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Molecular marker-based validation of blast resistance gene *Pi54* and identification of potential donors in temperate high altitude rice (*Oryza sativa* L.)

Gazala H. Khan, S. Najeeb[1], Zahoor A. Bhat[2], B. A. Padder[3], G. A. Parray[1], Shafiq A. Wani and Asif B. Shikari

Division of Biotechnology, [1]Mountain Research Centre for Field Crops, Khudwani; [2]Division of Genetics and Plant Breeding, [3]Division of Plant Pathology; Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir, Jammu and Kashmir 191 121

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Abstract

More than 100 genes have been reported to impart resistance against rice blast, however, not all are equally effective. Their effectiveness relies on factors such as the diversity in pathogen races prevailing in a certain area, rate of pathogen evolution, genetic background of a host and few others. *Pi54* is a major gene showing resistance to *Magnaporthe* populations in North-west Himalayas. In search of novel temperate donors suitable to high altitudes, a set of germplasm was screened using gene based markers for *Pi54*. Eighty three exotic and indigenous germplasm lines were genotyped using gene based markers and also validated for disease reaction using *Pi54* gene specific isolate namely, Mo-nwi-kash-32. Nine out of 83 germplasm lines amplified resistance specific alleles with both the markers Pi54 MAS and Pikh-STS. All these lines expressed resistance against the said diagnostic isolate, thereby validating the possible presence of gene in the lines. Further validation using more number of isolates and sequence analysis will help in mining useful alleles for this gene.

Key words: Rice, germplasm, blast, gene, *Pi54*, markers

Introduction

Rice (*Oryza sativa* L.) is the important staple food for more than half of the world's population, most of which live in developing countries (von Braun, 2007). In India, rice cultivation is mostly concentrated in the river valleys, deltas, hilly areas and low-lying coastal areas, which together contribute about 97% to the country's rice production. It gets exposed to varied types of stresses, of which blast of rice caused by *Magnaporthe oryzae* is one of the major production constraint. The disease has been reported from every rice growing region causing 10-30 % yield losses, however, uplands and cold areas encounter higher severity of the disease (Variar et al. 2009). More than 100 blast resistance genes have been mapped worldwide of which genomic locations of many are precisely known and 20 of them have already been cloned and characterized at sequence level (Sharma et al. 2012; Rathour et al. 2016). Out of these, genes like *Pi9* (Wang et al. 2013) and *Pi54* (Sharma et al. 2005) are attributed to confer broad spectrum resistance across parts to India.

India is endowed with enormous rice genetic diversity comprising of wild relatives, land races, exotic, indigenous, obsolete and high yielding cultivars, which happen to be the repositories of useful alleles for the traits of economic and biological importance. We in Kashmir maintain a huge germplasm repository of more than 1000 germplasm accessions, which are understood to possess tolerance to cold and are adapted to altitudes more than 2000m above mean sea level (Parray and Shikari 2008). Very little or no information has been generated on the collections regarding the possible alleles linked to blast resistance. With the advent of molecular markers, the identity of the genes in the germplasm can be verified precisely and quickly for those traits which are difficult to phenotype. Germplasm evaluation and characterization have been advocated as a pre-requisite for

*Corresponding author's e-mail: asifshikari@gmail.com
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identification of new genes and as a component to blast management programs by several authors (Li et al. 2005, Sharma et al. 2012; Skamnioti and Gurr 2009; Shikari et al. 2014). This has opened up the scope for discovery and identification of the novel genes/ alleles in hitherto unexplored genetic resources. Previously, the rice blast resistance gene *Pik*^{h} has been isolated from the *indica* rice, Tetep showing resistance to different *M. oryzae* strains in the North-Western Himalayan region of India (Sharma et al., 2005) and other parts of India and the US (Costanzo and Jia 2010; Rai et al. 2011). The gene was renamed *Pi54* after it was further re-alienated to a slightly distant location from the *Pik* locus (Sharma et al. 2010). The predicted *Pi54* protein contains NBS-LRR domain and the gene has been shown to induce the synthesis of callose (b-1, 3-glucan) in response to pathogen challenge (Sharma et al. 2005). Also, this gene has been included in combination with *Piz5* and few others to provide for broad spectrum resistance to blast in Basmati rice (Singh et al. 2011, Khanna et al. 2015). Given the importance of *Pi54* in being effective in NW-Himalayas, the present study was undertaken to assess the allelic profile and identify donors in diverse temperate rice germplasm collections.

Materials and methods

A total of 83 temperate rice genotypes procured from various global sources (Supplementary Table 1)were screened for *Pi54* specific allele alongwith *DHMAS 70Q 164-1b*, a doubled haploid line derived from *Tetep* as positive control.

Marker analysis

Genomic DNA of all the 83 accessions was extracted from fresh, healthy and young leaf tissue from fourteen day- old seedlings following Cetyl-Tri Methyl Ammonium Bromide method (Murray and Thompson 1980) with certain modifications. The DNA was purified by adding RNase (10 g/100ml) to the sample at the rate of 3 ul/ 100 ml of crude DNA. DNA quantification was done using 0.8% agarose gel. The λ uncut DNA was used as a comparison and the final concentration was adjusted to ~50 ng/µl. PCR assay was performed for blast resistance gene *Pi54* using two gene markers namely, *Pi54* MAS and Pikh-STS (Table 1). Besides, SSR marker RM224 (F: ATC GAT CGA TCT TCA CGA GG; R: TGC TAT AAA AGG CAT TCG GG) was used to confirm its association to two of these markers given above. Polymerase chain reaction (PCR) reaction mix comprised of ~50 ng of DNA, 10 x PCR buffer (10 mM Tris, pH 8.4, 50 mM KCl, 1.8 mM $MgCl_2$), 2 mM dNTPs (MBI, Fermentas, Lithuania, USA), 5

Table 1. The primer sequences used to screen for blast resistance gene *Pi54* in the temperate rice germplasm

Primer name	Sequences (5′ to 3′)	Map position (Mb)	Annealing	Ref.
Pi54 MAS	F: CAATCTCCAAAGTTTTCAGG R: GCTTCAATCACTGCTAGACC	24797978-24797997; 24798316-24798335	94^{o}C (30 s), 55^{o}C (30 s), 72^{o}C (1 m)	(Ramkumar et al. 2011)
Pikh STS	F: TTCTCTGCTTCTGATCACCAAA R: ATGCTGGAAATGCCAGTAGAAT	24796788-24796806; 24798037-24798058	94^{o}C (1 m), 55^{o}C (1 m), 72^{o}C (2 m)	(Thakur et al. 2015)

Table 2. Germplasm lines carrying resistance specific alleles and their disease reaction to diagnostic isolate of *M. oryzae*

S. No.	Genotype	Origin	Pi54 MAS	Pikh STS	RM224	Disease reaction
1	SKAU 360	J&K, India	R	R	150	1
2	K312-5-5-5-7-2	J&K, India	R	R	170	1
3	GS-116	J&K, India	R	R	165	1
4	DHMAS 70Q 164-1b	H.P., India	R	R	180	0
5	VL95-3337	VPKAS- Almora, India	R	R	180	0
6	IR61728-4B-2-1-1	Philippines	R	R	165	1
7	93037-IR1509-1-1-1	Turkey	R	R	165	1
8	YUNLU NO 56	China	R	R	165	1
9	HAMNAM15	Korea	R	R	165	0

R: Resistance specific allele; S: Susceptibility specific allele; H: Heterozygous fragment; Disease score: 0-2: Resistant; 3-5: Susceptible; Blast isolate used: Mo-nwi-kash-32 (*Avr-Pi54*)

pmol each of forward and reverse primer and 1 U of Taq DNA polymerase (Sigma Aldrich, India) in a reaction volume of 10 µl. PCR was performed in a thermal cycler (TaKara, Japan) with initial denaturation at 94^{o}C for 5 min; with varying cyclic regimes (Table 1) and final extension of 10 min at 72^{o}C. The 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).

Pathogenecity assay

The blast isolates, namely Mo-nwi-kash-32, avirulent to gene *Pi54*, was kindly provided by Dr. U. D. Singh, from Division of Plant Pathology, IARI, New Delhi and was used for pathogenecity assay under artificial conditions. Seeds were grown in 5 x 5 well pro-trays with 6 seedlings per well, replicated twice. The clean soil was fertilized with well decomposed organic matter, N_2 and P_2O_5 as per recommendation. The inoculum was prepared following protocol given by (Bonman et al. 1986). The seedlings were sprayed using hand atomizer (100 kPa) at 2-3 leaf stage with *M. oryzae* spore suspension adjusted to 5 x 10^4 spores per ml. Two drops of 0.02% Tween20 was added to the suspension prior to spray. The trays were kept in dark for 24 hours inside disinfected dew chambers and subsequently maintained at 25±1^{o}C and 85% RH under proper light regime. After 6 DAI, scoring was done following 0-5 scale of (Mackill and Bonmann 1992). This was followed by second scoring after 3 days interval. The whole experiment was repeated twice (Singh et al. 2013).

Table 3. Allelic distribution of markers for screening blast resistance gene *Pi54* in temperate rice germplasm

Germplasm source	Allele	Pi54 MAS	Pikh-STS
Indigenous	R	39	6
	S	8	43
	H	2	-
	Sub-total	49	49
Exotic	R	21	5
	S	13	29
	H	-	-
	Sub-total	34	34
Total	R	60	11
	S	21	72
	H	2	-
	Total	83	83

R: Resistance specific allele; S: Susceptibility specific allele; H: Heterozygous fragment

Results and discussion

Eighty three germplasm lines were screened for major blast resistance gene *Pi54*, using gene based markers Pi54 MAS (exon based co-dominant InDel marker) and Pikh-STS (a presence-absence gene based marker). Of these 49 were indigenous in origin and 34 were procured from various international sources (Supplementary Table 1). Thirty nine of 49 indigenous germplasm lines amplified resistance specific allele (216 bp) using marker Pi54 MAS, eight showed susceptibility specific fragment (359 bp) and two had heterozygosity at a given locus. The given set amplified resistance specific fragment in six germplasm lines against marker Pikh-STS and rest showed absence of band. Of the 34 exotic germplasm lines, 21 and five lines were found to amplify resistance specific alleles with respect to markers *Pi54* MAS and *Pikh*-STS, respectively. Overall, 60 out of 83 germplasm lines amplified resistance alleles using Pi54 MAS and just 11 lines had *Pi54* specific alleles from marker Pikh-STS. There were nine such lines those had resistance specific alleles with respect to both the markers and included DHMAS 70Q 164-1b,SKAU 360, K312-5-5-5-7-2, GS-116, VL95-3337, IR61728-4B-2-1-1, 93037-IR1509-1-1-1, YUNLU NO 56 and HAMNAM15 (Table 2) (Fig. 1). Rai et al. (2011) after functional complementation studies have found *Pi54* to be effective to combat *M. oryzae* races in NW-Himalayas. Therefore, in pursuance to identify novel sources of *Pi54* which are adapted to high altitude regions of Kashmir, our preliminary exercise identified several lines with the help of above two markers. However, the marker Pi54 MAS classified higher number of genotypes as resistant ones as compared to marker Pikh-STS. Pi54 MAS targets Insertion-Deletion (InDel) segement in exonic region where 144 bp deletion has been found in resistant cultivars like Tetep (Ramkumar et al. 2011). The study validated 105 germplasm lines through Pi54 MAS and 216 bp allele co-segregated with *Pi54* resistance locus. In a separate study resistance specific allele for marker Pi54 MAS was found in 26 of 100 germplasm lines collected from various parts of India (Shikari et al. 2014). However, only seven expressed resistance response towards *Pi54* diagnostic isolate. In the present study, 34 (80%) of indigenous and 49 (62%) exotic collections were found to carry *Pi54* allele. However, only 13 (22%) lines namely, DHMAS 70Q

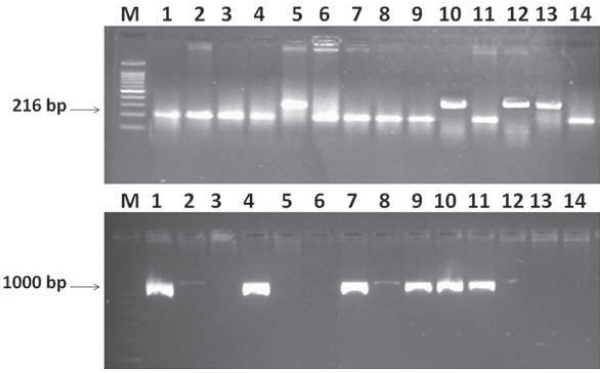

Fig. 1. Allelic profile of representative germplasm lines evaluated using gene based markers Pi54 MAS (above) and Pikh-STS (below). Lane M: 100 bp DNA Ladder (Fermentas, Luthuania); Lane 1 to 14: DHMAS 70Q 164-1b, Shalimar Rice-1, Jehlum, SKAU-360, SAW/GML/188, GS-200, VL95-3337, IR6833-R-R-B-19, IR61728-4B-2-1-1, WAB 881-10-37-18-13-PL-HB, YUNLU No. 56, WAB 880-1-38-20-14-BHB, HS-379, ECHVCA

164-1b, SKAU-284, SKAU-354, SKAU 360, K312-5-5-5-7-2, GS-116, GS-200, VL95-3337, IR61728-4B-2-1-1, 9107-TR1292-2-1-1-1, 93037-IR1509-1-1-1, YUNLU NO 56 and HAMNAM15 expressed resistant reaction to *Pi54*-specific isolate Mo-nwi-kash-32. The discrepancy between allelic profile and disease reaction may be attributed to the fac that the genic region based on which marker was developed carry additional three other InDels and 45 SNPs which possibly cause loss of functionality of putative *Pi54* allele in susceptible lines. Sharma et al. (2005) reported 39 SNPs, various repeats and InDels which markedly differentiate the *Pi54* region between *Tetep* and japonica (*Nipponbare*) genotypes. In our germplasm set, there are number of lines which belong to *japonica* and intermediates between *indica* and *japonica*. Such lines may carry dysfunctional *Pi54* even when the given marker gives a positive signal. Other marker namely Pikh-STS is again a gene based marker and was found to be more robust in identifying resistant lines in our set. Only two genotypes which carried resistance fragment using Pikh-STS, did not carry *Pi54* specific alleles using Pi54 MAS. Rest nine lines which had resistance alleles at both the marker loci, all of them depicted resistance response to diagnostic isolate. The lines exhibiting resistance reaction against isolate and found to have susceptible allele on marker analysis might be carrying an unknown gene.

Subsequently RM224 reported to be linked at 0.0 cM to *Pik* gene cluster (Fuentes et al. 2007) was used to screen a germplasm set and amplified six alleles across 83 lines. None of the alleles for RM224 showed association with resistance specific alleles amplified by the markers, Pi54 MAS and Pikh-STS. High PIC value of 0.68 was noted for RM224 across the whole germplasm collection which suggests relatively balanced distribution of the six identified alleles on agarose gel and also implies the usefulness of RM224 in marker-assisted selection for genes near *Pik*-cluster.RM224 has been extensively used in various studies to select for genes like *Pi1* (Prasad et al. 2009; Khanna et al. 2015) and *Pikh* (Shikari et al. 2014).

The study helped in identifying novel *Pi54* gene donors, as standard donors like, *Tetep* and *DHMAS 70Q 164-1b* pose limitations of lack of cold tolerance, asynchronity, late maturity, distinct grain type and few other traits which make them less preferable as parents in molecular breeding programs. Thus, novel donors identified here are expected to pave the way for resistance breeding programs aimed at improvement of temperate rice varieties. However, the putative *Pi54* harbouring lines identified here require to be verified at sequence level and may be screened using more number of gene specific isolates for further validation of resistance.

Authors' contribution

Conceptualization of research (ABS, GHK, SAW); Designing of the experiments (GHK, SN, ZAB, ABS); Contribution of experimental materials (GAP, ABS, ZAB); Execution of field/lab experiments and data collection (GHK, BAP); Analysis of data and interpretation (ABS, SAW, GHK); Preparation of manuscript (GHK, SAW, ABS).

Declaration

The authors declare no conflict of interest.

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Supplementary Table 1. Allelic profile for markers used to screen for blast resistance gene *Pi54* in a set of indigenous and exotic germplasm

S. No.	Genotype	Origin	Pi54 MAS	Pikh STS	RM224	Disease reaction
1	Shalimar Rice-1	J&K, India	R	S	165	4
2	Jehlum	J&K, India	R	S	165	4
3	SKAU-46	J&K, India	R	S	180	5
4	SKAU-280	J&K, India	R	S	180	4
5	SKAU-284	J&K, India	R	S	150	2
6	SKAU-324	J&K, India	S	S	150	4
7	SKAU- 338	J&K, India	H	R	150	4
8	SKAU-343	J&K, India	R	S	180	3
9	SKAU 353	J&K, India	R	S	180	4
10	SKAU- 354	J&K, India	R	S	150	1
11	SKAU- 357	J&K, India	H	S	165	4
12	SKAU 360	J&K, India	R	R	150	1
13	SKAU-380	J&K, India	R	S	180	4
14	K312-5-5-5-7-2	J&K, India	R	R	170	1
15	K312-15-4-2	J&K, India	R	S	165	4
16	K314-25-1-2-1-1	J&K, India	R	S	165	4
17	K312-30-2-2-3-1-3	J&K, India	R	S	150	4
18	K508	J&K, India	R	S	150	3
19	K696-10-2-1-2-2	J&K, India	R	S	200	4
20	K717-1-1-1	J&K, India	R	S	165	4
21	SAW/GML/8	J&K, India	R	S	165	4
22	SAW/PBG/50	J&K, India	R	S	200	4
23	SAW/PBG/51	J&K, India	R	S	165	4
24	SAW/PBG/56	J&K, India	R	S	165	4
25	SAW/PBG/58	J&K, India	R	S	165	4
26	SAW/PBG/62	J&K, India	R	S	200	4
27	SAW/GML/186	J&K, India	R	S	150	4
28	SAW/GML/188	J&K, India	S	S	165	4
29	SAW/GML/205	J&K, India	R	S	150	3
30	SAW/GML/234	J&K, India	R	S	150	4
31	SAW/GML/252	J&K, India	S	S	165	4
32	SAW/GML/270	J&K, India	R	S	165	4
33	SAW/GML/280	J&K, India	R	S	165	3
34	SAW/GML/286	J&K, India	R	S	180	3
35	SAW/GML/292	J&K, India	S	S	180	5
36	SAW/GML/294	J&K, India	R	S	180	4
37	SAW/GML/295	J&K, India	S	S	180	1
38	SAW/GML/310	J&K, India	R	S	165	4
39	SAW/GML/317	J&K, India	R	S	165	4

40	SAW/GML/327	J&K, India	R	S	150	4
41	GS-116	J&K, India	R	R	165	1
42	GS-200	J&K, India	R	S	180	2
43	GS-217	J&K, India	R	S	165	3
44	DHMAS 70Q 164-1b	H.P., India	R	R	180	0
45	RP 2421	H.P., India	S	S	165	5
46	Himdhan	VPKAS- Almora, India	S	S	165	3
47	VL91-192-3-IV1	VPKAS-Almora, India	R	S	165	3
48	VL94-3027	VPKAS-Almora, India	S	S	150	5
49	VL95-3337	VPKAS- Almora, India	R	R	180	0
50	IR68333-R-R-B-19	Philippines	R	S	165	3
51	IR57893-76	Philippines	S	S	150	4
52	IRBN 2008 V87	Philippines	S	S	150	4
53	IR61728-4B-2-1-1	Philippines	R	R	165	1
54	96004-TR1743-3-1-1	Turkey	R	S	165	4
55	95010-IR1644-4-1-1	Turkey	R	S	165	4
56	9107-TR1292-2-1-1-1	Turkey	R	S	180	2
57	SUREK-95	Turkey	R	S	180	4
58	93092-TR1564-4-1-1	Turkey	S	S	150	1
59	93037-IR1509-1-1-1	Turkey	R	R	165	1
60	87041-TR990-11-2-1	Turkey	S	S	165	4
61	HEXI 5	China	S	S	180	5
62	YUNLU NO 56	China	R	R	165	1
63	YUNLEN-19	China	R	S	180	5
64	L10573	China	S	S	165	5
65	Koshihikari	Japan	R	S	150	4
66	OLBYE-2	Korea	S	S	150	5
67	MILYANG 80	Korea	R	S	180	4
68	HAMNAM15	Korea	R	R	165	0
69	GILJU-1	Korea	R	S	180	3
70	DONGHAECHAL	Korea	R	S	180	3
71	OLBYE-1	Korea	S	S	165	4
72	H274-16-1-1	Argentina	S	S	180	4
73	H270-30-2-1	Argentina	R	S	165	4
74	H231-59-3-1	Argentina	R	S	165	4
75	H257-2-1-1	Argentina	R	S	180	5
76	WAB881-10-37-18-13-PL-HB	WARDA, Africa	S	R	150	4
77	WAB880-1-38-20-14-B-HB	WARDA, Africa	S	S	150	4
78	WAB450-11-1-P3'-1-1-HB	WARDA, Africa	R	S	150	4
79	GRALDO	Italy	R	S	180	5
80	MILLIN	Italy	S	S	165	4
81	GIGANTE VERCELLI	Italy	R	S	180	4
82	HS-379	Hungary	S	S	165	4
83	ECHVCA	Australia	R	S	165	4

R: Resistance specific allele; S: Susceptibility specific allele; H: Heterozygous fragment; Disease score: 0-2: Resistant; 3-5: Susceptible; Blast isolate used: Mo-nwi-kash-32 (*Avr-Pi54*)