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Identification and Mapping of *Pi1K* and *Pi2*, two Major Gene Conferring Resistance against Rice Blast in the progenies derived from Safri-17 × PR-122 × Safri-17 × Aganni; Safri-17 × PR-122; Dubraj × PR-122

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ABSTRACT: More than one-third of the world's population, including more than half of its population in India, depends on rice (*Oryza sativa* L.), which is also the most significant crop for making basic foods. In many agro-ecological settings, rice is farmed in India. By 2050, agricultural output on land that is now under cultivation must rise globally by 70% and in emerging nations by 100% if food insecurity is to be reduced. The host plant is infected by the rice blast fungus during many phases of crop growth, including the leaf, stem, neck, collar, node, and root. The largest problem facing rice breeders is the deterioration of resistance in the many rice types that have been produced throughout time. Consequently, developing resilient cultivars that are broad-spectrum resistance is a difficult undertaking. The major causes of the creation of virulent pathotypes of *Magnaporthe*, which make blast control a challenging work, are the wide host range, ongoing genetic variety, evolution, and host alterations. Because of this, and other factors, such as the economic significance of blast disease in rice production and human food, the Rice-*Magnaporthe* interaction pathosystem arose as a model system to research host-pathogen interaction. In our study, the progenies derived from crosses are showing complete resistance against rice blast fungus *Pyricularia oryzae*. The plant population are screened with natural inoculum under field condition in Raipur, Chhattisgarh. All the plants were highly resistance (HR reaction) to blast. The presence of genes is traced out using the linked molecular markers RM136 and RM7311 for *Pi2* and RM224 for tracing the presence of *Pi1K*. Identification of map position of these molecular markers was accomplished by identifying BAC or PAC clones that simultaneously contained a hit from the marker (Sequence of either forward or reverse Primer). Based on correlative in-silico mapping and blast analysis markers were landed to a physical positions on BAC/PAC clones, which demarcated BAC/PAC clones (pseudo molecules from Nipponbare) for reported genomic location.

Keywords: Rice blast, R gene, genetic mapping.

INTRODUCTION

Rice blast disease is one of the most destructive diseases of rice world-wide which causes approximately 30-40% of yield loss (Nalley *et al*., 2016; Annegowda *et al.,* 2021). Blast is caused by a filamentous ascomycete fungus *Magnaporthe oryzae* (Tel: *Pyricularia oryzae*) (Couch and Kohn 2002). Combating against rice blast, development of genetic resistance is most economic, effective and environmentally sustainable method for its management, but resistance genes (R genes) losses its effectivity with time due to evolution in pathogens. Marker aided selection is one of technique to achieve durable control with pyramiding the R genes (Hittalmani *et al*., 2000). There is well established molecular events that deals with the relationship of pathogen and host interaction and supports the rice-*M. oryzae* pathosystem (Valent, 1990). This work has also established gene-for-gene relationship is the governed resistance the plant progenies (Jia *et al*. 2002). The utilization of functional rice blast *R* genes is the most eco-friendly approach to prevent huge losses. At least 100 major genes have been identified so far that govern resistance against rice blast disease (Yin *et al*., 2021; Wang *et al*., 2022; Hassan *et al*., 2022). Over 30 resistant genes have been cloned including *Pish, Pi35, Pi37, Pi64, Pit, Pi-b, pi21, Pi63, PiPR1, Pi9, Pi2, Piz-t, Pi50, Pizh, Pigm, Pi-d2, Pi-d3, Pi25, Pi36, Pi5, Pii, Pi56, Pb1, Pik, Pik-p, Pikm, Pike, Pi1, Pik-h/Pi54, Pi54rh, Pi54of, Pia, Pi-CO39, Pi-ta, Ptr,* and *Pi65* (Liu

et al., 2005; Deng *et al*., 2006). *Pi-2* and *Piz-t* genes are isometric with a dissimilarity of having eight amino-acid in their leucine-rich repeats.

In this report we had described the presence of R gene into the introgressed lines of rice cultivars that provides resistance against leaf blast in elite rice varieties like Dubraj, Safri-17 and Aganni. The genes that confer resistance to the plant progenies were located with the help of high resolution in-silico mapping.

MATERIALS AND METHODS

Plant material Three crosses were used to study the genetic basis of the blast resistance by segregation analysis which was derived from crosses developed from elite varieties like Dubraj, Safri-17 and Aganni crossed with PR-122. The population was challenged with natural inoculum under field condition. Both the parents and the crosses were eliciting a differential response towards the blast inoculums. The seedlings were raised along with susceptible plant population. Disease incidence and disease severity were evaluated as described (Fukuta *et al*, 2009; Zhu *et al*., 2004). Both the parents and progenies derived from crosses (F7 generation) were evaluated against rice leaf blast incidence under field condition.

Evaluation of the disease. Standard scale for disease assessment was used as reference scale for disease assessment (Fukuta *et al*, 2009) which follows:

Table 1: Scoring of Leaf blast (Source: Fukuta *et al.*, 2009).

S. No.	Description	Reaction
0	No lesions observed	Highly resistant
1	Small brown specks of pin point size	Resistant
2	Small roundish to slightly elongated, necrotic lesions, about 1-2mm in diameter with distinct brown margin; lesions mostly found in lower leaves	Moderately Resistant
3	Same as 2, but significantly number in lesions over lower leaves	Moderately Resistant
4	Typical susceptible blast lesions, 3mm or longer infecting less than 4% of leaf area	Moderately susceptible
5	Typical susceptible blast lesions, 3mm or longer infecting 4-10% of leaf area	Moderately susceptible
6	Typical susceptible blast lesions, 3mm or longer infecting 11-25% of leaf area	Susceptible
7	Typical susceptible blast lesions, 3mm or longer infecting 26-50% of leaf area	Susceptible
8	Typical susceptible blast lesions, 3mm or longer infecting 51-75% of leaf area	Highly Susceptible
9	Typical susceptible blast lesions, 3mm or longer infecting more than75 % of leaf area	Highly Susceptible

Molecular mapping of the population

Mapping population. One thousand two hundred sixty in F7 individuals derived from Safri 17 × PR 122 × Safri17 × Aganni, Safri 17 × PR122, Dubraj × PR122 were screened for blast resistance under natural conditions at Raipur. All the plants were highly resistance (HR reaction) to blast. The presence of genes was traced out using the linked molecular markers RM136 and RM7311 for *Pi2* and RM224 for tracing the presence of *Pi1K*. Identification of map position of these molecular markers was accomplished by identifying BAC or PAC clones that simultaneously contained a hit from the marker (Sequence of either forward or reverse Primer). Based on correlative in-silicomapping and blast analysis markers were landed to a physical positions on BAC/PAC clones, which demarcated BAC / PAC clones (pseudo molecules from Nipponbare) for reported genomic location.

DNA preparation Genomic DNA was isolated from parental includes Safri-17, Dubraj Aganni and PR-122, while progenies derived from Safri-17 × PR-122 × Safri-17 × Aganni, Safri-17 × PR-122 and Dubraj × PR-122 were also screened genotypically. Fresh leaf tissue was used for isolation of genomic DNA. The genotyping of F7 generation population, where 180 plants progenies were assessed based on the resistant, susceptible and heterozygous reaction, following rapid method of plant genomic DNA extraction (Zheng *et al*., 2008) for isolation of DNA from rice leaves.

Analysis of simple sequence repeat. Three SSR primers were used to screened the polymorphism of parental lines and determine resistant and susceptible parents along with progenies from crosses derived.

(i) For *Pi1k* gene: RM-224 was screened for parental polymorphism. All the four parents were amplified, where at 150bp PR-122 shown resistant whereas Safri-17 and Dubraj shown susceptible reaction.

(ii) For *Pi2* gene: RM-136 and RM-7311 were screened for parental polymorphism. Both the markers were shown evident difference between the susceptible and resistant parents.

RESULTS AND DISCUSSION

Phenotypic reaction of rice blast. The parental lines and progenies were raised in nursery along with susceptible checks *i.e*., Swarna which initiates the disease incidence and progression. The plants were transplanted to the field to check the phenotypic reaction of leaf blast in the field (Asfaha *et al.,* 2015). The parental lines and progenies were evaluated and scored as per the reaction of *Pyricularia oryzae*.

Table 2: Evaluation for rice blast resistance in introgressed lines scored for leaf blast and calculation of disease severity.

Code	Genotype	Leaf blast scoring			Disease severity		
		Mean	SD	SeM	Mean	SD	SeM
IL-1	Safri-17 × PR-122 × Safri-17 × Aganni	39.33	30.73	34.77	7.37	3.56	4.03
IL-2	Safri-17 × PR-122	41.87	31.16	35.27	7.77	4.47	5.06
IL-3	Dubraj × PR-122	40.03	16.73	18.94	9.60	3.98	4.50
P 1	Safri-17 (elite variety)	159.67	55.1	62.35	22.35	2.10	2.38
P 2	PR-122 (monogenic line)	36.00	16.75	18.95	8.56	3.99	4.51
P 3	Aganni (elite variety)	161.17	90.26	102.13	17.71	3.80	4.30
P 4	Dubraj (elite variety)	159.00	58.67	66.39	21.98	2.79	3.16
	Swarna (susceptible check)	439.00	103.87	117.54	42.52	3.05	3.45

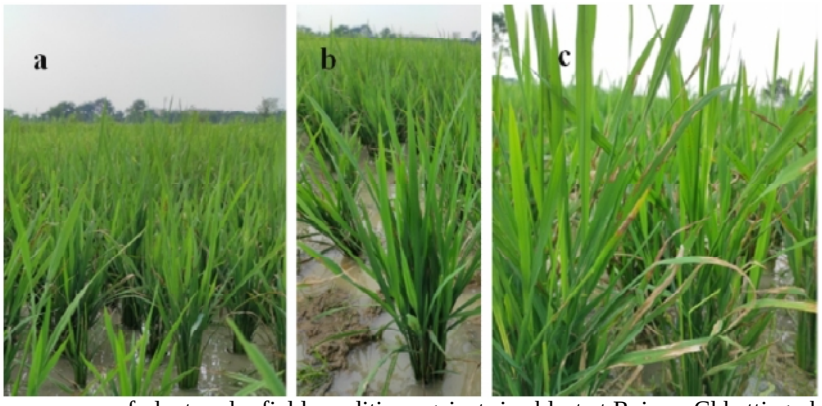

Fig. 1. Phenotypic responses of plant under field condition against rice blast at Raipur, Chhattisgarh. a & b). Crossed plant population showing resistance and c) Susceptible plant showing blast reaction.

Genetic mapping of three different crosses. Three DNA molecular markers were used to markers linked to *Pi2* and *Pi1k*, respectively obtained from the public database released by Gramene (http://www.gramene.org/) to identify additional markers linked to *Pi2*. These SSR markers were selected from BAC clones (table 3) which had map position on BAC or PAC clones corresponding to the reported genomic location of *Pi2* (Liu *et al.*, 2005; Jeon *et al*., 2003) was selected for validation with and aim that a number of DNA markers were closely linked to selection of this important gene in rice breeding programs.

Table 3: List of markers used during the genotypic analysis.

Gene	Marker	Primer sequence	Motif	Type	Chromosome	Linkage distance
Pi2	**RM 136**	F: 5'GAGAGCTCAGCTGCTGCCTCTAGC3'	(AGG)7	SSR	6	1.50cM
		R: 5'GAGGAGCGCCACGGTGTACGCC3'				
PiK	**RM 224**	F: 5' ATCGATCGATCTTCACGAGG3'	(AAG)8(AG)13	SSR	11	0 cM
		R: 5' TGCTATAAAAGGCATTCGGG3'				
Pi2	**RM 7311**	F: 5'AGTGGTCGTTGAACTCGCAG3'	(CAAT)6	SSR	6	1.2 cM
		R: 5'TCGTGGCGCCTTTAATCTC3'				

One thousand two hundred sixty in F9 individuals derived from Safri 17 × PR-122 × Safri17 × Aganni, Safri 17 × PR-122, Dubraj × PR-122 were screened for blast resistance under natural conditions at Raipur. Selective genotyping was performed on the progenies derived from crosses expressing highly resistant/ hypersensitive reaction (HR). Molecular markers were found to effective in order to trace the presence of introgressed gene *Pi1k* and *Pi2* in homozygous/ heterozygous/ absence and in some cases the markers were unable to amplify the product. The detailed study of the genotyping of the progenies were explained the following table:

Table 4: Traceability of introgressed gene *Pi1k* and *Pi 2* in homozygous/heterozygous/absence/not amplify by the linked molecular markers.

Molecular markers	Homozygous state	Heterozygous state	Absence	Marker did not amplify
Cross I (Safri-17 × PR-122 × Safri-17 × Aganni)				
RM136	1257	3	--	--
RM 224	1180	56	13	11
RM 7311	1188	47	20	5
Cross II (Safri-17 × PR-122)				
RM136	1251	9	--	--
RM 224	1250	10	--	--
RM 7311	1175	10	--	75
Cross III (Dubraj × PR-122)				
RM136	1208	2	--	50
RM 224	1241	19	--	--
RM 7311	1189	47	--	24

Analysis of gene combination. The polymorphism between parental lines were examined with the help of SSR markers present in the Chromosome 6 for *Pi2* gene and chromosome 11 for *Pi1k* gene. This polymorphism helped in detection among parents with susceptible and resistant reactions, respectively. Thus, these SSR markers were helpful in distinguishing the resistant bulk and susceptible bulk.

The presence or absence of the introgressed gene *Pi1k* and *Pi2* traced using linked molecular markers indicated that progenies containing two gene combination for blast resistant gene provides more resistance for blast infection as compared to single gene introgression. Pyramiding of resistant gene *i.e.*, *Pi1k* and *Pi2* in the rice plant is responsible to withstand the infection of different physiological races of *Pyricularia oryzae* over the area.

Mapping of resistant gene for blast using gene-based markers. Three markers were used for detection of polymorphism in parental lines which was further used for progenies in F7 generation. RM 136, RM 224 and RM7311 were three molecular markers that help to detect the resistant and susceptibility segregation reactions among progenies.

Genotyping of introgressed lines were useful in order to understand the allelic status of the gene of interest which is associated to resistant gene for blast. The introgressed lines which shown positive checks were given as score 1 which represents resistant reactions whereas the introgressed lines with negative checks were scored as 0. In the present study two important blast gene were identified among the resistant parent and the progenies.

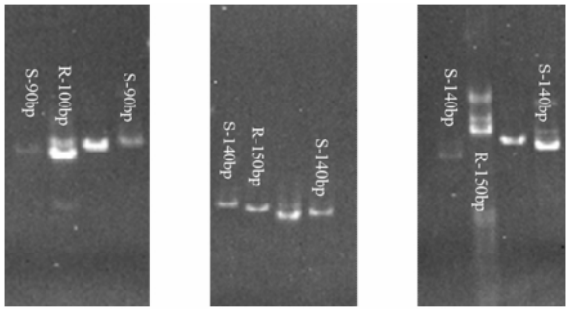

Fig 2. DNA molecular markers with notation S for susceptible parents- Safri-17 and Dubraj and R for resistant parent- PR-122.

Table 5: Traceability of gene combination in introgressed line-I derived from Safri-17 × PR-122 × Safri-17 × Aganni.

Sr. No.	Molecular marker	Gene combination	No. of progeny
Two gene combination			
1.	RM136, RM 7311, RM 224	*Pi1k+Pi 2*	1084
2.	RM 136, RM 224	*Pi 2+Pi1k*	43
3.	RM 7311, RM 224	*Pi 2+Pi1k*	2
Single gene			
4.	RM 224	*Pi1k*	--
5.	RM 136	*Pi 2*	54
6.	RM 7311	*Pi 2*	1
7.	RM 136/ RM 7311	*Pi 2*	76

Table 6: Traceability of gene combination in introgressed line-II derived from Safri-17 × PR-122.

Sr. No.	Molecular marker	Gene combination	No. of progeny
Two gene combination			
1.	RM136, RM 7311, RM 224	*Pi1k+Pi 2*	1162
2.	RM 136, RM 224	*Pi 2+Pi1k*	80
3.	RM 7311, RM 224	*Pi 2+Pi1k*	6
Single gene			
4.	RM 224	*Pi1k*	2
5.	RM 136	*Pi 2*	3
6.	RM 7311	*Pi 2*	1
7.	RM 136/ RM 7311	*Pi 2*	6

Table 7: Traceability of gene combination in introgressed line-III derived from Dubraj × PR-122.

Sr. No.	Molecular marker	Gene combination	No. of progeny
Two gene combination			
1.	RM136, RM 7311, RM 224	*Pi1k+Pi 2*	1140
2.	RM 136, RM 224	*Pi 2+Pi1k*	65
3.	RM 7311, RM 224	*Pi 2+Pi1k*	35
Single gene			
4.	RM 224	*Pi1k*	2
5.	RM 136	*Pi 2*	--
6.	RM 7311	*Pi 2*	11
7.	RM 136/ RM 7311	*Pi 2*	3

Introgressed lines have proved to be an effective methodology which helped in pyramiding the resistant genes among the elite rice cultivar which provides resistance against severity of leaf blast incidence. Marker assisted selection had proven to be an important tool that directly helped to find out potential genetic resources which helped in detection of rice blast. Upon stringent screening of introgressed lines in filed condition the population can be also validated with the help of DNA molecular markers which helped in identification of the introgressed resistant gene in the plants.

Apart from stringent phenotypic screening of rice leaf blast in field condition, its genotypic screening was also performed which is based on resistant gene identification or known R alleles (Hayashi *et al*., 2006). PCR based approach (Mahender *et al.*, 2012) helped in setup the direct influence of the alleles in case of resistance. SSR markers spreads uniformly in the genome which can easily identify the presence of introgressed R gene in the introgressed lines.

On further amplification of *in-silico* analysis, it can be made clear that within the genetic map there were several gene that can be located which will help to identify more disease related genes in the introgressed lines. This information can be useful in mapping of gene associated to resistance. These lines which were evaluated can be a good source of resistance against leaf and neck blast disease.

Fukuoka *et al*. (2014) highlighted that allelic variation mining for crop breeding may be utilised to produce resistance for the creation of long-lasting resistance to rice blast. With the use of fine-scale mapping, complementation testing, and progeny tests, they discovered the existence of *Pi35* at the Os01g0782100 locus and demonstrated that *Pi35* is an allelic to Pish, which confers race-specific resistance to *Magnaporthe oryzae*. Their research revealed that Os01g0782100, which is located on chromosomal number 1, has two novel loci that contribute to the resistance to *Magnaporthe oryzae*. They tested KS-*Pi35*, which are almost isogenic *Pi35* lines.

Xiao *et al.* (2019) explained about one of the most detrimental diseases to rice production is rice blast, which is brought on by *Magnaporthe oryzae.* A successful approach to combat the disease is thought to be the development of resistant types through the pyramiding of resistant (R) genes. But is it actually necessary to pyramid more R genes in particular ecological regions? In order to respond to this query, a collection of enhanced rice lines was created. After that, the recurrent parent (RP), donor parents (DPs), and enhanced lines were examined for their agronomic features and resistance to blast disease. They created seven better lines by introducing the R gene(s) into a shared genetic background via marker-assisted backcross breeding, including three monogenic lines, three two-gene pyramids, and one three-gene pyramid (MABB). The recurrent genome of the seven improved lines ranged from 89.1 to 95.5% based on 302 SSR markers, with an average genome recovery of 92.9%.

CONCLUSION

In the present study the progenies were validated with the presence of two resistant genes against rice blast which confer broad-spectrum resistance to the population. The introgression of resistant genes into the elite varieties and with the help of marker assisted selection, resistant plants can be easily derived which is incorporated with R gene for *P. oryzae.* Further with the help of bioinformatics, more primers can be developed that can co-segregate with the resistant gene.

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Conflict of Interest. None.

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