Molecular Screening of Blast Resistance Genes in Rice using SSR Markers

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(Received on June 12, 2014; Revised on December 9, 2014; Accepted on November 17, 2014)

Rice Blast is the most devastating disease causing major yield losses in every year worldwide. It had been proved that using resistant rice varieties would be the most effective way to control this disease. Molecular screening and genetic diversities of major rice blast resistance genes were determined in 192 rice germplasm accessions using simple sequence repeat (SSR) markers. The genetic frequencies of the 10 major rice blast resistance genes varied from 19.79% to 54.69%. Seven accessions IC337593, IC346002, IC346004, IC346813, IC356117, IC356422 and IC383441 had maximum eight blast resistance gene, while FR13B, Hourakani, Kala Rata 1-24, Lemont, Brown Gora, IR87756-20-2-2-3, IC282418, IC356419, PKSLGR-1 and PKSLGR-39 had seven blast resistance genes. Twenty accessions possessed six genes, 36 accessions had five genes, 41 accessions had four genes, 38 accessions had three genes, 26 accessions had two genes, 13 accessions had single R gene and only one accession IC438644 does not possess any one blast resistant gene. Out of 192 accessions only 17 accessions harboured 7 to 8 blast resistance genes.

Keywords : blast, marker assisted selection, resistance genes, rice, SSR markers

Rice blast is one of the most destructive diseases affecting rice production worldwide, which caused an economic loss up to 65% yield in susceptible cultivars (Li et al., 2007). Losses are dependent on the growth stage of the plant at which infection occurs, level of resistance and prevailing environmental conditions. It occurs more frequently in rain-fed areas in wet season due to favourable environmental

conditions for disease development. The identification and isolation of additional host blast resistance (R) genes and pathogen avirulence gene are now required to deepen understanding of molecular mechanisms involved in the host-pathogen interaction (Valent, 1990). Generally R genes are identified in land races, cultivars and wild rice collections using differential physiological races of *Magnaporthe oryzae* (Tanksley et al., 1997). With fine mapping and cloning of many blast resistance genes, many PCR-based markers have been developed to screen and identify different blast resistance genes. DNA markers closely linked to a blast R gene that confers resistance to a particular race of the pathogen can be effectively employed for marker assisted selection (MAS), which is much faster than traditional pathogenicity assays. Accurate identification of a particular R gene in diverse elite germplasm using DNA markers and differential blast races is an essential step for ensuring the accuracy of R gene utilization in using MAS for different rice breeding programs (Roy-Chowdhury et al., 2012a).

Recently, more than 100 major blast resistance genes from *japonica* (45%), *indica* (51%) and other (4%) genotypes have been identified and documented (Ballini et al., 2008; Berruyer et al., 2003; Chauhan et al., 2002; Chen et al., 2002; Huang et al., 2010; Liu et al., 2004; Liu et al., 2005; Sharma et al., 2012; Xiao et al., 2010; Zhu et al., 2004); and many rice varieties with complete resistance to *M. grisea* have been developed, but in many cases this resistance has been breakdown within a few years of the initial cultivation owing to the emergence of stronger virulent isolates of rice blast fungus (Han et al., 2001). Partial and field resistance of rice blast has received much attention as a means of effective control of a parasite under natural field condition and conferring durable blast resistance when exposed to new races of that parasite (Hittalmani et al., 2000; Liu et al., 2005). These R genes are distributed throughout the 12 rice chromosomes except chromosome 3 (Liu et al., 2010; Yang et al., 2008). Out of them, 22 have been

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Table 1. List of blast resistance genes, markers and germplasm used as check variety

S.No.	Gene	Chromosome locus	Check variety	Linked marker	Linkage distance (cM)	Expected PCR product size (bp)	SSR motif	Annealing temp. (°C)	References
1	Pi-9	6	PB-1460	RM 541	0.6	158	(TC)16	55	Cho et al., 2008
2	Pi-1	11	PB-1460	RM 224	0.0	157	(AAG)8(AG)13	56.4	Fuentes et al., 2007
3	Pi5-(t)	11	PB-1460	RM 21	0.0	157	(GA)18	60.40	Cuong et al., 2006
4	Piz-5	6	PB-1460	RM 527	0.3	233	(GA)17	55	Fjellstrom et al., 2006
5	Pi-b	2	PB-1460	RM 208	1.2	173	(CT)17	57.85	Hayashi et al., 2006
6	Pi-ta	12	PB-1460	RM 247	5.0	131	(CT)16	55.25	Eizenga et al., 2006
7	Pi33	8	IR-64	RM 72	11.5	166	(TAT)5C(ATT)15	56.95	Berruyer et al., 2003
8	Pi-27(t)	1	IR-64	RM 259	9.7	162	(CT)17	56.65	Zhu et al., 2004
9	Pitp(t)	1	Tetep	RM 246	0.0	116	(CT)20	58.50	Barman et al., 2004
10	Pi-k ^h	11	Tetep	RM 206	0.6	147	(CT)21	56.30	Sharma et al., 2005

cloned namely *Pib*, *Pita*, *Pik-h*, *Pi9*, *Pi2*, *Piz-t*, *Pid2*, *Pi36*, *Pi37*, *Pik-m*, *Pit*, *Pi5*, *Pid3*, *pi21*, *Pb1*, *Pish*, *Pik*, *Pik-p*, *Pia*, *NLS1*, *Pi25* and *Pi54rh* (Ashikawa et al., 2008; Bryan et al., 2000; Chen et al., 2006; Chen et al., 2011; Das et al., 2012; Fukuoka et al., 2009; Hayashi et al., 2010; Hayashi and Yoshida, 2009; Lin et al., 2007; Lee et al., 2009; Liu et al., 2007; Okuyama et al., 2011; Qu et al., 2006; Sharma et al., 2005; Shang et al., 2009; Takahashi et al., 2010; Tang et al., 2011; Wang et al., 1999; Yuan et al., 2011; Zhou et al., 2006; Zhai et al., 2011). However, it is imperative to identify broad-spectrum blast resistance genes for effective protection against dynamic blast isolates of *M. grisea*. Highly adaptive virulent isolates/races of the pathogen often challenge the effectiveness of deployed R genes and thus urge the need for the positive screening and identification of different blast R genes in the germplasm collection (Wang et al., 2010).

The identification and isolation of additional host resistance genes and pathogen avirulence genes is now required to deepen understanding of molecular mechanisms involved in the host-pathogen interaction and strategic deployment of resistance genes in commercial cultivars. Molecular markers are now widely used to characterize gene bank collections that contain untapped resources of distinct alleles which will remain hidden unless efforts are initiated to screen them for their potential use and function. Thus, this study was carried out to acquire the information for genetic diversities of blast resistance genes in rice germplasm accessions, so that efforts can be utilized to develop high yielding rice cultivars with resistance to blast through markers assisted selection.

Materials and Methods

Plant materials. The experimental materials comprised of 192 rice germplasm accessions received from Networking Project National Research Centre on Plant Biotechnology, New Delhi, Birsa Agricultural University, Ranchi and Department of Genetics and Plant Breeding, Institute of Agricultural Sciences, Banaras Hindu Varanasi, India, and their seeds were multiplied in wet season 2013. The details of the source of 192 rice accessions are presented in Table 2.

DNA extraction. Young leaves were collected from two week old plantlets of each germplasm grown in the plant growth chamber. The 40 mg leaves were taken from each accession placed in 1.2 ml collection microtubes (Qiagen Tissue Lyser II, Qiagen, U.S.A.) and in each microtube 3 mm tungsten beads were dispensed by bead dispenser and kept at -80°C for 4 hrs. Tissues were disrupted and homogenized by qiagen tissue lyser to a fine powder at frequency of 30 vibrations/seconds for 30 seconds. Fine powdered leaf samples were used for isolation of genomic DNA using CTAB (hexadecyl trimethyl ammonium bromide) method (Doyle and Doyle, 1987). The DNA was quantified spectrophotometrically (Perkin Elmer, Singapore) by measuring A260/A280, and DNA quality was checked by electrophoresis in 0.8% agarose gel.

SSR analysis. Ten previously reported SSR markers synthesized by Eurofins Genomics (Bangalore, India), were used to analyze the status of the blast resistance genes (Table 1). The amplification was carried out in 15 μl of reaction

Table 2. List of one hundred ninety two rice germplasm accessions, their source and genotypic screening for blast resistance gene with SSR markers

S.No.	Accession Number	Source	Blast resistance genes										Total number of genes
			<i>Piz-5</i> (RM 527)	<i>Pi-9</i> (RM 541)	<i>Pitp(t)</i> (RM 246)	<i>Pi-1</i> (RM 224)	<i>Pi-5(t)</i> (RM 21)	<i>Pi-33</i> (RM 72)	<i>Pi-b</i> (RM 208)	<i>Pi27(t)</i> (RM 259)	<i>Pik-h</i> (RM 206)	<i>Pi-ta</i> (RM 247)	
1	Dular	IRRI, Philippines	0	1	0	0	1	0	0	0	1	0	3
2	FR13A	IRRI, Philippines	1	1	1	0	0	1	0	0	1	0	5
3	FR13B	IRRI, Philippines	1	1	0	1	1	1	1	0	1	0	7
4	Karkati 87	IRRI, Philippines	0	0	1	1	1	0	1	0	0	0	4
5	Birpala	IRRI, Philippines	1	0	1	1	1	0	0	0	1	0	5
6	Goda Heenati	IRRI, Philippines	1	0	1	0	0	0	0	1	0	0	3
7	IR-64 Sub-1	NRCPB, New Delhi, India	1	0	0	0	0	1	1	0	0	0	3
8	Heensulai	IRRI, Philippines	1	0	1	1	0	0	1	0	0	0	4
9	Madal	IRRI, Philippines	1	0	1	1	0	1	1	0	0	0	5
10	Mahadikwee	IRRI, Philippines	1	0	0	1	1	1	0	0	0	0	4
11	Ausboro	IRRI, Philippines	1	0	0	0	0	1	1	1	1	0	5
12	Jaldungi	IRRI, Philippines	1	0	0	0	1	0	1	1	1	0	5
13	Nirboi	IRRI, Philippines	1	1	0	0	0	0	0	1	0	0	3
14	CR-1009	IRRI, Philippines	1	1	0	0	0	1	0	1	1	0	5
15	Hourakani	IRRI, Philippines	1	0	0	1	1	1	1	1	1	0	7
16	Naldak	IRRI, Philippines	1	1	0	0	1	0	1	1	0	0	5
17	Lunishree	CRRRI, Cuttack, India	1	1	0	1	0	0	1	1	0	1	6
18	Swarna Sub-1	NRCPB, New Delhi, India	1	1	0	0	0	1	1	1	1	0	6
19	Sambha Sub-1	NRCPB, New Delhi, India	1	0	1	1	0	1	1	1	0	0	6
20	Kariywa	NRCPB, New Delhi, India	1	1	0	1	0	1	1	0	1	0	6
21	S-155	CRRRI, Cuttack, India	0	1	0	0	0	1	0	1	0	0	3
22	Tsao Wanching	IRRI, Philippines	1	1	1	1	0	1	0	1	0	0	6
23	JC-1	IRRI, Philippines	0	1	1	0	0	1	0	1	0	0	4
24	Lumbini	IRRI, Philippines	0	0	0	0	1	0	1	1	1	1	5
25	Madhukar	IRRI, Philippines	1	1	1	0	0	1	0	0	0	0	4
26	N-22	IRRI, Philippines	0	0	0	0	1	1	1	1	1	0	5
27	Binulawan	IRRI, Philippines	0	1	0	1	0	0	1	0	0	0	3
28	Kala Rata 1-24	IRRI, Philippines	1	1	0	1	1	1	1	1	0	0	7
29	Tatan	IRRI, Philippines	1	0	1	0	0	0	0	0	1	1	4
30	Bapakribuna	IRRI, Philippines	1	0	1	0	0	1	0	0	0	0	3
31	Gota	IRRI, Philippines	1	0	0	1	1	1	1	0	1	0	6
32	Khaiyen	IRRI, Philippines	0	0	0	1	0	1	1	1	1	0	5
33	Kamnan	IRRI, Philippines	1	1	1	0	0	0	0	0	0	0	3
34	Lemont	IRRI, Philippines	1	1	1	1	1	1	0	0	1	0	7
35	Latsika	IRRI, Philippines	0	0	1	0	1	1	0	1	0	0	4
36	Akita Komachi	IRRI, Philippines	1	1	0	0	0	0	0	0	0	0	2
37	M-202	IRRI, Philippines	0	1	1	0	0	1	1	0	1	0	5
38	Amaroo	IRRI, Philippines	0	1	1	0	1	0	1	0	0	0	4
39	Nipponbare	IRRI, Philippines	0	1	1	0	1	0	1	0	0	0	4
40	Asami Dhan	IRRI, Philippines	0	0	1	0	1	0	1	0	0	0	3
41	Chanyngat	IRRI, Philippines	0	0	1	1	1	1	0	1	0	0	5
42	Budkodi	IRRI, Philippines	1	0	1	1	1	1	0	1	0	0	6
43	Dub Gelong	IRRI, Philippines	0	0	1	0	1	1	1	0	1	0	5
44	Sawitri	IRRI, Philippines	0	0	0	0	0	1	1	1	0	0	3

Table 2. Continued

S.No.	Accession Number	Source	Blast resistance genes										Total number of genes
			<i>Piz-5</i> (RM 527)	<i>Pi-9</i> (RM 541)	<i>Pitp(t)</i> (RM 246)	<i>Pi-1</i> (RM 224)	<i>Pi-5(t)</i> (RM 21)	<i>Pi-33</i> (RM 72)	<i>Pi-b</i> (RM 208)	<i>Pi27(t)</i> (RM 259)	<i>Pik-h</i> (RM 206)	<i>Pi-ta</i> (RM 247)	
45	Asthu Bhejna	IRRI, Philippines	0	0	0	0	0	0	1	0	0	0	1
46	Tundaniya	IRRI, Philippines	1	0	1	0	0	0	1	0	1	0	4
47	MTU-7029	BHU, Varanasi, India	1	0	1	0	1	1	0	1	1	0	6
48	Sarjoo-52	BHU, Varanasi, India	1	0	0	1	0	1	0	0	1	0	4
49	HUR-105	BHU, Varanasi, India	1	0	1	0	0	1	1	0	0	0	4
50	HUR-3022	BHU, Varanasi, India	1	0	0	0	0	1	0	0	0	0	2
51	HUR-38B	BHU, Varanasi, India	1	0	0	0	1	1	1	0	0	0	4
52	HUBR-40	BHU, Varanasi, India	1	1	0	0	0	1	1	0	0	1	5
53	HUBR 2-1	BHU, Varanasi, India	1	1	1	0	0	0	1	0	0	0	4
54	CB05-753-3	BAU, Ranchi, India	1	1	1	0	0	0	1	0	1	0	5
55	IR73000-98-1-2-1	BAU, Ranchi, India	1	1	0	0	0	1	0	1	1	0	5
56	Brown Gora	BAU, Ranchi, India	0	1	1	1	1	1	1	1	0	0	7
57	IR82912-B-B-10	BAU, Ranchi, India	0	1	1	0	0	0	0	0	0	0	2
58	IR82910-B-B-67-2	BAU, Ranchi, India	1	0	1	1	0	0	0	0	0	0	3
59	UPRI2012-16	BAU, Ranchi, India	0	1	0	1	0	0	0	0	0	0	2
60	CR3488-1-2-1-2	BAU, Ranchi, India	1	1	1	0	1	0	0	0	0	0	4
61	IR82912-B-B-13	BAU, Ranchi, India	0	1	0	0	0	0	0	0	0	0	1
62	IR83926-B-B-71-4	BAU, Ranchi, India	1	1	1	0	0	0	1	0	0	0	4
63	CT15678-2-3-3-1	BAU, Ranchi, India	1	0	1	0	0	0	1	0	0	0	3
64	B10580E-KN-28	BAU, Ranchi, India	1	1	1	1	0	0	0	0	1	0	5
65	IR82635-B-B-93-2	BAU, Ranchi, India	1	1	1	0	0	0	0	0	0	0	3
66	BVD109	BAU, Ranchi, India	1	1	1	1	0	0	0	0	1	0	5
67	IR71146-97-1-2-1-3	BAU, Ranchi, India	1	1	1	0	0	0	0	0	0	0	3
68	IR82635-B-B-143-1	BAU, Ranchi, India	1	1	1	0	1	0	0	0	0	0	4
69	BAU408-05	BAU, Ranchi, India	0	1	0	0	0	0	0	0	0	0	1
70	IR82912-B-B-2	BAU, Ranchi, India	0	1	0	0	0	0	0	0	0	0	1
71	IR82912-B-B-7	BAU, Ranchi, India	1	1	1	0	0	0	0	0	0	0	3
72	IR83929-B-B-132-3	BAU, Ranchi, India	0	0	0	0	0	0	0	0	0	1	1
73	IRAT-112	BAU, Ranchi, India	1	1	0	0	0	0	0	1	1	0	4
74	IR87756-20-2-2-3	BAU, Ranchi, India	1	1	1	0	0	1	0	1	1	1	7
75	IR67017-124-2-4	BAU, Ranchi, India	0	0	0	1	0	0	0	1	1	0	3
76	B3688-TB-25-MR-2	BAU, Ranchi, India	1	0	0	0	1	0	0	1	1	0	4
77	BP19768-2-3-7-78-1-1	BAU, Ranchi, India	1	0	0	1	1	0	0	1	1	0	5
78	IR78933-B-24-B-B-4	BAU, Ranchi, India	1	0	0	1	1	0	0	1	1	0	5
79	BP13510-1-2-PK-3-1	BAU, Ranchi, India	1	0	1	0	0	0	1	1	1	0	5
80	RR617-B-B-3	BAU, Ranchi, India	1	0	1	0	1	0	0	1	0	0	4
81	CR3423-1	BAU, Ranchi, India	0	0	1	0	0	0	0	1	0	0	2
82	CR1946-2-1	BAU, Ranchi, India	0	0	1	0	0	0	0	1	0	0	2
83	Kala Bunde	NRCPB, New Delhi, India	0	0	0	0	0	0	1	0	1	0	2
84	IC260937	DBT, New Delhi, India	1	0	0	0	1	0	0	0	0	0	2
85	IC260961	DBT, New Delhi, India	1	0	0	0	1	0	0	0	0	0	2
86	IC260964	DBT, New Delhi, India	1	0	0	1	1	0	0	0	0	0	3
87	IC267416	DBT, New Delhi, India	0	0	0	0	1	0	1	0	0	0	2
88	IC278777	DBT, New Delhi, India	1	0	0	0	0	0	0	0	0	0	1
89	IC280466	DBT, New Delhi, India	1	0	1	0	0	0	0	0	0	0	2

Table 2. Continued

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			<i>Piz-5</i> (RM 527)	<i>Pi-9</i> (RM 541)	<i>Pitp(t)</i> (RM 246)	<i>Pi-1</i> (RM 224)	<i>Pi-5(t)</i> (RM 21)	<i>Pi-33</i> (RM 72)	<i>Pi-b</i> (RM 208)	<i>Pi27(t)</i> (RM 259)	<i>Pik-h</i> (RM 206)	<i>Pi-ta</i> (RM 247)	
90	IC281785	DBT, New Delhi, India	1	0	0	0	1	0	0	0	1	0	3
91	IC281786	DBT, New Delhi, India	0	1	1	1	0	1	0	1	0	1	6
92	IC282418	DBT, New Delhi, India	0	1	0	1	1	1	1	1	0	1	7
93	IC282443	DBT, New Delhi, India	0	1	0	1	1	0	0	0	0	1	4
94	IC282463	DBT, New Delhi, India	1	1	1	1	0	0	0	1	0	1	6
95	IC282471	DBT, New Delhi, India	1	1	1	1	0	0	0	1	0	1	6
96	IC282514	DBT, New Delhi, India	1	1	1	1	0	0	0	0	0	1	5
97	IC282808	DBT, New Delhi, India	1	1	0	1	0	0	0	0	0	1	4
98	IC282815	DBT, New Delhi, India	1	1	0	1	1	0	0	0	0	1	5
99	IC282816	DBT, New Delhi, India	1	1	0	1	1	0	0	0	0	0	4
100	IC282822	DBT, New Delhi, India	1	1	0	1	0	0	0	0	0	0	3
101	IC282824	DBT, New Delhi, India	1	1	0	1	0	0	0	0	0	0	3
102	IC331196	DBT, New Delhi, India	0	1	0	0	0	0	0	0	0	0	1
103	IC334180	DBT, New Delhi, India	0	1	1	1	1	0	0	0	0	1	5
104	IC337051	DBT, New Delhi, India	0	0	0	0	1	0	1	0	0	1	3
105	IC337367	DBT, New Delhi, India	0	0	0	0	0	1	0	0	0	1	2
106	IC337558	DBT, New Delhi, India	0	0	1	0	0	0	0	0	0	1	2
107	IC337578	DBT, New Delhi, India	0	0	0	1	1	1	1	0	0	0	4
108	IC337582	DBT, New Delhi, India	0	0	0	1	0	0	1	0	0	0	2
109	IC337588	DBT, New Delhi, India	1	1	1	0	0	1	1	0	0	1	6
110	IC337593	DBT, New Delhi, India	1	1	0	1	0	1	1	1	1	1	8
111	IC341351	DBT, New Delhi, India	1	1	1	0	0	1	0	1	0	1	6
112	IC346002	DBT, New Delhi, India	1	1	1	0	1	1	1	1	0	1	8
113	IC346004	DBT, New Delhi, India	1	1	1	1	0	1	1	1	0	1	8
114	IC346813	DBT, New Delhi, India	1	1	1	1	0	1	1	1	0	1	8
115	IC346880	DBT, New Delhi, India	1	1	0	0	0	1	0	1	0	1	5
116	IC356117	DBT, New Delhi, India	1	1	1	1	0	1	1	1	0	1	8
117	IC356419	DBT, New Delhi, India	1	1	0	1	0	1	0	1	1	1	7
118	IC356422	DBT, New Delhi, India	1	1	1	1	1	0	1	1	0	1	8
119	IC356429	DBT, New Delhi, India	1	1	0	1	0	0	0	1	0	1	5
120	IC356431	DBT, New Delhi, India	1	1	0	1	0	0	0	1	0	1	5
121	IC356432	DBT, New Delhi, India	1	1	0	1	0	0	0	1	0	1	5
122	IC356448	DBT, New Delhi, India	0	1	0	1	0	0	0	1	0	1	4
123	IC362206	DBT, New Delhi, India	0	1	0	0	1	0	0	1	0	1	4
124	IC382604	DBT, New Delhi, India	0	1	1	1	0	0	0	1	1	1	6
125	IC383396	DBT, New Delhi, India	0	1	0	0	1	0	0	1	0	1	4
126	IC383404	DBT, New Delhi, India	0	1	0	1	0	0	0	1	0	1	4
127	IC383441	DBT, New Delhi, India	1	1	1	1	1	1	0	1	0	1	8
128	IC383559	DBT, New Delhi, India	0	1	0	0	0	1	0	1	0	0	3
129	IC384176	DBT, New Delhi, India	0	0	1	0	0	1	0	1	0	0	3
130	IC384190	DBT, New Delhi, India	1	0	1	1	1	1	0	0	0	0	5
131	IC384260	DBT, New Delhi, India	1	1	0	0	0	1	0	0	0	0	3
132	IC391524	DBT, New Delhi, India	0	1	1	1	0	0	1	0	0	0	4
133	IC418382	DBT, New Delhi, India	0	0	0	0	0	0	1	0	1	0	2
134	IC426012	DBT, New Delhi, India	1	1	1	0	1	0	1	0	0	0	5

Table 2. Continued

S.No.	Accession Number	Source	Blast resistance genes										Total number of genes
			<i>Piz-5</i> (RM 527)	<i>Pi-9</i> (RM 541)	<i>Pitp(t)</i> (RM 246)	<i>Pi-1</i> (RM 224)	<i>Pi-5(t)</i> (RM 21)	<i>Pi-33</i> (RM 72)	<i>Pi-b</i> (RM 208)	<i>Pi27(t)</i> (RM 259)	<i>Pik-h</i> (RM 206)	<i>Pi-ta</i> (RM 247)	
135	IC426013	DBT, New Delhi, India	1	1	1	0	1	0	1	0	0	0	5
136	IC426017	DBT, New Delhi, India	1	1	1	0	1	1	1	0	0	0	6
137	IC426058	DBT, New Delhi, India	1	1	1	0	1	0	1	0	0	0	5
138	IC426060	DBT, New Delhi, India	0	1	0	1	0	0	1	0	0	0	3
139	IC426061	DBT, New Delhi, India	0	0	1	0	0	0	1	0	1	0	3
140	IC426137	DBT, New Delhi, India	0	0	1	0	0	0	0	0	0	0	1
141	IC438644	DBT, New Delhi, India	0	0	0	0	0	0	0	0	0	0	0
142	IC446975	DBT, New Delhi, India	0	0	1	0	0	0	1	0	0	0	2
143	EC545051	DBT, New Delhi, India	0	0	1	1	0	0	1	0	0	0	3
144	EC545061	DBT, New Delhi, India	0	0	1	0	0	1	1	0	0	0	3
145	PKSLGR-1	BHU, Varanasi, India	1	1	1	1	1	1	1	0	0	0	7
146	PKSLGR-2	BHU, Varanasi, India	1	1	1	0	1	0	0	0	0	0	4
147	PKSLGR-3	BHU, Varanasi, India	1	1	1	0	0	1	1	0	0	0	5
148	PKSLGR-4	BHU, Varanasi, India	0	1	0	1	1	0	0	0	0	0	3
149	PKSLGR-5	BHU, Varanasi, India	0	1	1	0	0	1	1	0	0	0	4
150	PKSLGR-6	BHU, Varanasi, India	0	1	0	1	1	0	0	0	0	0	3
151	PKSLGR-7	BHU, Varanasi, India	1	0	1	0	0	1	1	1	0	0	5
152	PKSLGR-8	BHU, Varanasi, India	1	0	1	0	1	1	1	1	0	0	6
153	PKSLGR-9	BHU, Varanasi, India	0	0	0	1	1	0	0	0	0	0	2
154	PKSLGR-10	BHU, Varanasi, India	0	0	0	1	1	0	0	0	0	0	2
155	PKSLGR-11	BHU, Varanasi, India	0	0	0	1	1	0	0	0	0	0	2
156	PKSLGR-12	BHU, Varanasi, India	0	0	0	1	1	1	0	0	0	0	3
157	PKSLGR-13	BHU, Varanasi, India	0	0	0	1	0	0	0	0	0	0	1
158	PKSLGR-14	BHU, Varanasi, India	0	0	0	1	0	0	0	0	0	0	1
159	PKSLGR-15	BHU, Varanasi, India	0	0	0	0	1	0	1	0	0	0	2
160	PKSLGR-16	BHU, Varanasi, India	0	0	0	1	1	0	0	0	0	0	2
161	PKSLGR-17	BHU, Varanasi, India	0	0	1	0	1	1	0	0	0	0	3
162	PKSLGR-18	BHU, Varanasi, India	0	0	1	0	1	0	0	0	0	0	2
163	PKSLGR-19	BHU, Varanasi, India	0	1	0	1	0	0	0	0	0	0	2
164	PKSLGR-20	BHU, Varanasi, India	1	1	0	0	0	1	0	0	1	0	4
165	PKSLGR-21	BHU, Varanasi, India	0	1	1	0	1	0	1	0	0	0	4
166	PKSLGR-22	BHU, Varanasi, India	0	1	0	1	0	0	0	0	0	0	2
167	PKSLGR-23	BHU, Varanasi, India	0	1	0	1	0	0	0	0	0	0	2
168	PKSLGR-24	BHU, Varanasi, India	0	1	1	1	0	0	1	0	0	0	4
169	PKSLGR-25	BHU, Varanasi, India	1	1	0	1	1	1	1	0	0	0	6
170	PKSLGR-26	BHU, Varanasi, India	0	0	1	0	1	0	1	0	1	0	4
171	PKSLGR-27	BHU, Varanasi, India	0	0	1	1	1	0	1	0	1	0	5
172	PKSLGR-28	BHU, Varanasi, India	0	1	1	0	1	0	1	0	0	0	4
173	PKSLGR-29	BHU, Varanasi, India	1	0	0	0	0	0	0	1	1	0	3
174	PKSLGR-30	BHU, Varanasi, India	1	1	1	1	1	0	1	0	0	0	6
175	PKSLGR-31	BHU, Varanasi, India	0	0	0	1	0	0	0	0	0	0	1
176	PKSLGR-32	BHU, Varanasi, India	0	0	0	0	0	0	0	0	1	0	1
177	PKSLGR-33	BHU, Varanasi, India	1	0	0	1	1	0	0	0	0	0	3
178	PKSLGR-34	BHU, Varanasi, India	1	0	0	0	1	0	0	1	0	0	3
179	PKSLGR-35	BHU, Varanasi, India	0	0	0	1	1	1	1	0	1	0	5

Table 2. Continued

S.No.	Accession Number	Source	Blast resistance genes										Total number of genes
			<i>Piz-5</i> (RM 527)	<i>Pi-9</i> (RM 541)	<i>Pitp(t)</i> (RM 246)	<i>Pi-1</i> (RM 224)	<i>Pi-5(t)</i> (RM 21)	<i>Pi-33</i> (RM 72)	<i>Pi-b</i> (RM 208)	<i>Pi27(t)</i> (RM 259)	<i>Pik-h</i> (RM 206)	<i>Pi-ta</i> (RM 247)	
180	PKSLGR-36	BHU, Varanasi, India	0	0	0	0	1	1	1	0	1	0	4
181	PKSLGR-37	BHU, Varanasi, India	1	0	1	1	0	1	0	0	0	0	4
182	PKSLGR-38	BHU, Varanasi, India	1	1	0	1	0	1	0	1	1	0	6
183	PKSLGR-39	BHU, Varanasi, India	1	1	0	1	1	1	0	1	0	1	7
184	PKSLGR-40	BHU, Varanasi, India	1	1	0	1	0	1	0	1	1	0	6
185	PKSLGR-41	BHU, Varanasi, India	1	0	1	0	0	1	0	0	1	0	4
186	PKSLGR-42	BHU, Varanasi, India	0	1	1	0	0	1	0	0	1	0	4
187	PKSLGR-43	BHU, Varanasi, India	0	1	0	1	0	0	0	0	1	0	3
188	PKSLGR-44	BHU, Varanasi, India	0	0	0	0	1	0	0	0	0	0	1
189	PKSLGR-45	BHU, Varanasi, India	0	0	1	0	1	1	0	0	0	0	3
190	PKSLGR-46	BHU, Varanasi, India	0	0	1	0	0	1	0	0	0	0	2
191	PKSLGR-47	BHU, Varanasi, India	0	0	1	0	0	1	0	0	0	0	2
192	PKSLGR-48	BHU, Varanasi, India	0	0	0	1	1	1	0	0	1	0	4
Frequency (%)			54.69	52.60	47.92	44.27	40.63	40.10	39.06	33.85	27.08	19.79	
Approx. size (bp)			216	170	120	140	147	160	170	150	130	126	

The rice blast resistance gene scored as the presence (1) and absence (0) of amplicon linked to ten of allele specific SSR markers.

mixture containing 30 ng genomic DNA, 1.5 mM PCR buffer (MBI Fermentas, USA), 400 μ M dNTPs (MBI Fermentas), 1 U Taq DNA polymerase (MBI Fermentas) and 0.4 μ M primer using a thermal cycler (Mastercycler gradient, Eppendorf). Thermal cycling program involved an initial denaturation at 94°C for 4 min, followed by 34 cycles of denaturation at 94°C for 45 sec, annealing at 2°C below T_m of respective primers for 30 sec, primer extension at 72°C for 30 sec, followed by a final extension at 72°C for 8 min. SSR markers (Co-dominant) show specific site banding pattern on chromosome for specific traits. The 2.5% agarose gel was used for visualising banding pattern >20 bp product size between the alleles. The amplified PCR products with a 50 bp DNA marker ladder (MBI Fermentas) were size fractionated by electrophoresis in 2.5% agarose gel prepared in TAE buffer and visualized by staining with ethidium bromide (0.5 μ g/ml) in a gel documentation system (BIO-RAD, USA).

Results

Allelic diversity of rice blast resistance genes. The results of genotypic screening of 192 accessions for the presence or absence of 10 major rice blast resistance genes using SSR markers are presented in Table 2 and electrophoresis pattern of each SSR marker linked to blast resistant gene with few accessions are shown in Fig. 1A&B. The germ-

plasms PB-1460 for *Pi-9*, *Pi-1*, *Pi-5(t)*, *Piz-5*, *Pi-b*, *Pi-ta*; IR-64 for *Pi-33*, *Pi27(t)* and Tetep for *Pitp(t)*, *Pi-k^h* were used as gene differential lines. Estimation of PCR results for 10 blast resistance genes viz. *Piz-5*, *Pi-9*, *Pitp(t)*, *Pi-1*, *Pi-5(t)*, *Pi-33*, *Pi-b*, *Pi27(t)*, *Pi-k^h* and *Pi-ta* were determined by visualization of amplicons on near 216 bp, 170 bp, 120 bp, 140 bp, 147 bp, 160 bp, 170 bp, 150 bp, 130 bp and 126 bp of positive fragments, respectively. The genetic frequencies of the 10 major rice blast resistance genes were ranged from 19.79% to 54.69%. Seventy three accessions containing at least five positive bands of the 10 rice blast resistance markers. The blast resistance gene *Piz-5* was widely distributed in 54.69% accessions followed by *Pi-9* in 52.60%, *Pitp(t)* in 47.92%, *Pi-1* in 44.27%, *Pi-5(t)* in 40.63%, *Pi-33* in 40.10%, *Pi-b* in 39.06%, *Pi-27(t)* in 33.85%, *Pik-h* in 27.08% and *Pi-ta* in only 19.79% accessions. Seven accessions IC337593, IC346002, IC346004, IC346813, IC356117, IC356422 and IC383441 had maximum eight blast resistance gene, while FR13B, Hourakani, Kala Rata 1-24, Lemont, Brown Gora, IR87756-20-2-2-3, IC282418, IC356419, PKSLGR-1 and PKSLGR-39 possessed seven blast resistance genes, and 20 accessions had six genes, 36 accessions had five genes, 41 accessions had four genes, 38 accessions had three genes, 26 accessions had two genes, 13 accessions had single *R* gene and only one accession IC438644 does not possess any one blast resistant gene.

Fig. 1. (A) Agarose gel electrophoretic pattern of some selected rice germplasm accessions generated by using SSR markers (1) RM 541, (2) RM 224, (3) RM 21, (4) RM 527, (5) RM 208, where M is 50 bp DNA size marker, C is check variety and numbers 1-192 represent rice germplasm accessions as described in Table 2.

Genetic diversity of *Pi-1* and *Pi-9* gene. Estimation of PCR results for the *Pi-1* and *Pi-9* rice blast resistance genes were determined by visualization of amplicons on 140 bp and 170 bp of positive fragments using SSR primer RM 224 and RM 541 on the chromosome number 11 and 6, respectively. *Pi-1* gene was scored on 85 accessions and

Pi-9 gene in 101 accessions. *Pi-9* gene fragment was the second most prevalent among the germplasm accessions studied. Fifty one accessions amplify both SSR markers corresponding to the resistance check (PB 1460) while 57 accessions did not amplify either of the two markers and hence negative for these two genes.

Fig. 1. (B) Agarose gel electrophoretic pattern of some selected rice germplasm accessions generated by using SSR markers (6) RM 247, (7) RM 72, (8) RM 259, (9) RM 246, (10) RM 206, where M is 50 bp DNA size marker, C is check variety and numbers 1-192 represent rice germplasm accessions as described in Table 2.

Genetic diversity of *Pi-5(t)* and *Piz-5* gene. The SSR marker RM 21 is linked to blast resistance gene *Pi5-(t)* on chromosome no. 11, revealed the presence of a 147 bp fragment specific for *Pi5-(t)* mediated blast resistance in the differential line PB 1460. Presence of rice blast resistance gene *Piz-5* on chromosome 6 was determined by visualization of positive fragments using SSR primer RM

527 on 216 bp of positive fragment corresponding to the resistance differential line PB 1460. *Piz-5* gene fragment was the most prevalent among the accessions studied. Forty accessions possessing both genes corresponding to the resistance check (PB 1460) while 51 accessions did not amplify either of the two markers and hence negative for the two genes.

Genetic diversity of *Pi-ta* and *Pi-b* gene: PCR based screening of *Pi-ta* and *Pi-b* genes on chromosome 12 and 2 showed that only 38 and 75 accessions under study produced positive bands on 126 bp and 170 bp with SSR marker RM 247 and RM 208, respectively. Eighty nine accessions possessed at least one of *Pi-ta*/*Pi-b* genes. Twelve accessions amplify both SSR markers corresponding the resistance check (PB 1460) while 91 accessions did not amplify either of the two markers and hence negative for the two genes.

Genetic diversity of *Pi-27(t)* and *Pi-33* gene. The *Pi-27(t)* and *Pi-33* specific PCR primer RM 259 and RM 72 were produced 150 bp and 160 bp amplicon based on its sequence on chromosome 6 and 8, respectively. Screening of *Pi-27(t)* and *Pi-33* blast resistance gene were determined by visualization of positive fragments from 65 and 77 accessions with SSR marker RM 259 and RM 72, respectively. The 37 accessions amplify both SSR markers corresponding to the resistance check (IR 64) while 87 accessions did not amplify either of the two SSR markers which showed negative for the two genes.

Genetic diversity of *Pi-k^h* and *Pitp(t)* gene. Fifty two accessions show the positive fragment of *Pi-k^h* gene located on chromosome 11 with tightly linked SSR markers RM 206, while 92 accessions show the positive fragment of *Pitp(t)* located on chromosome 11 with tightly linked SSR markers RM 246. Result indicates the presence of an approx 130 bp and 120 bp fragment specific for blast resistance genes *Pi-k^h* and *Pitp(t)* in the differential Tetep, respectively. Eighteen accessions showed positive bands for both genes while 68 accessions did not amplify any of the two genes.

Discussion

The marker-assisted selection of rice blast resistance genes will help in the breeding program in multi-diseases resistant rice varieties in genetic resources of rice. Some of these germplasm accessions may have special properties that are important to breeding program. In the present study, the genetic frequencies of the 10 major rice blast resistance genes *Piz-5*, *Pi-9*, *Pitp(t)*, *Pi-1*, *Pi-5(t)*, *Pi-33*, *Pi-b*, *Pi27(t)*, *Pi-k^h* and *Pi-ta* were ranged from 19.79% to 54.69%. Similar results were reported by Kim et al. (2010) in 84 accessions of rice germplasms possessed more than three positive bands of the eight rice blast resistance genes, and Imam et al. (2014) reported the genetic frequency of the nine major rice blast resistance genes *Piz*, *Piz-t*, *Pik*,

Pik-p, *Pik-h*, *Pita/Pita-2*, *pita*, *Pi9* and *Pib*, ranged from 6 to 97% in the select set of rice germplasms. Our result also showed that the analysis of the distribution of resistance genes in ancient populations of landraces can direct the rice blast resistance breeding program and rice blast control by genetic diversity. Many rice varieties have been developed as completely resistant to *M. oryzae* strains, but soon breakdown of rice blast resistant genes occurred because of the emergence of stronger virulent isolates of rice blast fungus (Mackill and Bonman, 1992). Genotyping of the accessions with allele specific markers helped to identify 10 major blast resistance genes in 192 rice germplasm accessions from different ecological regions.

The 54.69% accessions possessing resistance gene *Piz-5* on chromosome 6. These accessions were distributed in different ecosystem across the globe. Similarly, Yan et al. (2007) evaluated a core subset of the USDA 1790 rice germplasms and they found some accessions contained *Piz-5* gene with additional R genes. In addition we verified accuracy of our results, 105 accessions possessed identical alleles for *Piz-5* gene at approximate 216 bp fragments corresponding to the check (PB 1460). The presence of the same marker alleles in these accessions suggests that they contain a *Piz-5* gene. This finding is important because these 105 accessions were collected from different geographic regions. The most likely reason for this similarity is that the original donor parent for the *Piz-5* gene may contain the same genomic fragment for all these cultivars. In contrast, 87 accessions do not showed similar marker allele suggesting that these accessions presumably either inherited from different donors or the result of recombination during the breeding process. In conclusion, we not only verified the *Piz-5* gene in 105 accessions using previously identified DNA markers but also demonstrated the usefulness of DNA markers with differential blast resistance gene for germplasm characterization. Similar report also made earlier by Roy-Chowdhury et al. (2012b).

The resistance pattern of the accessions is examined for the presence of amplicon products of the major genes. It was noted that though resistance is generally proportional to the frequency of the resistant gene(s). The present study was taken up with a selected set of rice accessions covering a wider geographical region where certain genes (*Piz-5*, *Pi9*, *Pitp(t)* and *Pi-1*) were more diverse than others and these were identified in 105, 101, 92 and 85 accessions on chromosome 6, 6, 1 and 11, respectively. Similarly, Imam et al. (2014) reported in his study that the genes (*Pi9*, *Pita-2*, *Piz-t*) were more effective than others in thwarting infection. Thirty eight accessions produced positive bands near 126 bp fragments corresponding to the check (PB 1460) for

blast resistance gene *Pi-ta*, which has been located near the centromere of chromosome 12 (Wang et al., 2002). Identification and validation of *Pi-ta* genes reveals that the Indian rice germplasm are diverse and potential source of blast resistant lines which can be exploited in rice blast breeding programs (Shikari et al., 2013). The *Pi-ta* genes commonly used in rice breeding programs worldwide have originated from several traditional *indica* cultivars, including Tetep from Vietnam and Tadukan from the Philippines (Cho et al., 2008). Transferring blast resistance genes to different genetic backgrounds is very cumbersome and tedious. Since, it would be difficult to identify under field conditions using conventional approaches in order for marker-assisted selection to facilitate at early selection phase with greater accuracy (Gu et al., 2005).

Molecular screening of *Pik-h* and *Pi-b* blast resistance gene were determined by visualization of positive fragments from 52 and 75 accessions, respectively. Members of the *Pik-h* multi-gene family and *Pi-b* were moderately distributed genes in the present study but neither the germplasm possessing them nor the isogenic lines in the previous evaluations had exhibited resistance (Variar et al., 2009). Presence of major rice blast resistance gene *Pitp(t)* and *Pi-27(t)* on chromosome 1 and *Pi-33* on chromosome 8 was determined by visualization of 120 bp, 150 bp and 160 bp positive fragments, respectively. The gene-specific marker RM 246 for resistance gene *Pitp(t)* amplified positive bands in 85 accessions, while *Pi-27(t)* and *Pi-33* genes were identified in 65 and 77 accessions, respectively. This study illustrated the utility of SSR markers to identify rice varieties likely carried the same *R* genes with potentially novel resistance. Rice varieties with a number of alleles in common with any specific resistance might have a similar blast *R* gene, and understanding the natural diversity at the specific gene is important for incorporation of specific *R* gene using DNA marker into rice breeding program (Jia et al., 2003).

Genetic diversity among the rice accessions and within the pathogen often leads to inconsistent marker and phenotype analysis. MAS have the advantage in identifying *R* genes, but its power lies in the robustness of the markers used. The identification and analysis of rice blast resistance genes suggests that DNA primers derived from the gene is a valuable tool for blast gene identification and screening among the rice germplasm (Roy-Chowdhury et al., 2012a, b). In this study, the PCR based markers employed for screening of different blast resistance genes are well established and effective. The consistent results showed with the selected SSR markers for respective genes was highly reliable and make them the marker of choice for molecu-

lar screening of rice blast resistance genes among the rice accessions. Plant breeders often use cultivars developed in other countries to broaden the genetic background of the improved cultivars being developed such as the major fungal diseases of blast, but most breeding programs of rice have a narrow genetic diversity of breeding resources. Our results showed that 17 accessions harboured 7 to 8 blast resistance genes, which can be suggested that these accessions could be used as sources of resistance genes in designing future breeding programmes, and there is good possibility of obtaining enhanced resistance through gene pyramiding.

Acknowledgement

Authors are thankful to Department of Biotechnology, New Delhi, Ministry of Agriculture, Government of India for their financial support through Grant No. 07/473, BHU Varanasi India.

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