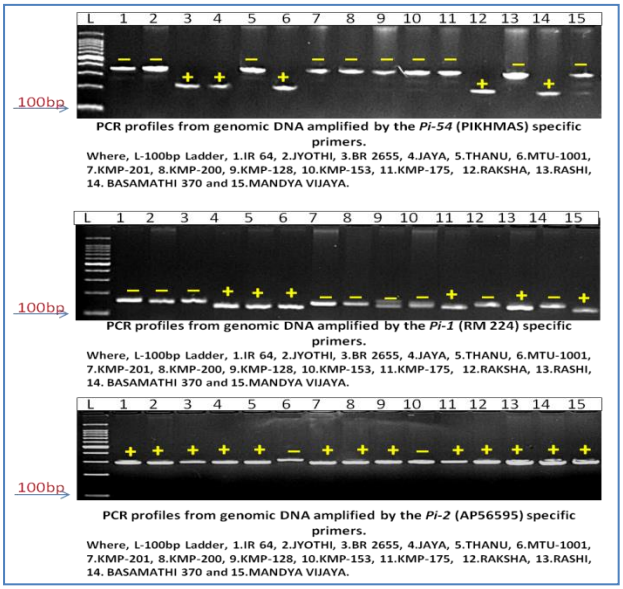


**Fig-1 Gel profiles of different markers used in this study**

**Cluster analysis**

The dendrogram was generated based on similarity degree coefficient from 0.3 to 1.0. The present study, involving 15 accessions of rice collected form Rice Breeding department, AICRP on Rice, ZARS, V.C. FARM, Mandya. Six distinct lineages in different similarity index were found. All the varieties were shown significant variation. However, KMP-153 formed a separate lineage which is represented in [Fig-2]. SSR analysis grouped the lines independently in two major clusters, cluster I and Cluster II. KMP-173 alone comes under cluster I. Cluster II again divided into two groups cluster IIA and cluster IIB. In cluster IIA comprises MTU 1001 variety and cluster IIB again subdivided into several subgroups.

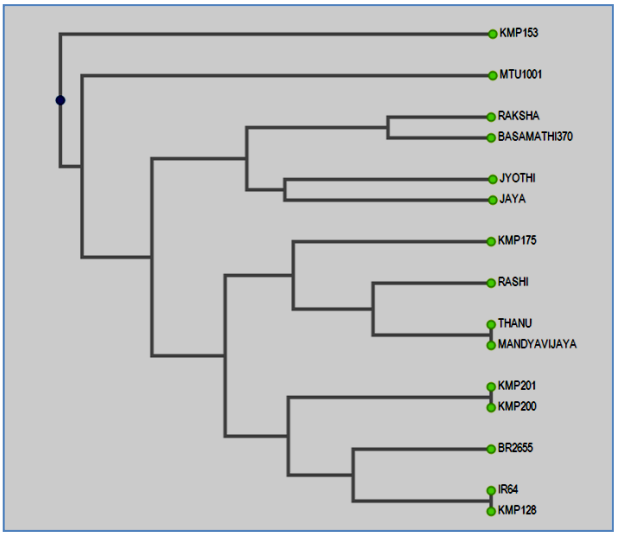


**Fig-2 Unweighted pair group method arithmetic averages among fifteen rice germplasms based on Jaccard's distance**

**Conclusion**

Understanding of the genetic background of rice with respect to blast resistant genes and pathogen dynamics to blast disease incidence can help to formulate strategies to development of resistant cultivars in rice for blast disease. This research finding will be helpful guidance about the application of different molecular approaches that can be used for accelerating the development of new disease resistant rice cultivars by sustaining rice yields to meet up the demand and food security in India.

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| | Similarity Matrix computed with Jaccard's coefficient | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | **13** | **14** | **15** |
| **1** | 1 | 0.571 | 0.833 | 0.444 | 0.833 | 0.429 | 0.8 | 0.8 | 1 | 0.571 | 0.571 | 0.714 | 0.714 | 0.625 | 0.833 |
| **2** | | 1 | 0.5 | 0.75 | 0.5 | 0.375 | 0.667 | 0.667 | 0.571 | 0.5 | 0.714 | 0.625 | 0.625 | 0.75 | 0.5 |
| **3** | | | 1 | 0.556 | 0.714 | 0.571 | 0.667 | 0.667 | 0.833 | 0.5 | 0.5 | 0.857 | 0.625 | 0.75 | 0.714 |
| **4** | | | | 1 | 0.556 | 0.625 | 0.5 | 0.5 | 0.444 | 0.4 | 0.75 | 0.667 | 0.667 | 0.778 | 0.556 |
| **5** | | | | | 1 | 0.571 | 0.667 | 0.667 | 0.833 | 0.5 | 0.714 | 0.625 | 0.857 | 0.556 | 1 |
| **6** | | | | | | 1 | 0.5 | 0.5 | 0.429 | 0.375 | 0.571 | 0.5 | 0.5 | 0.444 | 0.571 |
| **7** | | | | | | | 1 | 1 | 0.8 | 0.429 | 0.667 | 0.571 | 0.571 | 0.5 | 0.667 |
| **8** | | | | | | | | 1 | 0.8 | 0.429 | 0.667 | 0.571 | 0.571 | 0.5 | 0.667 |
| **9** | | | | | | | | | 1 | 0.571 | 0.571 | 0.714 | 0.714 | 0.625 | 0.833 |
| **10** | | | | | | | | | | 1 | 0.333 | 0.625 | 0.444 | 0.556 | 0.5 |
| **11** | | | | | | | | | | | 1 | 0.444 | 0.857 | 0.556 | 0.714 |
| **12** | | | | | | | | | | | | 1 | 0.556 | 0.875 | 0.625 |
| **13** | | | | | | | | | | | | | 1 | 0.667 | 0.857 |
| **14** | | | | | | | | | | | | | | 1 | 0.556 |
| **15** | | | | | | | | | | | | | | | 1 |

| | Distance matrix based on Jaccard's coefficient | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
| 1 | 0 | 0.429 | 0.167 | 0.556 | 0.167 | 0.571 | 0.2 | 0.2 | 0 | 0.429 | 0.429 | 0.286 | 0.286 | 0.375 | 0.167 |
| 2 | | 0 | 0.5 | 0.25 | 0.5 | 0.625 | 0.333 | 0.333 | 0.429 | 0.5 | 0.286 | 0.375 | 0.375 | 0.25 | 0.5 |
| 3 | | | 0 | 0.444 | 0.286 | 0.429 | 0.333 | 0.333 | 0.167 | 0.5 | 0.5 | 0.143 | 0.375 | 0.25 | 0.286 |
| 4 | | | | 0 | 0.444 | 0.375 | 0.5 | 0.5 | 0.556 | 0.6 | 0.25 | 0.333 | 0.333 | 0.222 | 0.444 |
| 5 | | | | | 0 | 0.429 | 0.333 | 0.333 | 0.167 | 0.5 | 0.286 | 0.375 | 0.143 | 0.444 | 0 |
| 6 | | | | | | 0 | 0.5 | 0.5 | 0.571 | 0.625 | 0.429 | 0.5 | 0.5 | 0.556 | 0.429 |
| 7 | | | | | | | 0 | 0 | 0.2 | 0.571 | 0.333 | 0.429 | 0.429 | 0.5 | 0.333 |
| 8 | | | | | | | | 0 | 0.2 | 0.571 | 0.333 | 0.429 | 0.429 | 0.5 | 0.333 |
| 9 | | | | | | | | | 0 | 0.429 | 0.429 | 0.286 | 0.286 | 0.375 | 0.167 |
| 10 | | | | | | | | | | 0 | 0.667 | 0.375 | 0.556 | 0.444 | 0.5 |
| 11 | | | | | | | | | | | 0 | 0.556 | 0.143 | 0.444 | 0.286 |
| 12 | | | | | | | | | | | | 0 | 0.444 | 0.125 | 0.375 |
| 13 | | | | | | | | | | | | | 0 | 0.333 | 0.143 |
| 14 | | | | | | | | | | | | | | 0 | 0.444 |
| 15 | | | | | | | | | | | | | | | 0 |

**Author Contributions:**
Experiment was designed by Deepak, C.A. Rajanna, M.P. and Harini Kumar, K.M Complete lab work has been performed by Manoj Kumar, H.B. and Ayeesha Munawery. All authors read and approved the final manuscript.

**Abbreviations:**
**CTAB-**Cetyl trimethyl ammonium bromide and **MAS-** Marker Assisted Selection

**Ethical approval:** This article does not contain any studies with human participants or animals performed by any of the authors.

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