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Determination and expression of genes for resistance to blast (*Magnaporthe oryza*) in Basmati and non-Basmati *indica* rices (*Oryza sativa* L.)

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One hundred and twenty two (122) genotypes of Basmati and non-Basmati *Indica* rice genotypes were evaluated for expression of resistance against blast disease under induced epiphytotic conditions. Disease severity (%) and area under disease progress curve (AUDPC) parameters were used for screening the blast resistance. Only 13 genotypes expressed resistance against the blast disease. Nine genotypes carried blast resistance genes but, were susceptible under induced epiphytotic conditions. The rice genotype VLD-61 had no resistance genes; however, it expressed strong resistance against blast. An empirical breeding strategy for development of blast resistant improved varieties of rice was also discussed.

Key words: *Magnaporthe oryzae*, restriction digestion, molecular breeding, Basmati rice

INTRODUCTION

Rice (*Oryza sativa* L.) is the staple food for more than half of the world population of Asia. Also, the genetic and functional syntenies observed among cereal crops over the years has made rice the most important cereal crop for the discovery and utilization of agronomically important genes for crop improvement. Blast caused by *Magnaporthe oryzae* (Couch and Kohn, 2002), is one of the most devastating diseases of rice (*O. sativa* L.) worldwide. Its frequent appearance at all stages of plant growth greatly decreases yield and grain quality of Basmati and non-Basmati (Ou, 1985; Singh, 2008) *Indica* rices.

The rice varieties developed after green revolution which could withstand higher levels of crop management

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Abbreviations: SFR, Super fine resolution; **PCR,** polymerase chain reaction; **CTAB,** cetyl trimethyl ammonium bromide; **AUDPC,** area under disease progress curve.

that is, new cultural practices (increased doses of fertilizers and irrigation), were generally prone to diseases. The narrow genetic base seems to be one of the reasons for their more vulnerability to rice blast (Naqvi and Chattoo, 1996). Even after identification of more than 60 resistant genes to blast disease and availability of complete genome map of rice, still, blast is considered as a big challenge for the successful cultivation of rice in many parts of the world.

Keeping this in view, the present study was undertaken to 1) characterize rice genotypes for blast resistance under induced epiphytotic conditions and, 2) detect genes responsible for blast using gene specific markers in order to develop breeding strategies for development of blast resistant improved varieties of rice.

MATERIALS AND METHODS

Plant material and field trials

One hundred and twenty two (122) *Indica* rice genotypes collected from different sources were evaluated for resistance against blast disease under induced artificial epiphytotic conditions at SVP University farm, Meerut (Indo-Gangetic Plains/North West Plains Zone, India, 28.99°N and 77.70°E) during 2009 *Kharif* (rainy) season. For the sake of brevity, results of only 22 promising varieties are given presently (Table 1).

Standard agronomic and management practices were followed to raise 22 varieties. Inoculums were prepared from infected leaf samples having conidia and mycelium of blast pathogen. Inoculums having concentration of 10×10^4 to 50×10^4 conidia per ml were used for inoculation of plants. Fields were frequently irrigated to induce environmental conditions conducive to blast pathogen to multiply well at faster rate. Genomic DNA was isolated from leaves harvested from 15 days old seedling taken from individual plants using modified cetyl trimethyl ammonium bromide (CTAB) method (Moller et al., 1992).

Field data analysis and evaluation for the presence of gene specific markers

Disease severity (%) was recorded at three stages *viz.,* late anthesis, late milking and dough stages following the methodology of Jeger (2004). Area under disease progress curve (AUDPC) based on disease severity over time, which has been suggested to be a pragmatic approach for disease assessment was estimated using the formula:

$$\mathrm{AUDPC}=\sum_{i=1}^{i}[\{(Y_i+Y_{(i+1)})/2\}\times(t_{(i+1)}-t_i)]$$

Where, Y is the disease level at time $t_{i;}$ $t_{(i+1)}$ - t_i duration (days) between two disease score.

Analysis of variance (ANOVA) for the disease severity (%) at dough stage and AUDPC was performed using SAS software (version 603; SAS Institute Inc; CaryNC 1997). For the purpose of identification of blast resistance genes, with the help of gene specific markers, all the rice genotypes were evaluated for the presence of marker bands supposed to be linked with b last resistant gene(s).

DNA Amplification and gel electrophoresis

Genotypes were subjected to identify for the blast resistance genes with the help of ten gene specific blast resistance primers (Table 2) procured from Integrated DNA Technologies, USA (IDT). Each reaction mixture (20 µl), used for genes specific amplification consisted of assay buffer (10 mM Tris HCl, pH 8.0, 50 mM KCl), 15 mM $MgCl_2$, 1 U of Red Taq DNA polymerase, 1.0 mM each of dATP, dTTP, dCTP and dGTP,5 µm of genes specific primers (IDT, USA) and approximately 50 ng of genomic DNA for genes specific primers, respectively. The polymerase chain reaction (PCR) amplification conditions for genes specific markers analysis were as follows: initial extended step of denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 1 min, primer annealing at 50°C for 1 min, elongation at 72°C for 2 min, followed by extension step at 72°C for 7 min and then final hold at 4°C till electrophoresis. PCR products was mixed with 5 µl of gel loading dye (1x buffer, Bromophenol blue, 0.1%; xylene cyanol 0.1%; and glycerol in water, 50%).

The amplification products were electrophoresed on 2.0% super fine resolution (SFR) gel at 3 to 5 volts/cm in 1x TAE buffer. Genomic DNA was quantified by UV absorbance at 260 and 280 nm, using Bio Rad smart tech™ spectrophotometer. The ratio of OD 260/280 was also calculated to estimate the purity of nucleic acid. Genomic DNA was also quantified by agarose gel electrophoresis as the size of genomic DNA is quite big, a 0.8% gel was used to visualize the genomic DNA, as it can resolves DNA molecules in the range of 0.7 to 8.5 kb.

Restriction digestion

The PCR products of the different gene specific markers were digested by *Eco RI* restriction enzyme. Each reaction mixture (20 µl), used for restriction digestion of different genes specific amplification products consisted of 2.0 ul of 10x restriction buffer, 1.0 ul of 10u/ul *Eco RI*, 7.0ul of dd H_2O and 5.0 ul of PCR product. The reactions were incubated at 37°C for 2 to 3 h.

RESULTS AND DISCUSSION

The presence of several blast resistance genes (*Pi1, Pib, Pi2, Pik^h, Pi5-1, Pi9 and Piz-MRG-4963*) in rice genotypes revealed that resistance sources are allelic for resistance genes indicating the possibility of obtaining transgressive segregants, if crosses are made between these resistant genotypes. Higher levels of resistance under induced epiphytotic conditions and presence of blast resistance gene specific marker bands with at least 2 to 3 blast resistance genes indicated the presence of many other minor genes (Table 2).

The appearance of blast resistance gene linked markers for example, *Pi1*$_{350bp}$ in 11 rice genotypes (VLD-85,Vivekdhan154,VLD-221, VLD-65, Vivekdhan-82, VLD-86, Vallabh basmati-21, MAUB-13, Haryana Mahak and Sathi white), *Pi2*$_{1400bp}$ (Figure 1) in 5 rice genotypes (Vivekdhan-62, VLD-65, VLD-86, MAUB-171 and Sathi white), Pik$^h_{990bp}$ in 3 rice genotypes (VLD-85, Vivekdhan-15 4and Vivekdhan-81), Pi9$_{1600bp}$ (Figure 2) in 6 rice genotypes (Vivekdhan-154, VLD-81, VLD-65, Vivekdhan-82, MAUB-57 and PB-1), *Pib*$_{365bp}$ in 5 rice genotypes (Vivekdhan154,VLD-81, VLD-65, Vivekdhan-82, MAUB-57 and PB-1), *Piz-MRG4963*$_{325bp}$ in 6 rice genotypes

Table 1. Characteristics of rice varieties included in the pathogenecity testing (Vegetos, 2010).

S/N	Genotype	Source/Origin	Duration	Height	Quality	Mean disease response to blast	
						% Severity	AUDPC
1	VLD-85	VL, Almora	Short duration	Semi dwarf	Coarse	25.00± 2.58	648.75± 90.22
2	VL-62	VL, Almora	Short duration	Semi dwarf	Coarse,	45.00 ± 2.77	1085.23± 99.62
3	Vivekdhan-62	VL, Almora	Short duration	Semi dwarf	Coarse,	35.00 ± 4.80	925.43 ± 98.97
4	VLD-61	VL, Almora	Short duration	Semi dwarf	Coarse,	30.00 ± 3.30	816.22 ± 85.22
5	Vivekdhan-154	VL, Almora	Short duration	Semi dwarf	Coarse,	25.00 ± 2.25	734.29 ± 88.86
6	VLD-81	VL, Almora	Short duration	Semi dwarf	Coarse,	25.00 ± 2.28	567.43 ± 70.21
7	VLD-221	VL, Almora	Short duration	Semi dwarf	Coarse,	35.00 ± 3.50	923.54 ± 76.26
8	VLD-65	VL, Almora	Short duration	Semi dwarf	Coarse,	20.00 ± 2.60	456.09 ± 56.43
9	Vivekdhan-82	VL, Almora	Short duration	Semi dwarf	Coarse,	25.00 ± 2.22	568.53 ± 45.25
10	VLD-86	VL, Almora	Short duration	Semi dwarf	Coarse,	20.00 ± 2.15	531.34 ± 51.25
11	Sathi White	Farmers' variety, Uttar Pradesh	Short duration	Semi dwarf	Coarse	85.00 ± 4.65	2645.31±224.51
12	Haryana Basmati	CCSHAU, KAUL, Haryana	Long duration	Tall	Basmati	80.00 ± 4.51	2078.34±198.37
13	Ranvir Basmati	Jammu and Kashmir	Medium duration	Tall	Basmati	75.00 ± 4.79	1958.54 ±197.39
14	Vallabh Basmati-21	SVPUAT, Meerut Uttar Pradesh	Short duration	Semi dwarf	Basmati	25.00 ± 2.25	525.00 ± 40.89
15	MAUB-57	SVPUAT, Meerut,	Long duration	Semi dwarf	Basmati	75.00 ± 4.16	1783.31 ±178.33
16	Haryana Mahak	CCSHAU, KAUL, Haryana	Long duration	Semi dwarf	Super Fine	80.00 ± 4.50	2097.67 ±195.80
17	MAUB-13	SVPUAT, Meerut,	Long duration	Semi dwarf	Super Fine	65.00 ± 3.56	1431.09 ±107.90
18	PB-1	IARI, New Delhi	Long duration	Semi dwarf	Basmati	85.00 ± 4.65	2564.35 ±208.49
19	Punjab Basmati	PAU, Ludhiana	Long duration	Tall	Basmati	55.00 ± 4.26	2025.00 ±195.51
20	Vallabh Basmati-22	SVPUAT, Meerut, Uttar Pradesh	Long duration	Semi dwarf	Basmati	20.00 ± 2.15	516.65 ± 45.16
21	Taroari Basmati	CCSHAU, KAUL, Haryana	Long duration	Tall	Basmati	75.00 ± 4.20	1876.57 ±176.29
22	MAUB-171	SVPUAT, Meerut, Uttar Pradesh	Long duration	Tall	Basmati	45.00 ± 5.39	1087.50± 98.89

(VL-31077, Vivekdhan-62, vivekdhan-154, VLD-81 and VLD-221). Contrarily, *Pi5-2, Piz-2341,* and *Piz-5836* blast resistance genes were not able to indicate their presence, as no amplification was observed with the corresponding primers in any of the rice genotypes used. Presence of common marker bands in most of the rice genotypes was an indication that common resistance genes were present in all the resistant genotypes. The genotypes showing presence of marker bands with maximum number of blast resistance genes' primers and also expressed resistance in the field conditions have been selected for further verification and exploitation in future breeding programmes.

Results of evaluation of 22 rice genotypes for disease severity (%) are discussed presently. On the basis of phenotypic expression of resistance mechanism towards disease reactions observed under induced epiphytotic conditions, these genotypes were classified into two groups, resistant (R) and susceptible (S) as given in Table 3. Artificial disease pressure was created by inoculating all rice genotypes with most aggressive isolates identified at the Molecular Biology Laboratory, SVP University of Agriculture and Technology, Meerut, India. Rice genotypes were characterized on the basis of disease severity (%) at dough stage as well as AUDPC based on disease severity at different growth stages. Occurrence of disease during grain formation affects the yield maximum as observed in many other cases. However, AUDPC is

Table 2. Ten blast resistance genes specific markers used for the identification of blast resistance rice genotype.

S/N	Resistance genes for blast	Sequence of forward (5'-3') primer	Sequence of reverse (3'-5') primer	Make
1	Pi-1	AGGGAGATTTGACCATCGTG	CCTGATTGCAAGAGAGGTAGGC	IDT
2	Pi-2	GTTGTTTGAGCTCTCCAATGCCTGTTC	CTGCAGTGCAATGTACGGCCAGG	IDT
3	Pik^h	ATGGTTCTTTAAAATTGGGGC	ATGGCAAAACTTCAAGAGAAA	IDT
4	Pi-5-1	TACAAGTTGGCAGCTTTATCTGAG	TCAGAAGCACTGGATCTTTCTGCA	IDT
5	Pi-5-2	AGTGAACTCCAAACATGTGAACAC	TCATACCTGTTGCGGTTTCTGCCT	IDT
6	Pi-9	GTAGGTACATCAAGGACGAG	AGGTGTTCGCCCCGCAGGT	IDT
7	Pi-b	GAACAATGCCCAAACTTGAGA	GGGTCCACATGTCAGTGAGC	IDT
8	Pi-Z (MRG4963)	CGAAAAGTGGGAAGCAAATG	GCGTACCCCTAGTGGCTGTA	IDT
9	Pi-Z (MRG5836)	TATAAGCCGCAGCCAATTC	AAAAACCTAGAAAATGGGAAAATG	IDT
10	Pi-Z (MRG2431)	ATCCAAATCCAATGGTGCAG	GTGGCGAAAGGGAACATTCT	IDT

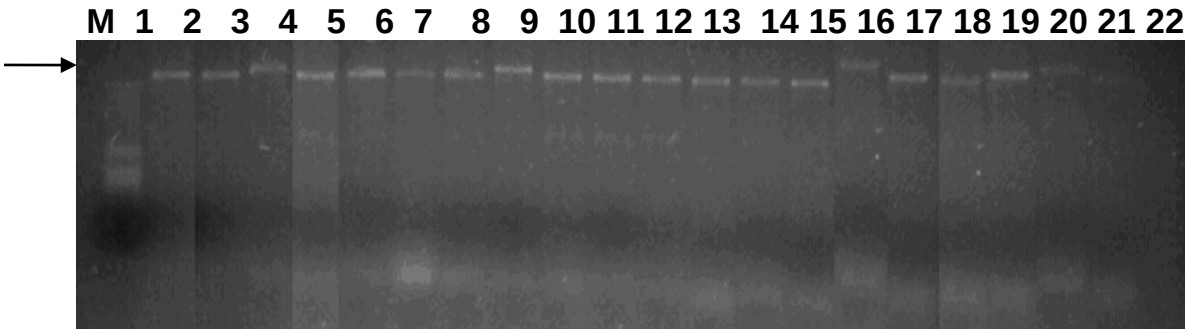

Figure 1. Amplification products resulting from the *Pi2)* primer in 22 rice genotypes on a 1.5 % SFR (Super Fine Resolution). 1400bp amplification product of *Pi2* gene indicated by an arrow. M is 100 bp ladder.

considered as the best parameter to declare a variety resistant or susceptible (Jeger, 2004). AUDPC provide more precise and pragmatic classification of resistance and susceptible genotypes (Van der Plank, 1963; Chaurasia et al., 1999; Jeger, 2004) than that based on the percentage disease score of each genotype.

Presence of a continuous range of variation, for disease severity in the present study indicated that blast resistance is under the control of several additive genes having small, but cumulative effect to disease resistance. Singh and Rajaram (1991) reported similar additive gene action for the leaf rust of wheat. Sandhu et al. (2003) suggested a polygenic control for blast resistance. Naqvi and Chattoo (1996) also reported polygenic control of resistance in case of blast disease of rice caused by *Pyricularia oryzae*. With increasing number of additive genes, variation among genotypes would increase. Additive gene action is always an attraction for breeders to exploit traits and bring desirable changes in population through selection and accumulation of resistance genes into a single genotype. However, the breeding progress using available resistant genotypes have not been satisfactory (Ahn and Ou, 1982; Ou, 1980; Villarreal et al., 1981) indicating the practical difficulty in enhancing the blast resistance. One of the important reasons of slow breeding in blast resistance has been suggested to be non-availability of reliable molecular markers linked to blast resistance (Pengyuan, 2004) as selection for disease resistance at morphological level is not very promising due to changes in environmental factors such as temperature, humidity and growth stages, disease expression can be altered and selection for disease resistant genotypes can be biased.

In the present investigation, out of 22 rice genotypes only 13 genotypes were resistant for the blast disease. The rest nine rice genotypes, which were susceptible under artificial epiphytotic conditions, carried blast resistance genes as indicated by gene linked markers. Though, out of these, only one genotype VLD-61 had no resistance genes, however, it expressed strong resistance against blast. This might be explained that some other genes which could not be detected herein were also responsible for resistance against the disease. The rest eleven resistant genotypes had varying number of resistant genes. Maximum, six genes (*Pi1, Pik^h Pi5-1, Pi9, Pib, Piz-MRG-4963)* were detected in the genotype Vivekdhan-154. The genotype VLD-65 had four resistance genes and expressed better resistance

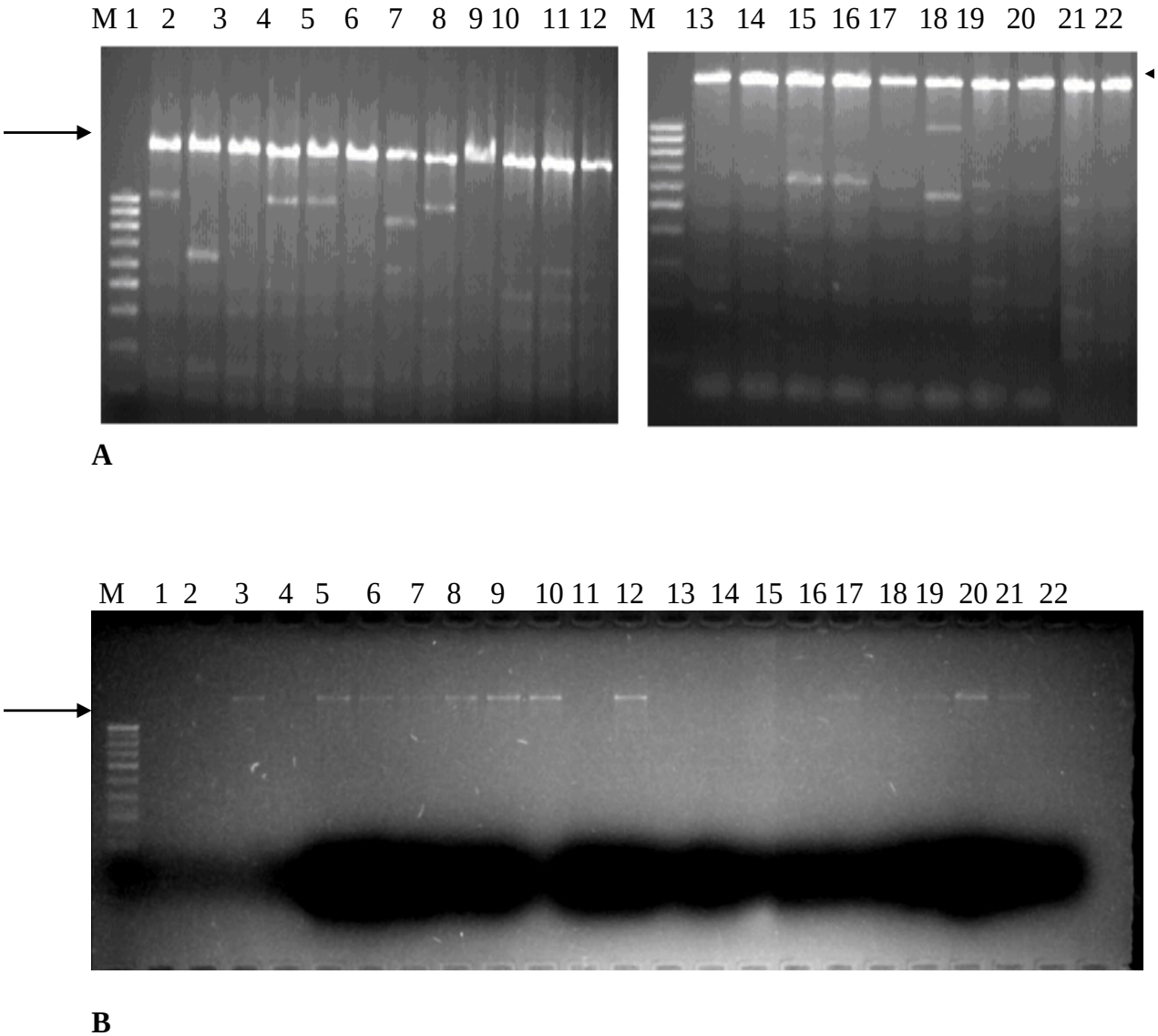

Figure 2. Amplification products resulting from the *Pi9* primer in 22 rice genotypes on a 1.5 % SFR (Super Fine Resolution). A, Aproximate1800-bp monomorphic amplification product of *Pi9* gene ndicated by an arrow. B, Approximate 1600 bp gene size of *Pi9* after restriction digestion by *EcoRI* indicated by an arrow. M is 100 bp ladder.

against the disease than that by Vivekdhan-154. It may be ascribed to the contribution of the gene *Pi2* which was additionally present in VLD-65. The statement is also supported by the resistance expressed by MAUB-171 which had only one gene *Pi2.*

Contrarily, most of the nine rice genotypes which were found to be susceptible under artificial epiphytotic conditions, had some genes for resistance against the disease such as *Pi5-1, Pi1, Pi2, Pi9*. But these genes did not express resistance well in the given environment. The only genotype Tarori basmati had none of the resistance gene considered in the present investigation. However, despite having gene(s) for resistance, the genotypes Sathi-white, PB-1, Haryana Mahak, Ranvir basmati expressed more susceptibility than that expressed by Taroari basmati (Table 3).The statement that some additional gene(s) were/might be involved in the mechanism controlling resistance could be forwarded to explain this situation. The only genotype Sathi white despite having *Pi2* gene for blast resistance was included in the list of susceptible varieties. It could be explained that perhaps some common genes were required to make *Pi2* express against blast disease. In cases described above, such genes would have been present for making it resistant.

The most popular variety of basmati rice acceptable for export, Pusa Basmati-1, despite having two genes *Pi5-1*and *Pi9* for resistance was included in the category of

Table 3. Reactions of blast (*Magnaporthe oryzae*) disease (a) in induced epiphytotic conditions (b) observed in PCR studies as indicated below in the columns 3 and 4, respectively.

S/N	Genotypes	Disease reactions in artificial epiphytotic conditions and PCR studies											
		Artificial		Amplification of blast resistance gene-linked markers in PCR studies									
		R	S	Pi1	Pi2	PiK^h	Pi51	Pi52	Pi9	Pib	Piz-MRG 4963	Piz (MRG 5836)	Piz (MRG 2431)
1	VLD-85	+	-	+	-	+	+	-	-	-	-	-	-
2	VL-31077	+	-	-	-	-	-	-	-	+	+	-	-
3	Vivek Dhan-62	+	-		+	-	-	-	-	-	+	-	-
4	VLD-61	+	-		-	-	-	-	-	-	-	-	-
5	Vivek Dhan -154	+	-	+	-	+	+	-	+	+	+	-	-
6	VLD-81	+	-	+	-	+	+	-	+	-	+	-	-
7	VLD-221	+	-	+	-	-	-	-	-	+	+	-	-
8	VLD-65	+	-	+	+	-	+	-	+	-	-	-	-
9	Vivek Dhan -82	+	-	+	-	-	-	-	+	+	-	-	-
10	VLD-86	+	-	+	+	-	+	-	-	+	-	-	-
11	Vallabh-21	+	-	+	-	-	+	-	-	-	-	-	-
12	MAUB-57	-	+	-	-	-	+	-	+	-	-	-	-
13	MAUB-13	-	+	+	-	-	+	-	-	-	-	-	-
14	Vallabh Basmati-22	+	-	-	-	-	-	-	-	-	-	-	-
15	MAUB-171	+	-	-	+	-	-	-	-	-	-	-	-
16	Haryana Basmati	-	+	-	-	-	+	-	-	-	-	-	-
17	Ranbir Basmati	-	+		-	-	+	-	-	-	-	-	-
18	Haryana Mahak	-	+	+		-	-	-	-	-	-	-	-
19	Sathi White	-	+	+	+	-	+	-	-	-	-	-	-
20	Pusa Basmati-1	-	+	-		-	+	-	+	-	-	-	-
21	Punjab Basmati	-	+	-		-	+	-	-	-	-	-	-
22	Taroari Basmati	-	+	-		-	-	-	-	-	-		-

+, Indicates the presence of marker band; -, indicates the absence of marker band; R, indicates the resistant genotypes showing disease severity less than 46% and AUDPC less than 1000; S, indicates the susceptible genotypes showing disease severity more than 46% and AUDPC more than 1000.

most susceptible varieties. Perhaps it may be argued that resistance resulted due to interactions between both the genes (*Pi5-1*and *Pi9*) has now been broken down. The resistant cultivars having the same gene combination (*Pi5-1* and *Pi9*) perhaps also had other resistance genes in different combinations to make the genotypes resistant. Therefore, there is a need to initiate research programmes to incorporate genes for resistance in such mega varieties without losing their desirable characteristics such as duration, yield, quality among others.

On the basis of the results obtained in the present study, following empirical strategy could be suggested for development of blast resistant varieties of rice. Firstly, the blast resistant promising genotypes VLD-85, VL-31077, Vivekdhan-62 Vivekdhan-154, VLD-81 VLD-221, VLD-65, Vivekdhan-82, VLD-86, and Vallabh Basmati-21 can be used as male as well as female parents in crossing with blast susceptible

varieties still popular among farmers in order to obtain transgressive segregants. The blast resistant varieties mentioned above can be crossed among themselves in all possible combinations. Secondly, the same blast resistant varieties can be used as non-recurring donor parents in the back cross method with the traditional varieties of Basmati rice viz. Type-3, Taroari basmati, Ranvir Basmati, Basmati-370 and evolved basmati varieties viz. Vallabh Basmati- 22, Pusa Basmati-1, PS-4 among others. and thirdly, the blast resistant variety Vallabh Basmati-21 can be crossed with traditional varieties of Basmati rice viz. Type-3, Tarori basmati, Ranvir basmati, Basmati-370 and evolved basmati varieties. The blast resistant variety MAUB-171 can be crossed with traditional varieties of Basmati rice viz. Type-3, Taroari basmati, Ranbir basmati, Basmati-370 and evolved basmati varieties to transfer the *Pi2* gene. The segregating populations thus obtained should be handled as per pedigree method (Allard, 1960). Tightly linked DNA markers may facilitate early selection for blast resistance genes in breeding programs. These markers may also be useful to map new genes for resistance to blast isolates. Closely linked molecular markers are likely to enhance (Mehla et al., 2011) the efficiency of selection of resistant genotypes in rice breeding programs.

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