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Identification and validation of rice blast resistance genes in Indian rice germplasm

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Abstract

Blast disease caused by *Magnaporthe oryzae* is a major constraint in rice production. Identification of new donors for blast resistance is a pre-requisite for effective utilization of diverse germplasm for marker assisted incorporation of blast resistance into improved varieties. Therefore, in the present study, a set of 100 diverse rice germplasm accessions were evaluated for 11 blast resistance genes namely *Pik*^{m}, *Pik*, *Pik*^{h}, *Pi1*, *Pi5*, *Pi54*, *Pib*, *Piz5*, *Piz*, *Pi9* and *Pish*, both at genotypic and phenotypic level. Genotyping with gene based/ gene linked markers could identify six genotypes from the germplasm possessing as many as six resistance specific alleles. A total of 34 and 67 germplasm lines were found to possess resistance alleles for two genes, *Pik*^{m} and *Pik*, respectively. Phenotypic validation using artificial inoculation in the germplasm was carried out with 4 diverse isolates under controlled conditions. The congruence between marker genotype and disease phenotype on a set of monogenic lines for blast resistance in the LTH background was used to compute Disease Resistance Index (DRI) in the germplasm. Cumulative DRI for each genotype was computed over all the marker loci. The genotypes Heibao, Kalinga-I, Vijetha, Anjali, Bhaubhog, Sada Kaijam, Kala Jeera had high cumulative resistance score. Allelic Cumulative Disease Resistance Index (ACDRI), a measure for comparing the effectiveness of markers was calculated and markers linked to *Pik*^{m}, *Pik, Piz5, Pi1* were found to possess higher accuracy and better correlation with expected patterns of resistance under artificial inoculation. Based on disease resistance index, 25 germplasm accessions were found carrying blast resistance specific alleles at different loci and were fully validated for disease phenotype, which are valuable in breeding for resistance, allele mining and functional genomics studies.

Key words: Rice, blast disease, germplasm characterization, resistance genes, gene specific markers

Introduction

Rice (*Oryza sativa* L.) is a source of food to over three and a half billion people and contributes more than 20% of total dietary calories [1]. India, a country near Centre of origin of rice, is endowed with enormous rice genetic diversity and is a home to at least 50,000 landraces which are repositories of useful alleles for the traits of economic and biological importance [2]. Conservation of plant genetic resource is a very important activity but remains as 'gene morgues' unless they are characterized and utilized to 'virtue some marrows'. Rice being grown under varied ecologies, confronts several biotic stresses. The emergence of new virulent pathotypes has resulted in evolution of novel allelic forms of genes which is necessary for survival of genotypes and *vice versa*. One of the important biotic stresses affecting rice is blast disease caused by *Magnaporthe oryzae* and it accounts for 10-30% yield losses, even may be higher under uplands and cold areas [3]. Incorporation of genetic resistance in cultivars is an effective strategy to manage the disease. Rice blast follows the classical gene-for-gene system [4]. At host-pathogen interface, *Avr*-gene when recognized by the plant, R-gene triggers rapid and robust suite of cellular defense, which gets manifested as hypersensitive response at the infection site. Around 100 blast resistance genes have been genetically mapped [5] and 22 of them have already been cloned and characterized at sequence level. Some have broad resistance spectrum like *Pi9* [6], and *Pi54* [7] while some confer immunity towards

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predominant race groups in specific regions, such as *Pita* in US [8]. Further, the characterization of these genes in distinct genetic backgrounds relies heavily on the availability of the well defined differential stocks and diagnostic pathogen races which are though limited in number. However, availability of gene based markers helps in validating the presence of genes if present in the germplasm. Pertinently, it becomes necessary to validate the markers between susceptible and resistant backgrounds [9, 10]. Even though world over, many cultivars have been bred for blast resistance; the genes have been derived from a few donors. This may be because of the effectiveness of standard donors against the pathotypes with wide geographical distribution and also due to the fact that a large proportion of germplasm (> 90%) remains uncharacterized in the gene banks [11]. There is a reasonable scope for discovery and identification of the novel genes/ alleles in hitherto unexplored genetic resources. Also, the precise information vis-à-vis the nature of genes and reaction to most virulent isolates of the important rice collections is inadequate in India. Therefore, the present study was undertaken (i) to decipher the presence of genes governing blast resistance in diverse germplasm collection and (ii) their validation through phenotyping for disease reaction against the isolates representing different lineages for their potential utility in MAS programs.

Materials and methods

Plant material

A total of 100 rice germplasm accessions including 46 aromatic/non-aromatic varieties and 54 popular landraces uncharacterized for blast resistance and collected from various parts of India were used in the present study.

Magnaporthe oryzae isolates

A set of four isolates representing distinct pathotypes collected from different geographical locations namely, Mo-ni-0066, Mo-ni-0052, Mo-nwi-127 and Mo-nwi-31 were used to screen the germplasm. The isolates Mo-ni-0066 and Mo-ni-0052 are maintained at Indian Type Culture Collection (ITCC), Division of Plant Pathology, IARI, New Delhi, India whereas, Mo-nwi-31 and Mo-nwi-127 have been deposited in microbial type culture collection of Institute of Microbial Technology (IMTECH), Chandigarh, India vide accession number MTCC-11056 and -11510, respectively.

Culture preparation, inoculation and blast disease scoring

Stored cultures of each pathogen isolate were revived by inoculating the colonized filter discs on oatmeal agar slants. Mycelia from 10-day-old slants were macerated in 5 ml of distilled water and plated onto Mathur's medium (glucose 2.80 g, $MgSO_4.7H_2O$ 1.23 g, KH_2PO_4 2.72 g, neopeptone 2 g, agar 20 g, distilled water 1L) [12] for sporulation. After 8 to 10 days of incubation at 25 ± 1^oC, the plates were washed with 10 ml of distilled water to make a spore suspension. Each spore suspension was filtered through two layers of muslin cloth and the spore concentration was adjusted to 10^5 spores/ml. About 30-40 ml of the spore suspension containing gelatin (0.1%) and Tween-20 (0.02%) was sprayed onto 21-day-old seedlings using a glass atomizer. Inoculated seedlings were kept in a humidity chamber maintained at 25 ± 1^oC and sprayed three to four times a day with distilled water to maintain high humidity.

Seedlings of all the rice genotypes were raised in 5x4 well plastic pro-trays each well having diameter of 5 cm holding 6 seedlings per well. The clean soil was fertilized one week prior to sowing with well decomposed organic matter, N_2 and P_2O_5 as per recommendation. Pots were kept in proper and healthy environment to ensure luxuriant growth. Twenty-one-day old seedlings (3-4 leaf stage) were inoculated following the method of Bonman [13]. Seedlings were sprayed uniformly with hand atomizer (100 kpa). About 40 ml spore suspension per fungal isolate was sprayed over each pro-tray. The inoculated plants were transferred to dew chambers and kept for 24 h, at 25^oC and 95-100% RH. Subsequently, the plants were transferred to a mist room and kept at $25\text{-}28^oC$ for 6-7 days.

Disease scoring was done seven days after inoculation (DAI) on a 0-5 disease rating scale [14]. After four days interval, second reading was taken. The germplasm with disease rating 0-2 was rated as resistant and 3-5 were grouped as susceptible. The experiment was conducted with two replications and repeated twice.

Marker analysis

Genomic DNA of all the 100 accessions was extracted from fresh, healthy and young leaf tissue from eighteen-day-old seedlings following CTAB (Cetyl-Tri Methyl Ammonium Bromide) method [15]. The DNA was purified by adding RNase (10μg/100ml) to the sample

at the rate of 1 µl/100 ml of crude DNA. DNA quantification was done using 0.8% Agarose gel. The λ uncut DNA was used as a standard and the final concentration was adjusted to ~25 ng/µl. PCR assay was performed for 11 blast resistance genes using gene based /gene linked InDel/STMS markers (Table 1). The markers Pi54 MAS (*Pi54*), Ckm-1, Ckm-2 (*Pik*^{m}), and Pibdom (*Pib*) are based on internal gene sequences. The markers k-2167, k-6816 (*Pik*), JJ-803 (*Pi5*) and AP-5659-5 (*Piz5, Piz* and *Pi9*) are part of sequences within 100-300 kb near the target genes; and 4 others namely RM224, RM1233 and OSR3 were closely linked to the genes *Pik*^{h}, *Pi1* and *Pish*, respectively. PCR reaction was carried out as described by Shikari et al [16], with minor modification in annealing temperatures for different primer pairs as indicated in Table 1. The PCR amplified products were resolved on 1.5% to 3.5% agarose gel, depending upon the primer used and the gel slabs were visualized in UV trans-illuminator and documented in gel documentation system (Bio-Rad Laboratories Inc., Hercules, CA).

Data analysis

For each marker data point (MDP) the amplified fragment was scored as resistant and susceptible allele according to its previously reported association with the disease (Table 1). LTH background monogenic lines along with the susceptible controls LTH and Co39 were placed as positive checks. The disease score against all the four isolates at each MDP was compared to the expectation on the basis of marker genotype specific to resistance allele. Disease score against four isolates was converted into an index named as Disease Resistance Index (DRI), which was obtained on dividing the number of isolates against which the genotype is resistant by number of *Avr*-isolates based on the analysis with monogenic differentials**.** Ultimately, these individual index scores at each marker genotype were summed to yield Genotypic Cumulative Disease Resistance Index (GCDI) across all markers (genes) for a given germplasm accession, $GCDI = \left[\sum_{gene=1}^{12} DRI\right]$ was calculated by including the reaction at *Pita* locus

Table 1. Molecular markers and the corresponding genes for resistance to blast assayed across 100 germplasm/ varieties

S.No.	Gene	Marker	Primer sequence	Chr.	Linkage distance	Ref
1	*Pi54*	Pi54 InDel	F: 5'CAATCTCCAAAGTTTTCAGG 3' R: 5'GCTTCAATCACTGCTAGACC3'	11	Gene based	[17]
2	*Pik*^{m}	Ckm-1	F: 5'TGAGCTCAAGGCAAGAGTTGAGGA3' R: 5'TGTTCCAGCAACTCGATGAG3'	11	Gene based	[20]
		Ckm-2	F: 5'CAGTAGCTGTGTCTCAGAACTATG3' R: 5'AAGGTACCTCTTTTCGGCCAG3'			
3	*Pik*	k-2167	F: 5'CGTGCTGTCGCCTGAATCTG3' R: 5'CACGAACAAGAGTGTGTCGG3'	11	0.1 cM	[21]
		k-6816	F: 5'TCGCCGATGCGGTTGATTTACTC3' R: 5'CGTATTTTGTGTTGTTAGGAGATAAGG3'		1.4 cM	
4	*Pi5*	JJ-803	F: 5'AAGTGAGCATCCAGTGCCTAATGA3' R: 5'AGCCGGTGCTCATAACACGTATTA3'	9	Gene based	[30]
5	*Pik*^{h}	RM224	F: 5'ATCGATCGATCTTCACGAGG3' R: 5'TGCTATAAAAGGCATTCGGG3'	11	0 cM	[23]
6	*Pi1*	RM1233	F: 5'TTCGTTTTCCTTGGTTAGTG3' R: 5'ATTGGCTCCTGAAGAAGG3'	11	0 cM	
7	*Pib*	Pibdom	F: 5'GAACAATGCCCAAACTTGAGA3' R: 5'GGGTCCACATGTCAGTGAGC3'	2	Gene based	
8	*Piz/ Pi9/ Piz5*	AP5659-5	F: 5'CTCCTTCAGCTGCTCCTC3' R: 5'TGATGACTTCCAAACGGTAG3'	6	0.05 cM	[36]
9	*Pish*	OSR-3	F: 5'AGCTAAGGTCTGGGAGAAACC3' R: 5'AAGTAGGATGGGGCACAAGCTC3'	1	5 cM	[41]

studied previously [16]. Another parameter, Allelic Cumulative Disease Resistance Index (ACDRI) was calculated based on the cumulative score across accessions for each marker (gene), $ACDRI = \left[\sum_{genotype=1}^{100} DRI\right]$. ACDRI was further used to calculate Percent Marker Efficiency (PME) of a given marker by dividing it by total number of genotypes with resistance allele at marker locus.

Results and Discussion

Virulence pattern of the M. oryzae isolates

Prior to the inoculation on germplasm, the pathotypes were validated for their reaction on 24 monogenic differentials. The isolates exhibited a virulence frequency of 68.0%, 65.3%, 8.0% and 72.0%, respectively across a set of monogenic differential lines representing 24 R-genes in the background of susceptible variety '*Lijiangxintuanheigu'*, hereafter referred as *LTH*. Each of the four pathotypes was characterized for *Avr-R*-gene combinations and based on incompatible (*Avr*-) reaction the inferences were drawn regarding the functions of the specific gene for the given germplasm. The isolate Mo-ni-0066 is avirulent to the genes *Pi54* and *Piz.* The genes *Pi54*, *Pik*, *Pik^h*, *Pib* and *Piz* showed resistant to Mo-ni-0052. Another isolate Mo-nwi-31 is virulent isolate against all the 24 monogenic differentials except the ones for *Pik^m*, *Pik^h*, *Pish*, *Pii* and *Pi5*. Mo-nwi-127 is a weak isolate as observed by its virulence against only one gene, *Pia*. The virulence/ avirulence gene constitution of the isolates on 12 blast resistance genes has been presented in Table 2.

Genotyping using gene based /linked markers

Out of 100 germplasm genotyped, six germplasm accessions were found having 6 genes each; four with 5 genes each; twenty five lines possessed 4 genes each, thirty three entries had 3 genes, twenty six were positive for 2 genes each and five with 1 gene each, as was inferred by the presence of resistance specific alleles on analysis with gene based/ linked markers (Table 3, Fig. 1.). The InDel marker Pi54 MAS for gene *Pi54*, amplified a resistance specific fragment of 216 bp in 26 germplasm lines. Similarly, through the PCR assay using above mentioned markers, the genes *Pik^m*, *Pik*, *Pik^h*, *Pi1*, *Pi5*, *Pib*, *Piz5*, *Piz*, *Pi9* and *Pish* were predicted to be present in 34, 67, 24, 19, 36, 25, 27, 8, 8 and 10 germplasm accessions, respectively. Similar inferences have been made based on the analysis of two gene based markers run on the same set of germplasm, where in 27 accessions were found to harbour dominant *Pita-Pita^2* resistance specific allele [16].

Table 2. Virulence pattern of isolates against a set of monogenic differentials with selected genes in the background of LTH

IRRI monogenic differentials	Gene	*M. oryzae* isolates				No. of isolates with *Avr*-reaction
		Mo-ni-0066	Mo-ni-0052	Mo-nwi-127	Mo-nwi-31	
IRBLkm-TS	*Pik^m*	*Vir*	*Vir*	*Avr*	*Avr*	2
IRBLk-Ka	*Pik*	*Vir*	*Avr*	*Avr*	*Vir*	2
IRBLkh-K3	*Pik^h*	*Vir*	*Avr*	*Avr*	*Avr*	3
IRBL1-CL	*Pi1*	*Vir*	*Vir*	*Avr*	*Vir*	1
IRBL5-M	*Pi5*	*Vir*	*Vir*	*Avr*	*Avr*	2
IRBLb-B	*Pib*	*Vir*	*Avr*	*Avr*	*Vir*	2
IRBLz-Fu	*Piz*	*Avr*	*Avr*	*Avr*	*Vir*	3
IRBL9-W	*Pi9*	*Vir*	*Vir*	*Avr*	*Vir*	1
IRBLz5-CA	*Piz5*	*Vir*	*Vir*	*Avr*	*Vir*	1
IRBLsh-S	*Pish*	*Vir*	*Vir*	*Avr*	*Avr*	2
IRBLta-K1	*Pita*	*Vir*	*Avr*	*Avr*	*Vir*	2
-	*Pi54*	*Avr*	*Avr*	-	-	2
Virulence of isolate on 24 monogenic differentials		68.2%	65.3%	8.0%	72.0%	

Table 3. Molecular marker analysis and disease reaction of germplasm accessions

S.No.	Germplasm	Disease reaction against isolates				No. of genes based on marker information	GCDI	Estimated alleles based on marker information and DRI
		Mo-ni-0066	Mo-ni-0052	Mo-nwi-127	Mo-nwi-31			
1	2	3	4	5	6	7	8	9
Varieties								
1	Jaldi Dhan 6	4	4	0	4	2	0.50	*Pi54 (0), Pik (0.5)*
2	CSR 10	4	4	1	4	6	2.83	*Pi54 (0), Pik^m (0.5), Pik^h (0.33), Pi5 (0.5), Pib (0.5), Piz5 (1.0)*
3	IR 50	0	2	0	4	4	3.50	*Pita (1.0), Pik (1.0), Pi5 (0.5), Pib (1.0)*
4	Sona Mahsuri	1	3	1	1	5	4.00	*Pi54 (0.5), Pik^m (1.0), Pi5 (1.0), Pib (0.5), Pish (1.0)*
5	Danteshwari	4	4	1	4	2	1.00	*Pik^m (0.5), Pib (0.5)*
6	Chaitanya	3	4	1	1	3	1.50	*Pi54 (0), Pik^m (1.0), Pib (0.5)*
7	Rasi	0	2	1	4	2	2.00	*Pik (1.0), Pib (1.0)*
8	K-429	3	4	4	5	3	0.00	*Pik^m (0), Pik^h (0), Pib (0)*
9	Pusa 33	4	4	1	5	3	1.00	*Pi54 (0), Pik^m (0.5), Pib (0.5)*
10	Tai Pei 309	4	3	5	3	6	0.00	*Pik^m (0), Pik^h (0), Pib (0), Piz (0), Pi9 (0), Pi19 (0), Pish (0)*
11	Heibao	1	2	1	1	5	5.00	*Pi54 (1.0), Pik (1.0), Pi1 (1.0), Pi5 (1.0), Pish (1.0)*
12	Kalinga-I	5	1	1	5	5	4.00	*Pita (1.0), Pik^m (0.5), Pik (1.0), Pi1 (1.0), Pi5 (0.5)*
13	Vijetha	0	2	0	1	4	4.00	*Pi54 (1.0), Pik^m (1.0), Pi1 (1.0), Pib (1.0)*
14	K 332	4	4	5	4	6	0.00	*Pi54 (0), Pik^m (0), Pik^h (0), Pi5 (0), Piz5 (0), Pi9 (0)*
15	Chandrahasini	0	1	1	5	4	3.50	*Pita (1.0),Pik (1.0), Pi5 (0.5), Pib (1.0)*
16	Varun Dhan	5	4	4	4	6	0.00	*Pi54 (0), Pik (0), Pik^h (0), Pib (0), Pi9 (0), Pish (0)*
17	Manhar	2	0	1	5	2	2.00	*Pik (1.0), Pi1 (1.0)*
18	Anjali	1	4	1	1	6	5.00	*Pik^m (1.0), Pik (0.5), Pi1 (1.0), Pi5 (1.0), Pib (0.5), Pish (1.0)*
19	ADT 37	1	0	1	4	3	3.00	*Pita (1.0), Pik (1.0), Pi1 (1.0)*
20	Suphala	4	2	1	4	6	4.66	*Pita (1.0), Pi54 (0.5), Pik^m (0.5), Pik (1.0), Piz (0.66), Pi9 (1.0)*
21	Subhadra	4	4	1	2	3	2.00	*Pik (0.5), Pi1 (1.0), Pib (0.5)*
22	Keshari	3	2	1	1	4	3.50	*Pi54 (0.5), Pik 9 (1.0), Pi1 (1.0), Pi5 (1.0)*
23	Rudra	3	3	1	2	4	2.50	*Pi54 (0), Pik^m (1.0), Pik (0.5), Pi1 (1.0)*
24	Shankar	4	4	2	5	3	1.00	*Pi54 (0), Pik^m (0.5), Pik (0.5)*
25	Pathara	1	2	1	5	4	4.00	*Pi54 (1.0), Pik (1.0), Pi1 (1.0), Pib (1.0)*
26	Badami	4	4	3	5	1	0.00	*Pik (0)*
27	Nilagiri	4	0	4	5	1	0.50	*Pik (0.5)*
28	IR-8	4	4	1	5	4	1.33	*Pi54 (0), Pik^m (0.5), Pik^h (0.33), Pib (0.5)*
29	IR-64	4	2	0	5	4	3.33	*Pita (1.0), Pik^m (0.5), Pik (1.0), Pib (1.0)*

(Contd ...)

1	2	3	4	5	6	7	8	9
30	Keshav	2	0	1	1	4	4.00	*Pita (1.0), Pik^m (1.0), Pik^h (1.0), Pib (1.0)*
31	Indira Sugandh Dhan-1	4	4	0	4	2	1.00	*Pik (0.5), Pib (0.5)*
32	Pant Dhan 12	5	2	1	4	3	2.50	*Pita (1.0), Pik^m (0.5), Pik (1.0)*
33	Prasad	2	0	1	1	2	2.00	*Pik (1.0), Pi5 (1.0)*
34	Swarnamukhi	0	0	0	1	4	4.00	*Pita (1.0), Pi54 (1.0), Pik^m (1.0), Pik^h (1.0)*
35	Sarathi	2	0	0	4	4	3.50	*Pi54 (1.0), Pik (1.0), Pi5 (0.5), Pib (1.0)*
36	PR118	3	0	0	4	4	2.66	*Pi54 (0.5), Pik^m (0.5), Pik^h (0.66), Pib (1.0)*
37	CSR23	4	0	0	4	3	2.50	*Pik^m (0.5), Pik (1.0), Pib (1.0)*
38	Pratiksha	4	0	1	1	2	2.00	*Pita (1.0), Pik (1.0)*
39	Samleshwari	2	2	0	4	3	3.00	*Pita (1.0), Pik (1.0), Pib (1.0)*
40	ND-118	4	0	2	5	3	2.50	*Pita (1.0), Pik^m (0.5), Pik (1.0)*
41	Haryana Basmati1	0	4	1	4	4	2.50	*Pi54 (0.5), Pik^m (0.5), Pi1 (1.0), Pi5 (0.5)*
42	Super Basmati	1	2	1	2	2	2.00	*Pi54 (1.0), Pik^m (1.0)*
Landraces								
43	Basmati (Orissa)	3	2	2	3	3	2.16	*Pik (1.0), Pik^h (0.66), Pi5 (0.5)*
44	Latasal	5	5	1	4	4	3.00	*Pita (0.5), Pik^m (0.5), Pi1 (1.0), Piz5 (1.0)*
45	Paani dooba	1	2	1	4	3	3.00	*Pik (1.0), Pi1 (1.0), Piz5 (1.0)*
46	Banstana	0	0	1	4	2	2.00	*Pik (1.0), Pib (1.0)*
47	Baubhog	5	2	1	4	5	4.66	*Pita (1.0), Pik (1.0), Pik^h (0.66), Pi1 (1.0), Pi5 (1.0)*
48	Kalo mota	1	1	2	3	3	2.66	*Pik (1.0), Pik^h (0.66), Piz5 (1.0)*
49	Mehandi	5	4	4	4	3	0.00	*Pik (0), Pi1 (0), Piz5 (0)*
50	Sonashree	5	4	2	1	2	1.50	*Pik (0.5), Piz5 (1.0)*
51	Jata Dhan	2	2	2	4	3	3.00	*Pita (1.0), Pik (1.0), Piz5 (1.0)*
52	Lal Dusari	5	4	1	4	4	3.50	*Pita (1.0), Pik (0.5), Pi1 (1.0), Piz5 (1.0)*
53	Tulsi Mukul	3	5	1	1	2	1.16	*Pik (0.5), Pik^h (0.66)*
54	Kishori	4	2	1	1	3	3.00	*Pik^m (1.0), Pik^h (1.0), Pi5 (1.0)*
55	Khaja	2	4	2	3	4	1.83	*Pita (0.5), Pik (0.5), Pik^h (0.33), Pi5 (0.5)*
56	Bangla Patni	4	3	1	4	2	1.00	*Pik (0.5), Pi5 (0.5)*
57	Lal Patri	2	5	1	4	3	1.50	*Pik^m (0.5), Pik (0.5), Pi5 (0.5)*
58	Dehraduni Gaudeshwari	5	5	2	3	2	0.50	*Pi54 (0), Pik (0.5)*
59	Kumargarh	4	0	1	4	2	1.50	*Pik (1.0), Pi5 (0.5)*
60	Tulsa	1	3	1	3	2	1.50	*Pik (0.5), Piz5 (1.0)*
61	Kalo Bhutia	2	0	1	1	4	4.00	*Pita (1.0), Pik^m (1.0), Pik^h (1.0), Pi5 (1.0)*
62	Birui	5	0	1	1	3	3.00	*Pita (1.0), Pik^h (1.0), Pi5 (1.0)*
63	Mayur Pankhi	4	4	1	4	3	1.83	*Pik (0.5), Piz (0.33), Pi9 (1.0)*
64	Boarti	5	4	1	4	3	1.50	*Pita (0.5), Pik (0.5), Pi5 (0.5)*
65	Sada Kaijam	1	4	1	2	4	4.00	*Pik^m (1.0), Pi1 (1.0), Piz5 (1.0), Pish (1.0)*
66	Paran Kalas	1	4	2	4	4	3.00	*Pik (0.5), Pi1 (1.0), Pi5 (0.5), Piz5 (1.0)*
67	Mourisal	4	2	2	1	3	2.50	*Pi54 (0.5), Pik^h (1.0), Pi5 (1.0)*
68	Sabita	1	4	1	4	4	2.00	*Pita (0.5), Pik (0.5), Pi5 (0.5), Pish (0.5)*
69	Lakki Kajal	1	0	1	2	2	2.00	*Pik^m (1.0), Pish (1.0)*
70	Kala Munia	4	4	2	1	4	3.66	*Pik^h (0.66), Pi5 (1.0), Piz5 (1.0), Pish (1.0)*
71	Lad Sal	3	4	4	4	3	0.00	*Pik (0), Piz5 (0), Pi9 (0)*
72	Agni Baou	4	2	2	4	3	3.00	*Pita (1.0), Pik (1.0), Piz5 (1.0)*

(Contd ...)

1	2	3	4	5	6	7	8	9
73	Durga Sudami	5	5	1	2	4	3.00	*Pita (0.5), Pik (0.5), Pi5 (1.0), Piz5 (1.0)*
74	Kakhow	4	5	2	2	4	3.00	*Pita (0.5), Pik (0.5), Piz5 (1.0), Pish(1.0)*
75	Chima Kamin	4	2	2	4	3	2.66	*Pik (1.0), Piz (0.66), Pi9 (1.0)*
76	Kala Jeera	1	2	1	1	4	4.00	*Pita (1.0), Pik^m (1.0), Pik^h (1.0), Pi5 (1.0)*
77	Lakhi Chura	2	0	1	3	2	1.50	*Pik (1.0), Pi5 (0.5)*
78	Pakhri	0	2	2	2	2	2.00	*Pik^h (1.0), Pi5 (1.0)*
79	Geetanjali	1	3	2	4	2	1.00	*Pik (0.5), Pi5 (0.5)*
80	Badshah Bhog	2	4	1	4	3	1.83	*Pik^h (0.33), Pi5 (0.5), Piz5 (1.0)*
81	Paoba	4	2	1	4	3	2.50	*Pita (1.0), Pik^m (0.5), Pik (1.0)*
82	Leela Bati	4	5	1	4	2	1.00	*Pik (0.5), Pi5 (0.5)*
83	Mala	4	0	1	4	1	1.00	*Pik (1.0)*
84	Sadhajhumur	4	0	2	4	2	2.00	*Pita (1.0), Pik (1.0)*
85	Super Sugan-dhamati	4	2	1	4	2	2.00	*Pik (1.0), Piz5 (1.0)*
86	Katori Bbhog	4	3	0	1	3	2.50	*Pik (0.5), Pi5 (1.0), Piz5 (1.0)*
87	Tangra	4	4	0	5	1	1.00	*Piz5 (1.0)*
88	Jugal	3	4	1	2	3	2.50	*Pik (0.5), Pi5 (1.0), Piz5 (1.0)*
89	Pak Basmati	3	4	1	4	3	2.00	*Pik (0.5), Pi5 (0.5), Piz5 (1.0)*
90	Bankra	5	2	2	4	3	2.50	*Pi54 (0.5), Pik (1.0), Piz5 (1.0)*
91	Poonti Kaami	2	3	2	1	1	1.00	*Piz5 (1.0)*
92	Lalmeeta	2	3	2	2	2	1.66	*Piz (0.66), Pi9 (1.0)*
93	Khayersal	1	4	2	1	3	3.00	*Pik^m (1.0), Pi1 (1.0), Piz5 (1.0)*
94	Janghi Jata	2	3	2	1	2	1.66	*Pik^m (1.0), Pik^h (0.66)*
95	Tuniaslet	2	3	2	4	2	1.66	*Piz (0.66), Pi9 (1.0)*
96	Randhuni Pagal	1	4	2	4	2	0.83	*Pik (0.50), Pik^h (0.33)*
Varieties								
97	Shalimar Rice 1	1	2	1	4	3	3.00	*Pi54 (1.0), Pik (1.0), Piz5 (1.0)*
98	Jhelum	4	3	1	5	0	0.00	-
99	Sneha	3	4	1	4	4	2.00	*Pi54 (0), Pik (0.5), Pib (0.5), Piz5 (1.0)*
100	Pusa Basmati 1	4	4	1	4	3	1.00	*Pi54 (0), Pik (0.5), Pi5 (0.5)*

Blast Score 0-2 = resistant, 3-5 = susceptible; Disease Resistance Index (DRI) = [No. of isolates against which a genotype is resistant] / [No. of isolates avirulent against particular gene based on analysis with monogenic differential lines]; Genotypic Cumulative Disease Resistance Index (GCDI) = DRI was calculated only for accessions with resistance specific allele under PCR assay. The reaction for genotypes previously [16] found amplifying *Pita-Pita*^{2} allele was included in working out DRI and GCDI. Twenty five lines of 100 recorded GCDI value equal to 1.00.

Phenotyping of rice lines

The four pathotypes used in present study i.e. Mo-ni-0066, Mo-ni-0052, Mo-nwi-127 and Mo-nwi-31 showed a virulence spectrum of 57, 51, 7 and 67%, respectively across all the germplasm lines.

Allelic distribution of Pi54 in rice

Out of 26 accessions which were *Pi54* positive, 7 expressed one-to-one relation (DRI=1.0) between marker genotype and the response from 2 *Avr*- isolates for *Pi54*, whereas, 7 marker positive genotypes showed DRI of 0.5 and 12 others expressed complete susceptibility with DRI equal to 0. The 7 entries with DRI of 1.0 included Heibao, Vijetha, Pathara, Swarnamukhi, Sarathi, Super Basmati and Shalimar Rice-1. Of these, the accessions Vijetha and Swarnamukhi were observed to show similar pattern in the validation studies reported earlier by Ramkumar *et al*. [17]. Besides they also included the accessions namely, Danteshwari (S), Haryana Basmati (R), Taipei 309 (S) and IR64 (S), which matched our results for gene *Pi54*, which has been found to be effective in NW Himalayas [18].

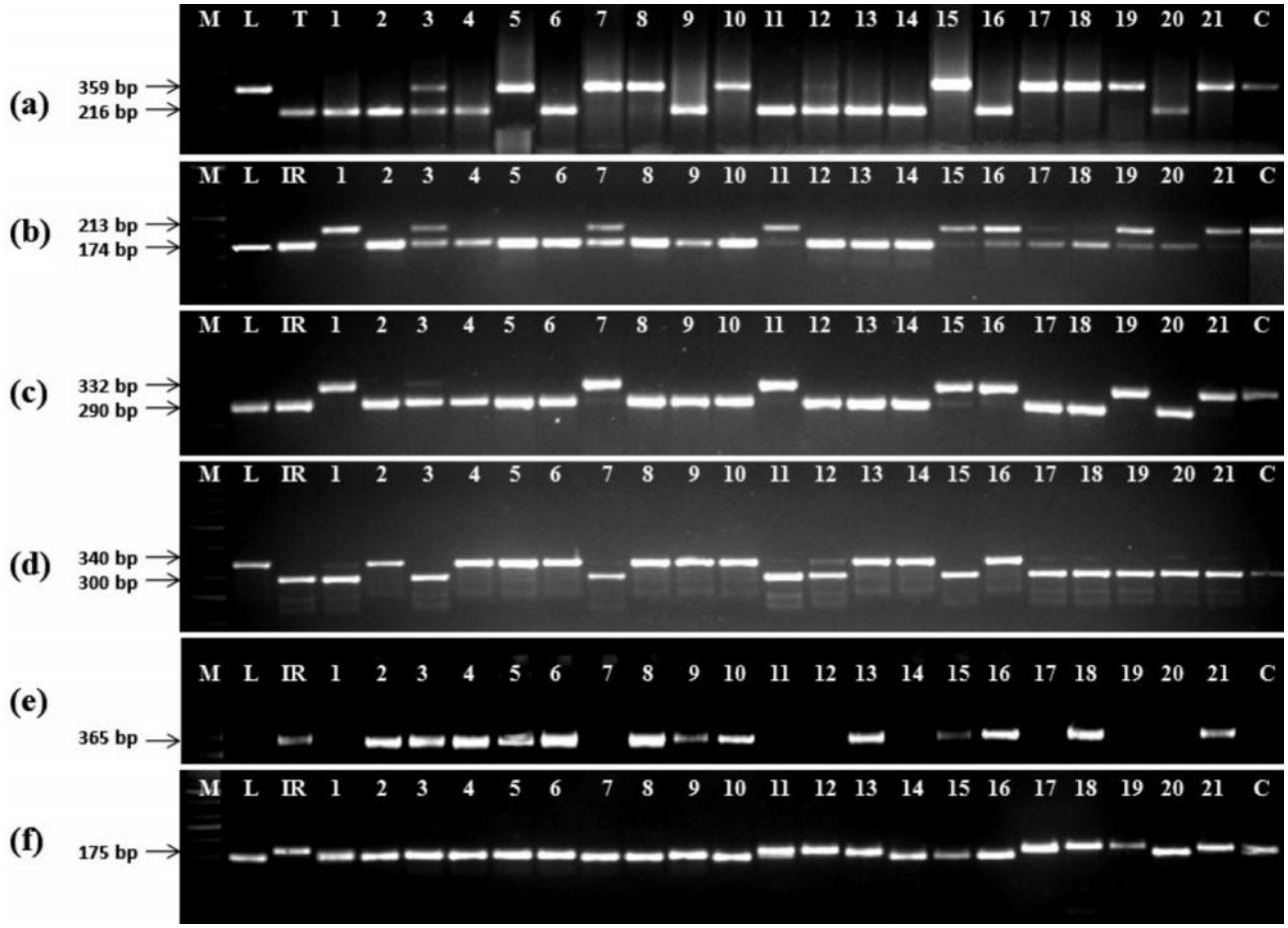

Fig. 1. A representative amplification profile generated using gene based/ gene linked markers: a = *Pi54* MAS (*Pi54*); b = Ckm-1 (*Pik^{m}1-TS*); c = Ckm-2 (*Pik^{m}2-TS*); d = k-6816 (*Pik*); e = Pibdom (*Pib*); f = RM1233 (*Pi1*); M = 50 bp ladder (Fermentas, Lithuania, USA); L = Blast susceptible check LTH; C = Susceptible check Co39; T = Tetep; IR = IRRI LTH background monogenic differential lines IRBLkm-TS (*Pik^m*) for (b) and (c); IRBLk-Ka (*Pik*) for d; IRBLb (*Pib*) for e and IRBL1-CL (*Pi1*) for f; Lanes 1-21: Germplasm accessions (Refer first column of Table 3); Arrow heads indicates fragment size in base pairs

Pik^m

Out of 34 genotypes positive for *Pik^m*, 15 were found to show complete match to the expectations based on *Pik^m* present in monogenic differential line IRBLKm-TS. These included the rice cultivars namely Sona Mahsuri, Chaitanya, Vijetha, Anjali, Rudra, Keshav, Swarnamukhi, Super Basmati, Kishori, Kalo Bhutia, Sada Kaijam, Lakki Kajal, Kala Jeera, Khayersal and Janghi Jata. Besides, 3 accessions expressed susceptibility both the diagnostic isolates, while, 16 genotypes showed DRI of 0.5. The *Pik^m* gene in the monogenic line IRBLKm-TS has been derived from cultivar, Tsuyuake. Ashikawa *et al*. [19] showed that the functional *Pik^m* gene belongs to NBS LRR class of R-genes and carries two sub sequences *Pik^m-TS* and *Pik^m-TS* which are present adjacent to each other but are oriented in opposite directions. Costanzo and Jia [20] found that both the sub-sequences had NBS-LRR with *Pik^{m}1-TS* being more divergent across germplasm while *Pik^{m}2-TS* happen to be conserved. Based on the sequence comparisons between Tsuyuake and cultivars, Nipponbare and 93-11, they developed two codominant markers Ckm-1 and Ckm-2 corresponding to *Pik^{m}1-TS* and *Pik^{m}2-TS*, respectively, which complement each other to confer *Pik^m* mediated resistance. Therefore, only the genotypes amplifying 174 bp and 290 bp alleles, respectively, for these two sub-sequences were expected to show *Pik^m* specific resistance and thus were classified to carry *Pik^m*. IR64 was one such

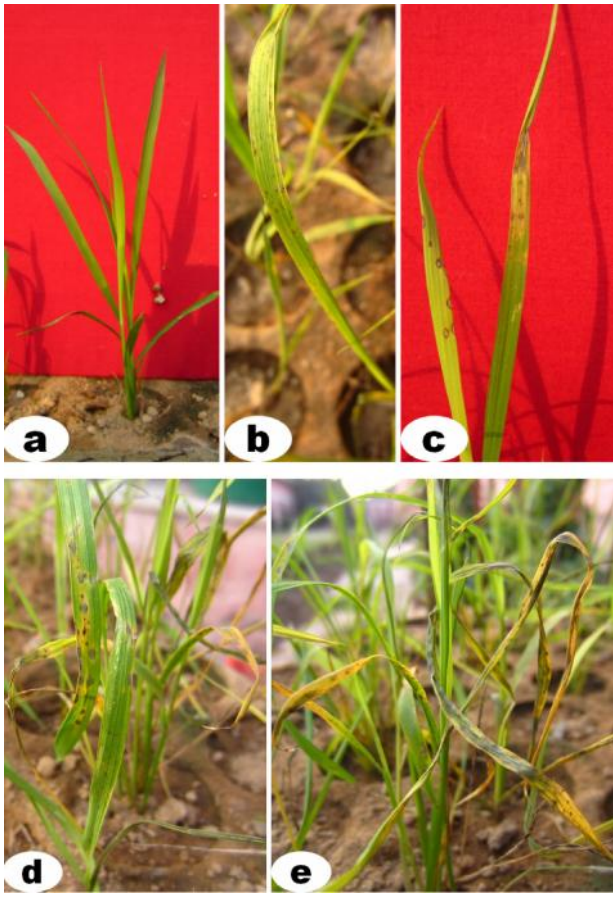

Fig. 2. Phenotypic reaction of representative germplasm lines against specific *Magnapor the oryzae* isolates under controlled conditions : a = Banstana (isolate: Mo-ni-0066; reaction score: 0); b = Kalo mota (isolate: Mo-ni-0052; reaction score: 1); c = Jhelum (isolate: Mo-ni-0066; reaction score: 4); d = Boarti (isolate: Mo-ni-0066; reaction score: 5); e = Tulsi mukul (isolate: Mo-ni-0052; reaction score: 5)

example identified here to possess *Pik*^{m} allele which is in conformation with the earlier validation studies [20]. Also, some entries in their analysis have shown resistance-susceptible alleles (+/-) similar to the present investigation, however, those genotypes were considered as having susceptibility specific genotype.

Pik

With respect to *Pik*, out of 67 genotypes carrying resistance specific alleles 34 were actually resistant to both the diagnostic isolates, 29 were resistant to either of them and 4 were susceptible to both the isolates. Hayashi *et al*. [21] found co-segregation of markers k-6816 and k-2167 with the gene *Pik* in F_2 population derived from Kanto 51 (*Pik*$^+$) x OISL 235 (*Pik*$^-$). Presently, both these markers are considered to identify the *Pik*$^+$ accessions. Thirty six germplasm accessions amplified k-2167 (619 bp) resistance allele and 59 had k-6816 (339 bp) allele specific to resistance phenotype. Of these, 25 accessions carried both k-2167 and k-6816 resistance specific alleles, while 11 and 34 amplified individually the respective alleles. The markers k-2167 and k-6816 were mapped at ~200 kb and ~350 kb from *Pik* with former falling in middle flanked by *Pik* and k6816. On comparing the marker profiles of Ckm-1 and Ckm-2 combination to that with k-2167 and k-6816, we found that the germplasm having resistant *Pik*^{m} alleles with respect to the markers Ckm-1 and -2, also possessed *Pik*^{m} specific (alternate) allele for k-2167 (~300 bp) and k-6816 (~380 bp). This can be explained as k-2167 and k-6816 also co-segregate with *Pik*^{m} with distinct fragment size as that of *Pik*. Hayashi *et al*. [21] found 12 SNPs and 2 InDels between Kanto 51 and Koshihikari. Therefore, polymorphism in other regions within and near the genes explains the differential interaction of isolates and loss of function of some marker positive accessions. Also, the presence of *Pik*^{p} in some of these accessions cannot be ruled out as all these genes are part of *Pik* cluster. The gene *Pik*^{p} reportedly map between *Pik*^{m} and *Pik* and the whole region falls in recombination suppressed region on the long arm of chromosome 11.

Pik^h

Twenty four genotypes amplified *Pik*^{h} resistance specific fragment out of which only 8 accessions showed perfect association between gene and the phenotype, while, 7, 5 and 4 genotypes had DRI of 0.66, 0.33 and 0.00, respectively. The microsatellite marker, RM224 was found to co-segregate with *Pik*^{h} at 0.9 cM in F_2 population of cross between IRBLKh-K3/Co39 [22] and at 0 cM in Maybelle 2 / Kaybonnet F_2 population [23]. A ~140 bp fragment is amplified in resistant genotypes and reported it as a most important marker. Since, *Pik*^{h} is a part of *Pik* cluster among which *Pik*^{m} is reported to show similar phenotype reaction pattern as *Pik*^{h} [22], therefore, the possibility of presence of *Pik*^{h} and *Pik*^{m} in *Pik*^{m} and *Pik*^{h} positive individuals seems likely. Infact, a number of germplasm accessions showed the presence of both the genes in our collections. The differential behavior to isolates wherever is present, may be attributed to dissimilar linkage blocks spanning *Pik* locus which might have been derived from the uncommon ancestral parents. Since, it was mentioned above that Tetep,

Tadukan and K3 all have contributed to resistance at *Pik* locus in specific geographical lineages.

Pi1

In case of gene *Pi1*, 18 out of 19 positive entries had resistance against one diagnostic isolate. *Pi1* gene maps near the telomeric region of chromosome 11L and has been found to co segregate with markers RM224 and RM1233 at 0 cM [23, 24]. In India, the gene has got low resistance spectrum and individually is not much effective. However, the combination with *Pita* and *Pi2* has been found promising [25]. RM224 is also closely linked to Pik^h in the near vicinity of *Pi1* and its phenotypic response is related with the same product size. However, in present study considering the differential response of Pik^h and *Pi1* monogenic differentials it can be concluded that entries like Nilagiri and Tulsimukul amplified RM224 resistance specific allele and are likely to carry *Pi1* as per the response against 4 isolates. The entries positive for RM1233 need to be tested for the presence of *Pi1* with additional isolates to gain more clarity.

Pi5

We amplified *Pi5* resistance allele from thirty six germplasm accessions using JJ803 marker. Of these, 15 expressed incompatibilities to the two isolates, 20 were found resistant to either of them and was susceptible. *Pi5* is allelic to the genes *Pi3* and *Pii* [26, 27, 28, 29]. Yi *et al*. [29] which has been shown by using three primer sets JJ113-T3, JJ81-T3 and JJ80-T3 amplified common fragment in Tetep, C104PKT (*Pi3*), RIL260 (*Pi5*) and the fragment co-segregated with PO6-6 resistance specific to *Pi5, Pii* and *Pi3*. Lee *et al*. [30] suggested that SCAR marker JJ803 (derived from dominant marker JJ80-T3) co-segregated with *Pi5* mediated resistance at 0 cM and the marker is a part of 90 kb sequence which spans *Pi5-1* and *Pi5-3* sub-sequences in Nipponbare which however, lacks *Pi5-2* present in resistant RIL260. Since, *Pi5-1* and *Pi5-2* complement each other and condition *Pi5* mediated resistance; the absence of any of these will make germplasm susceptible even in case if it is positive for the marker JJ803.

Pib

Out of 25 genotypes with *Pib* specific allele, 12 genotypes had matching genotype at marker allele and phenotype with 2 *Avr*-isolates; 10 had DRI of 0.5 and 3 were susceptible to both. *Pib* represents the first ever blast resistance gene to be cloned by Wang *et al*. [31] and the sequence information revealed was used by Fjellstrom et al. [23] for designing of dominant gene based marker Pibdom. The 365 bp allele of *Pib* is associated with blast resistance in the germplasm. Since, *Pib* belongs to a small gene family in *Pib*-cluster on chromosome 2, the probability of recombination between Pibdom and resistance governing modifiers within the cluster [23]. Moreover, *Pib* resistance is believed to be largely influenced by environmental conditions (temperature, humidity, etc.) prevailing at infection site. The resistance genotype in present germplasm accessions was confirmed by pathotype response with few anomalies which could be attributed to above mentioned reasons. Ebron et al. [32] analysed the genes in IRRI based varieties and reported the presence of *Pib* in dwarfing gene source DGWG and in Peta (parents to IR8). Since DGWG is a part of lineage to many of the HYVs, the predominance of *Pib* is expected as was reported for IRRI collections. In our investigations and in conformity to above expectations, IR8 and IR50, both amplified *Pib* resistance allele using Pibdom.

Piz5

For *Piz5*, where only one isolate was found to be avirulent, 26 out of 27 marker positive accessions expressed resistance. Of the eight genotypes which amplified resistance specific allele for *Piz*, none was found to be resistant to the three diagnostic isolates, however, DRI of 0.0, 0.33 and 0.66 was recorded for three, one and four accessions, respectively. Moreover, five out of 8 *Pi9* positive accessions were found to have resistance phenotype. The genes *Piz5*, *Piz* and *Pi9* map near the centromeric region of chromosome 6 [33]. The genetic distance of 1 cM equals 593 kb in this region [34], which makes it difficult to study allelism in segregating and natural populations. The marker AP-5659-5 has been found to share 279 bp allele for the germplasm carrying *Piz* and *Pi9* [35, 36]. The allele perfectly co-segregates with IC-17 resistance phenotype [34]. As in case of *Pita-*$Pita^2$ [16] and genes in *Pik* cluster, the use of diagnostic isolates for characterizing individual genes in *Piz5/Piz/Pi9* locus can assist the validation in marker assisted breeding programs. In the present investigations, IRBLz5-CA-1 carrying *Piz5* amplified 325 bp allele, which is widely distributed in our landrace collection analyzed in this study and with the exception of one, all the 25 landraces having such allele were also resistant to the isolate Mo-nwi-127 (DRI=1.0). *Piz5* gene is reported to be widespread in India and is characterized with high resistance spectrum [36, 37]. At the same time Roychowdhury *et al*. [36] found low

frequency of *Piz* in Indian germplasm represented in the USDA collection. The same trend was reflected in our data where none of the 8 genotypes possessing resistant allele expressed resistance to all the three isolates. *Piz* has been largely found in temperate *japonicas* [38]. *Pi9* gene is quite effective in Indian field conditions. Landraces, Mayur Pankhi, Chima Kamin, Lalmeeta and Tuniaslet showed *Piz-Pi9* specific allele with AP5659-5, however expressed resistant phenotype as was expected for the gene *Pi9*. For *Piz,* the four entries with exception of Mayur Pankhi showed DRI of 0.66 against 3 *Piz Avr*-isolates. The presence of *Piz* and/ *Pi9* can be un-ambiguously confirmed by using more number of diagnostic isolates or by DNA sequencing technologies [39].

Pish

The gene was confirmed in 7 out of 10 marker positive accessions which had DRI of 1.00. *Pish* has been found effective in NW Himalayan region where a combination of *Pish* with genes *Pi9, Piz5, Pita*^{2} is expected to confer durable and broad spectrum resistance to blast [40].

The correspondence between marker genotype and phenotypic reaction against specific isolates was measured by devising an index termed here as GCDI (Table 3, Fig. 2). The number of alleles predicted based on PCR assay for a given germplasm accession were equal to GCDI in case of resistance against specific isolates as expected based on reaction on differential set. For example, twenty five accessions in the present study recorded one-to-one relation between genotype and phenotype for varied loci as discussed above. GCDI equal to zero indicated complete disagreement between genotype and phenotype.

Validation of markers related to leaf blast resistance

Analysis was done to correlate marker data for each gene with phenotypic reaction of germplasm against diagnostic isolates. The summation of individual DRI values across array of germplasm for an individual marker, termed here as Allelic Cumulative Disease resistance Index (ACDRI), the maximum value of which would not exceed the total number of positive alleles for that particular marker (Table 4). The ACDRI reflects Per cent Marker Efficiency which is per centage of total number of positive genotypes showing expected disease response with diagnostic isolates. The maximum efficiency was recorded for the marker AP5659-5 linked to *Piz5* (96.3 %). The gene based markers for gene *Pik*^{m} (Ckm-1, Ckm-2) and for *Pib* (Pibdom) recorded marker efficiency of 67.6 and 68.0 %, respectively. The markers k-2167, k-6816 (*Pik*), JJ-803 (*Pi5*) and AP-5659 (*Pi9*) which map near to the corresponding gene sequence, had >60 % efficiency in detecting the resistance genotype.

Pi54 MAS (for *Pi54*) had a low efficiency of 40.4 % in the set of germplasm studied. The marker Pi54 MAS was derived from the largest InDel in exonic region of the gene between susceptible and resistant backgrounds (Ramkumar *et al*. 2011). In addition to this InDel, 3 other InDels and 45 SNPs have been reported by these authors which may be the cause for such a low efficiency. It is likely that the germplasm

Table 4. Allelic Cumulative Disease Resistance Index (ACDRI) and Marker Efficiency (%) as an index of association between marker genotype and pathotype response across 100 germplasm accessions

Gene	Marker	Total No. of resistance specific alleles	ACDRI	Per cent marker efficiency (%)	No. of *Avr*-isolates
Pi54	Pi54 MAS	26	10.5	40.4	2
Pik^m	Ckm-1, Ckm-2	34	23.0	67.6	2
Pik	k-2167	36	25.5	70.8	2
	k-6816	59	43.0	72.9	2
Pik^h	RM224	24	14.3	59.5	3
Pi1	RM1233	19	18.0	94.7	1
Pi5	JJ-803	36	25.0	69.4	2
Pib	Pibdom	25	17.0	68.0	2
Piz5	AP5659-5	27	26.0	96.3	3
Piz	AP5659-5	8	3.0	37.1	1
Pi9	AP5659-5	8	5.0	62.5	1
Pish	OSR-3	10	7.5	75.0	2

showing resistance specific allele with respect to Pi54 MAS may have some functional aberrations in any of these genomic regions which may result in loss of function at phenotypic level. Such instance was recorded in Pusa Basmati 1, a popular Basmati variety, which amplified 216 bp allele, but does not possess *Pi54* and was found highly susceptible. Actually, the basis of our hypothesis is the reaction of isolates on monogenic LTH background lines, however, for *Pi54,* we had to rely on donors like Tetep, where disease reactions might have been confounded in possible presence of certain unknown genes. To frame index on pretext of such host-isolate reactions may carry some doubt. Nevertheless, our results confirm the gene status for many of the entries dealt in earlier studies (Ramkumar *et al*. 2011; Sharma *et al*. 2005) and also some landraces were identified to carry the gene.

The approach of using PCR based marker analysis along with validation through phenotyping with diagnostic isolates helped us to identify the germplasm accessions harboring gene(s) for blast resistance. The genotypes like Suphala, Baubhog, Vijetha, Kalo Bhutia, Kala Jeera and Sada Kaijam each of which were found to carry 4 dominant blast resistance alleles. Five alleles were found in rice lines Heibao, Kalinga-I, Keshav, Pathara, IR-64, Kala munia, Samleshwari, Jata Dhan, Birui, Agni Baou, Khayersal and Paani Dooba had 3 genes each and expressed high degree of resistance to diagnostic isolates under controlled conditions. The germplasm identified here need to be evaluated with more number of isolates and can be tested under open field conditions in hot spot locations of the country. Also, detailed sequencing of target loci may lead to further insight into nature of resistance and will help in verification of functional domains of the gene responsible for host-isolate interactions. The information generated here has brought to our knowledge the potential utility of molecular markers *vis-a-vis* the identified gene sources which hold lot of promise in marker assisted breeding for improvement of blast resistance in rice.

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