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Evaluation and molecular characterization of 3K rice (*Oryza sativa*) germplasm subset for blast resistance

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ABSTRACT

Rice (*Oryza sativa* L.) is one of the most widely cultivated crop species in the world affected by many biotic stresses. Blast, a major biotic constraint in lowland and high altitude grown rice led to inconsistent yields in many parts of India and world. The present study was carried out during 2023 at ICAR-Indian Institute of Rice Research, Hyderabad, Telangana to identify blast resistant lines from 50 selected superior genotypes from 3K germplasm lines collected from Internation Rice Research Institute, Philippines. These lines were screened against SPI-40 blast isolate in uniform blast nursery to identify resistant lines during wet and dry seasons of 2023. Out of 50 lines, 22 were found to be resistant and genotyped using a set of 12 blast-linked/specific SSR markers covering *Pi1, Pi2, Pi9, Pi54, Pita, Pi40, Pi33, Pib, Pi20, Pi38, Piz,* and *Pitp* to determine the genetic basis of resistance. Genotyping of the 22 resistant lines confirmed the universality of *Pita* among the tested lines. Whereas, *Pi2* found to be least among the tested lines and *Pi40* was completely absent. Results revealed the cause of phenotypic resistance and several combinations of genes which work best against the tested pathotype were identified. The study of genetic diversity revealed that the RM1 marker had the highest PIC value of 0.95, indicating its strong polymorphic nature. Using the Unweighted Pair Group Method with arithmetic mean, the distance-based analysis categorized the accessions into five primary clusters. These resistant genotypes with different genetic cause of resistance can serve their role as source of donors or parental lines in development of blast resistant hybrids or varieties in future breeding programme for crop improvements.

Keywords: Blast resistance, Genotyping, PIC, Phenotyping, Rice

Rice (*Oryza sativa* L.) is an important cereal crop serving the hunger needs of world's population. Demand for rice is expected to increase as human population is booming over the years. Climate change is one of the major factors causing different kinds of abiotic stresses which in turn affects the evolution of virulent races of pest and diseases (Gautam *et al.* 2013). It is essential to raise crop productivity to increase the overall production by panoramic management practices along with identification and utilization of resistant/tolerant genetic sources for biotic and abiotic stresses. Of the biotic stresses affecting rice, blast disease caused by *Magnaporthe oryzae* is the most destructive. It has evolved into multiple virulent races leading to un-manageable damages to rice yields during favourable environmental conditions. It is observed that due to blast disease, yield decline was reported to be from 10–30% and in severe cases rice blast may turn deadly and cause up to 50–100% yield loss (Devanna *et al.* 2022, Gao *et al.* 2023). Identifying and utilizing the genetic resistance and their pyramiding in different genetic combination can give enduring resistance over a period of time and environments (Wu *et al.* 2015). So far, there have been 109 major genes for leaf blast resistance discovered, of which 25 genes/QTLs were molecularly characterized (Qu *et al.* 2006) and total of 23 independent genes have been cloned (consisting of ~35 different alleles) (Simon *et al.* 2023).

The majority of these genes are allelic or closely linked genes that encode disease-resistant NBS-LRR proteins and zinc finger domains (Korinsak *et al.* 2019, Mandal *et al.* 2023). Among the 109 identified blast genes, *Pi1*, *Pi2*, *Pi9*, *Piz*, *Pib*, *Pipt*, *Pi33*, *Pi20*, *Pi38*, *Pi54*, and *Pita* are part of the largest class of plant R genes that encode proteins with nucleotide-binding sites (NBS) (Kalia and Rathour 2019). Therefore, this study was focused to validate majority of these loci using linked/gene specific markers and to know their effect in different combinations through artificial screening using specific pathotype.

MATERIALS AND METHODS

Plant material: Germplasm consisting of 50 selected superior genotypes from 3K rice genome subset panel obtained from International Rice Research Institute,

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Philippines and maintained by ICAR-Indian Institute of Rice Research, Rajendranagar, Hyderabad, Telangana, (Table 1). These 50 lines were selected based on ideal plant type and mid early flowering duration, and rest of the lines were not included as they were photo sensitive (did not observe flowering in wet season) and the plant type was very tall and droopy.

Genomic DNA extraction: Genomic DNA was extracted using the Cetyl Trimethyl Ammonium Bromide (CTAB) method (Doyle and Doyle1987). Fresh leaves were collected from the plants at maximum tillering stage and leaf bits of around 1 cm were used for DNA extraction. The quality and quantity of extracted DNA were estimated using NanoDrop Spectrophotometer ND-1000. The DNA was diluted to a final concentration of ~ 30 ng/µl using 1XTE buffer.

Polymerase chain reaction (PCR): The PCR was performed as per the conditions described by Devi *et al.* (2015) and Singh *et al.* (2015) with minor modifications. PCR amplification was carried out with a 10 µl reaction mixture containing 30 ng (1µl) of genomic DNA, forward primer (0.5 µM) and reverse primer (0.5 µM), 4 µl of master mix (Takara) and 4 µl of nuclease-free water. The amplified products were electrophoretically resolved on 2.5% agarose gel and visualization was carried in a gel documentation system. The band size of the amplified fragments was designated using reference ladder of 100 bp (HiMedia-Mumbai) as a reference.

Statistical analysis: Genotype clustering was carried out by the distance-based approach through Power Marker V3.0 software. An unweighted neighbour-joining tree was constructed and Jaccard's coefficient with 1000 bootstraps was used to calculate the genetic distance.

Validation of blast resistance genes and estimation of genetic diversity: Twelve gene-specific SSR markers were used to validate resistance genes (*Pi1, Pi2, Pi9, Pi54, Pita, Pi40, Pi33, Pib, Pi20, Pi38, Piz* and *Pitp*) in 22 resistant genotypes, following established genotyping protocols (Supplementary Table 1). Genetic diversity was assessed using 96 SSR markers, including these 12 blast-resistant markers (Supplementary Table 2).

Phenotypic screening for blast resistance: Fifty accessions were screened for blast resistance in 2023 using the IIRR local isolate SPI-40, across both wet and dry seasons. Screening took place at ICAR-Indian Institute of Rice Research, Hyderabad, Telangana in a uniform blast nursery; along with highly susceptible check HR12, planted every five test entries to ensure even inoculum distribution. After 15 days of artificial inoculation, entries were scored on a 0–9 scale, with scores of 0–3 as resistant (R), 4–5 as moderately resistant (MR), and 6–9 as susceptible (S).

RESULTS AND DISCUSSION

Out of 50 genotypes tested against blast isolate SPI-40, 8 were highly resistant (R) with average scores of 0–3, 14 were moderately resistant (MR) with scores of 4–5, and 28 were susceptible with scores of 6–9 (Table 1). Genotyping of resistant accessions revealed varied resistance to the blast pathogen, with each of the 22 accessions having different R gene combinations. *Pita* was found to be the most prevalent gene, followed by *Pib* and *Pi38*, consistent with findings by Wang *et al.* (2015) and Miah *et al.* (2017) in indica germplasms. Disease reactions are illustrated in Fig. 1, showing resistant (R), moderately resistant (MR), and susceptible (S) responses.

Among the germplasm lines, 3 accessions found to have 7 blast resistant genes, 5 accessions with 6 blast resistance genes, 8 accessions with 5 blast resistance genes, 5 accessions with 4 blast resistant genes and only 1 accession found to have 3 blast genes (Table 2). Over the several decades, researchers have used mapping, isolation, and cloning to identify and characterize most blast resistance genes in rice. While disease resistance varies with genetic background and environmental conditions (Carrillo *et al.* 2021), some broad-spectrum genes show consistent effectiveness across different locations and seasons (Yadav *et al.* 2019, Singh *et al.* 2020). This study aimed to investigate the causes of resistance in selected germplasm lines through genotyping. Breeding efforts for blast resistance have typically focused on incorporating single or a few resistance genes alongside other stress-resistant genes. Varieties with only a few major resistance genes may lose effectiveness due to rapid changes in pathogen races (Ahn and Ou 1982). Resistance genes can act differently alone or in combination; some may provide resistance across various locations and races, while others require cooperation with additional genes for effective resistance (Younas *et al.* 2024).

In the current study, screening for blast resistance with local isolate SPI-40 revealed resistance levels of genes in different combinations. Three accessions (YANONGZA-04, BANCHI and KALAN) with seven gene combination showed different disease reactions. Accession YANONGZA-04 with seven resistant genes following the combination of *Pi9, Pita, Pib, Pipt, Pi33, Piz* and *Pi38* found

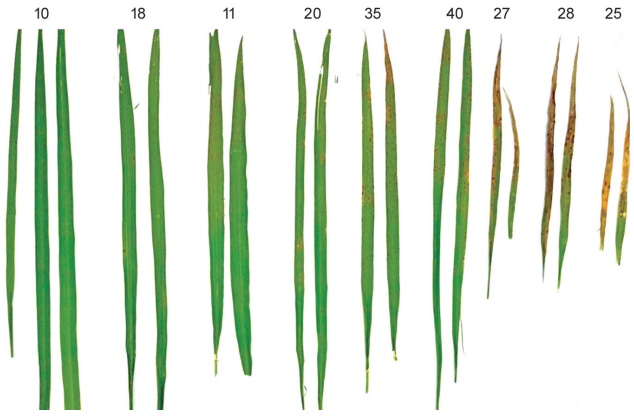

Fig. 1 Representation of blast reaction pattern among the accessions.
Note: *Numbering corresponds to genotypes listed in Table 1. 10, YANONGZA-04; 18, KALALAN; 11, PORASHI; 20, PALEPYU; 35, KULAKARUPPAN; 40, ASU; 27, HONGYANGZAO-3; 28, MAKROWAR 72-2-1-1; 25, KALOBONA.

Table 1 Evaluation and disease scoring of the accessions during wet and dry seasons of 2023

S.no.	Accession	Blast score (wet season 2023)	Blast score (dry season 2023)	Average disease score*	Disease reaction
Resistant accessions					
1.	NCS840	3	3	3	R
2.	BANCHI	1	3	2	R
3.	DA GANG ZHAN	4	5	4.5	MR
4.	LIMA	4	6	4	MR
5.	EPEAL 102	3	3	3	R
6.	BORO JYOT	3	4	3.5	MR
7.	KALO CHAKOL	5	4	4.5	MR
8.	GO CHIAGARI	4	4	4	MR
9.	KALARKAR	3	3	3	R
10.	YANONGZA-04	1	2	1.5	R
11.	PORASHI	4	4	4	MR
12.	PSBRC 88	4	4	4	MR
13.	KHAOCHUANCHOM 452	5	4	4.5	MR
14.	DAHARNAGRA	3	4	3.5	MR
15.	ARC 13591	2	2	2	R
16.	CICA 9	4	5	4.5	MR
17.	C 662083	4	5	4.5	MR
18.	KALALAN	2	3	2.5	R
19.	LAOZAOGU	4	5	4.5	MR
20.	PALEPYU	4	5	4.5	MR
21.	IRRI 123	2	4	3	R
22.	IRGA370-38-1-1F-C4-2	3	4	3.5	MR
Susceptible accessions					
23.	VARIRANGAHY	7	6	6.5	S
24.	SAHABAGAINDHAN	7	7	7	S
25.	KALOBONA	9	9	9	S
26.	DHANIYAPHOOL	7	6	6.5	S
27.	HONGYANGZAO-3	9	7	8	S
28.	MAKROWAR 72-2-1-1	7	7	7	S
29.	ARC 12576	7	5	6	S
30.	AUS 344	6	7	6.5	S
31.	B6136E3-T8-0-1-5	7	5	6	S
32.	BENGALYMORIMO	7	7	7	S
33.	TAICHUNGSENYO214	7	7	7	S
34.	ARC13919	7	7	7	S
35.	KULAKARUPPAN	7	5	6	S
36.	DAU NGHATHAIBINH	9	7	8	S
37.	ARC 12884	7	7	7	S
38.	LAMBRA	7	5	6	S
39.	C1016-1	6	6	6	S
40.	ASU	7	6	6.5	S
41.	IKUNGPAO	7	6	6.5	S
42.	WAR 72-2-1-1	7	7	7	S
43.	SIPULUTHITAMPENDEK	7	6	6.5	S
44.	DA-11	7	6	6.5	S
45.	CN44-40-7	9	7	8	S
46.	IKUNGPAO	7	6	6.5	S
47.	BINNAFUL	7	5	6	S
48.	SIPULUTHITAMPENDEK	7	6	6.5	S
49.	ARC 10754	6	6	6	S
50.	KHAOHAWM	7	5	6	S

*0–3, Resistant; 3.5–5, Moderately resistant; 6–9, Susceptible.

Table 2 Genotyping of germplasm lines for blast resistance genes using linked/trait specific SSR markers

Accession	Blast resistance genes												
	Pi1	*Pi2*	*Pi9*	*Pi54*	*Pita*	RM-166 (*Pib*)	RM-246 (*Pitp*)	RM-72 (*Pi33*)	RM-5364 (*Pi20*)	RM-19818 (*Piz*)	MSM6 (*Pi40*)	RM-3605 (*Pi38*)	Total no. of R genes
NCS840	1	1	0	0	0	0	0	1	1	1	1	0	6
BANCHI	1	0	0	1	0	0	0	1	1	0	1	0	7
DA GANG ZHAN	1	1	1	1	0	0	0	1	1	2	1	0	4
LIMA	1	1	1	1	0	0	1	1	1	0	1	0	4
EPEAL 102	1	1	1	0	0	0	1	1	1	1	1	0	4
BORO JYOT	1	1	1	1	0	0	0	0	1	0	1	0	6
KALO CHAKOL	1	0	0	1	0	0	1	1	1	1	1	0	5
GO CHIAGARI	1	1	1	1	0	0	0	1	1	0	1	0	5
KALARKAR	1	0	1	0	0	0	1	1	1	1	1	1	4
YANONGZA-04	1	1	0	1	0	0	0	0	1	0	1	0	7
PORASHI	1	1	0	1	0	1	0	1	1	0	1	0	5
PSBRC 88	1	1	1	1	0	0	1	0	1	0	1	0	5
KHAOCHUANCHOM 452	2	0	1	1	0	0	0	1	1	1	1	0	5
DAHARNAGRA	1	1	1	1	0	0	0	0	1	0	1	0	6
ARC 13591	1	1	0	0	0	1	0	1	0	0	1	1	6
CICA 9	1	0	1	1	0	0	1	1	0	1	1	0	5
C 662083	1	0	2	1	0	0	1	0	1	1	1	0	4
KALALAN	2	0	0	1	0	0	0	0	1	1	1	0	7
LAOZAOGU	2	1	0	1	0	0	1	1	0	1	1	0	5
PALEPYU	0	1	1	1	0	0	1	0	1	2	1	0	5
IRRI 123	0	1	1	0	0	0	0	1	1	2	1	0	6
IRGA370-38-1-1F-C4-2	1	1	1	1	0	0	1	1	1	2	1	0	3
Total	2	7	8	5	22	20	12	6	3	9	0	20	

0, Positive; 1 and 2, Negative.

to have lowest two season average disease reaction score (1.5), followed by BANCHI with *Pi2, Pi9, Pita, Pib, Pipt, Piz* and *Pi38* (average disease score 2) and KALAN with *Pi2*, *Pi9, Pita, Pib, Pipt, Pi33* and *Pi38* (average disease score 2.5) showcasing the importance of gene combination along with number of genes, these conclusion were in agreement with earlier reports that mentioned, resistance spectrum and degree of resistance of rice varieties were improved with multiple resistance genes (Liu *et al.* 2018, Yin *et al.* 2021). This study revealed that gene combination in accession YANONGZA-04 known to give utmost resistance compared to other available gene combinations to impart resistance against isolate SPI-40. Even though three accessions have seven gene combinations, quality of their combinations tend to show different level of resistance reaction.

Careful observation of association between genetic and phenotypic evaluation, highlights the effectiveness of *Piz* locus in imparting higher resistance in YANONGZA-04 and BANCHI accessions in comparison to KALAN accession where it lacks *Piz* in seven gene combination, which is supported by couple of studies (Wang and Valent 2009, Carrillo *et al.* 2021). Again YANONGZA-04 and BANCHI accessions have almost similar resistance gene combination except for the *Pi2* and *Pi33*, here *Pi33* showing resistance advantage over *Pi2* in series of other resistance gene combinations for the pathotype SPI-40. This is the first report citing the superior combination of genes while choosing *Pi33* and *Pi2* genes along with other resistance genes for MAS breeding targeting SPI-40 isolate. Similar results were stated by Wu *et al.* (2015) with the study targeting different accessions against 76 isolates collected across the country in China and concluded that particular *R* gene combinations were effective against defined isolates.

Accessions named NCS-840, EPEAL-102, KALARKAR, ARC-13591, IRRI-123, BORO JYOT and DAHARNAGRA showed average disease scores of 2–3.5 with the gene combination of 6, 4, 4, 6, 6, 6 and 6 genes respectively. BORO JYOT and DAHARNAGRA accessions found to have similar resistant genes and expressed similar individual, and average resistant pattern to blast disease

(Table 1). Among these seven accessions most commonly appeared gene was *Pi54* and *Pita*, except for BORO JYOT and DAHARNAGRA which have another broad spectrum gene *Piz* instead of *Pi54*. Despite presence of other genes among these genotypes resistance levels were found to be controlled mostly by *Pi54*, *Pita* and *Piz*.

ARC-13591 accession with all the three broad spectrum genes (*Pi54*, *Pita* and *Piz)* along with other 3 resistant genes found equally resistant as BANCHI with 7 resistant genes, indicates the quality of resistant gene contributing for blast resistance along with the number of resistant genes. These results were supported by some of the studies highlightening the broad-spectrum resistance governed by *Pi54* among several cultivars in India (Devanna *et al.* 2014, Vasudevan *et al.* 2015), for *Pita* (Meng *et al.* 2020, He *et al.* 2022) and *Piz* (Wang and Valent 2009, Carrillo *et al.* 2021).

Out of 8 accessions possessing five R genes, lowest average disease scores were recorded by PSBRC 88, GO CHIAGARI and PORASHI. These three accessions have *Piz*, *Pita* and *Pi38* in common. To know the real cause of differential resistance among 5 gene combination lines, these three accessions with remaining 5 gene combination accessions were screened for two seasons. Results have clearly indicated that accessions without *Piz* have shown higher disease score compared to lines with *Piz* in 5 gene combinations. Accessions with *Piz* locus known to show higher form of resistance compared to other accessions which lacks *Piz* locus in five gene combinations as evidence of our results (Table 1 and Table 2). This concludes the superior effect of *Piz* in series of gene combinations in imparting higher leaf blast resistance.

DA GANG ZHAN, C 662083, and LIMA, each with four R genes, showed resistance similar to lines with 5 R genes. Similarly, IRGA370-38-1-1F-C4-2, with 3 R genes, was as resistant as BORO JYOT and DAHARNAGRA with six R genes. This variation in resistance is due to interactions among R genes, as noted by Tabien *et al.* (2000) and Ning *et al.* (2020), which can both enhance and diminish resistance based on genetic background. Studies reported by Xiao *et al.* (2018) and Hittalmani *et al.* (2000) demonstrate that the effectiveness of R gene combinations varies. For example, plants with *Pi2+Pita* showed lower resistance than those with only *Pi2*, while japonica 07GY31 with *Pizt+Pi54* had higher resistance than lines with a single R gene. These findings emphasize the importance of selecting effective R gene combinations for developing broad-spectrum resistance.

Genetic diversity among 22 resistant accessions was assessed using 96 SSR markers, including 12 related to blast resistance. Distance-based analysis with the Un-Weighted Pair Group Method with Arithmetic Mean (UPGMA) clustered the accessions into five major groups (Fig. 2). The average allelic frequency was 0.34, with major and minor alleles ranging from 0.95–0.15. The PIC values, indicating marker utility for genetic diversity analysis, were highest for RM234 (0.77), reflecting greater diversity and polymorphism, and lowest for RM20023 (0.02), with an average of 0.44 (Supplementary Table 2).

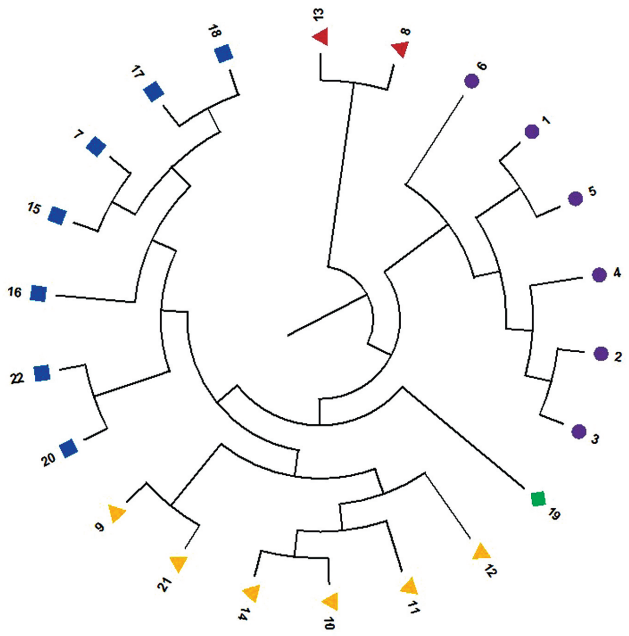

Fig. 2 Cluster diagram grouping 22 blast resistant lines into different clusters.

Genotypes 1, 2, 3, 4, 5 and 6 clustered together due to their shared possession of blast resistance genes (*Pita*, *Pib* and *Pi38*). Similarly, genotypes 8 and 13 clustered together due to their presence of genes *Pita*, *Pib* and *Pipt*. Whereas the genotypes 9, 10, 11, 12, 14 and 21 clustered together due to their shared possession of *Pita* gene (Fig. 2). Notably, these genotypes display identical gene combinations, which likely accounts for their grouping within the same cluster.

Our results indicate that a higher number of resistant genes correspond to a lower disease score, as shown in Table 1. However, adding too many resistant genes can be cumbersome and may impact other important traits. Notably, accessions EPEAL 102 and KALARKAR, with four resistant genes each (*Pi54+Pita+Pib+Pi38* and *Pi54+Pita+Pib+Pi2*), demonstrated lower disease scores. Breeders should consider using combinations involving *Pi54, Pita*, and *Pib* for a broad spectrum of resistance against *M. oryzae.* The study concludes that to develop stable, disease-resistant varieties, it is crucial to analyze effective gene combinations rather than simply accumulating resistance genes.

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