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Molecular Screening Of Traditional Rice Varieties (Trvs) For Blast Resistance Genes Using SSR Markers

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ABSTRACT
Rice is a staple and most important security food crop consumed by almost half of the world's population. More rice production is needed due to the rapid population growth in the world. Rice blast caused by the fungus, Magnaportheoryzaeis one of the most destructive diseases of this crop in different part of the world. It is difficult to control rice blast because of high pathogen plasticity, mutation rate and breakdown of blast resistance. 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 32 rice germplasms which includes 30 Traditional rice varieties (TRVs), a resistance (Tetep) and susceptible (Susceptible) checks using five simple sequence repeat (SSR) markers.The genetic frequencies of the five major rice blast resistance genes varied from 25% (Pi1) to 90.6% (Pi2).A TRV, Mugadsuganda had maximum of five resistance genes, nine TRVs possess four blast resistance genes, while four TRVs like, Naweli, ThornadaBatta, Adribatta and Mullubatta had only one blast resistance gene. Tetep and HR 12 possess three and two resistance genes respectively. Most of the TRVs were shown to be rich source R genes for rice blast, could be utilized for future breeding works to develop blast resistance varieties.
***Key Words**: Rice, TRVs, Magnaportheoryzae, Genetic Frequency, SSR markers*

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INTRODUCTION

Rice (*Oryzasativa* L.) is one of the most important cereal crops belonging to the family *Poaceae*, which contributes significantly to global food security and is the staple food for more than half of the world's population. With China and India as lead producers, more than 90 % of rice is produced in Asia (Kumar *et al*., 2011). India has the largest acreage under rice with an area of 44.1 million hectare and stands 2nd in production with 105.5 million tonnes, next to China and productivity is 2391 Kg/ha. Karnataka is one of the major rice growing states in India with an area of 13 lakh ha and production of 25.73 lakh tonnes and productivity of 2573 Kg/ha (www.indiastat.com). Global rice demand is estimated to rise from 6.76×10^8 tonnes in 2010 to 8.52×10^8 tonnes in 2035 (Khush, 2013). To produce 1.76×10^8 tonnes additional rice, it is needed to increase the yield and also minimize the yield loss caused by various diseases and insect pests. Between 10 and 30 per cent of the annual rice harvest is lost due to infection by many diseases (Skamnioti and Gurr, 2009).

More than 70 diseases are caused by fungi, bacteria, viruses and nematodes in rice (Sattari*et al*., 2014). Among these diseases, rice blast caused by *Magnaportheoryzae* is major serious threat to the world food security as rice is the staple food for majority of population. It is a potentially damaging disease in upland environment where drought and soil stress predispose the rice crop to severe attacks by the pathogen. This fungus infects during nearly all growth stages on all aerial parts of rice, leading to leaf blast, neck and panicle blast and nodal blast. The yield loss due to this disease ranged from 1 to 50 %, meaning each year it destroys abundant rice amounting to economic loss of over $70 billion of dollar (Scheuermann*et al.,* 2012). In 2015, The Hindu newspaper reported that in four rice growing areas of Mysore district of

Karnataka (Hunsur, Narasipur, K R Nagar, and Nanjangud), rice crop in an area of 21,330 hectares was affected by rice blast (The Hindu, 2015).

The genus *Magnaporthe* collectively parasitizes more than 50 hosts with limited host range of individual isolates and relatively rare cross-infectivity. The ability of this fungus to quickly overcome resistance within a short time after the release of a new cultivar has made breeding for resistance a constant challenge (Babujee and Gnanamanickam, 2000). Hence, development of broad spectrum and durable blast resistant varieties is essential for combating this disease.

The host plant resistance is considered as a best way to control the blast disease. So, 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. Generally R genes are identified in land races, cultivars and wild rice collections using differential physiological races of *Magnaportheoryzae.* 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.

To date, around 100 blast resistance genes have been identified (Devanna*et al.*, 2014), and 22 of them have been cloned and characterized *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).Except chromosome 3 These R genes are distributed throughout the 12 rice chromosomes. Though many resistant varieties to *Magnaportheoryzae*have been developed, the resistance is not long lasting because of high pathogen plasticity in the fields making single resistance gene break down after three to five years of the cultivar release (Devi *et al*., 2015). Hence, development of broad spectrum and durable blast resistant varieties is essential for combating this disease.

Traditional rice varieties (TRVs) are rich source of resistance genes and are unexploited and characterized. So there is need for collection, documentation and screening of such rice varieties and identification of novel genes and their functional alleles which effectively tackle the disease.

Thus, this study was carried out to acquire the information for genetic diversities of blast resistance genes in TRVs, 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

A set of 30 TRVs that are popular in Karnataka and other parts of south India and some improved varieties were obtained from germplasm collection of Division of Rice Breeding, AICRP (Rice), Zonal Agricultural Research Station along with checks were evaluated in the present study during 2015-2017, to identify blast resistant genes of paddy against *Magnaportheoryzae*at ZARS, V. C. Farm, Mandya. The details of the source of 32 rice varieties were presented in Table 1

DNA Isolation

Leaf samples were collected from 20-25 days old seedlings and were stored immediately at –80°C till DNA was isolated. Genomic DNA was isolated from fresh, healthy and young leaves from 30 traditional rice varieties along with two checks following CTAB (Cetyl-Tri Methyl Ammonium Bromide) method (Murray and Thompson, 1980).

The DNA purity was analysed by estimating the ratios between 260 nm and 280 nm using advanced automated DNA quantifier, Biospec-nano (Spectrophotometer for life science) pure DNA samples will normally have a ratio between1.8 and 2.0. If the sample is contaminated by proteins, the ratio will be significantly less than 1.8. Ratio of 2.0 or more indicates a high proportion of RNA in the sample. Following this, all the DNA samples were diluted to a final concentration of 40 ng/µl using TE buffer. The quality and quantity of DNA was also analyzed by running the genomic DNA samples on 0.8% agarose gels. This additional step would give us an idea on the extent of DNA shearing.

PCR and SSR marker analysis

Five specific SSR markers were used for molecular validation of 30 TRVs, Tetep and HR 12 for rice blast resistance (Table 2). The primer sequences were obtained from www.graminae.org and other previously published research work sheath blight resistant QTLs and their associated markers. The primer sequence

was used and the oligos were synthesized from Sigma Inc. Each polymerase chain reaction (PCR) was conducted in 10 µL reaction volume containing 2.0 µl of TAKARA mix (The TAKARA mix, the master mix for the PCR, containing Taq polymerase, $MgCl_2$, buffer and loading dye was obtained from TAKARA Inc.), 1 µL of each primer (10 µM), 1µL of template DNA (40 ng/ µL), and 5 µL of ddH_2O. The following temperature profiles and cycles were maintained by using a thermal cycler from Applied Biosystems. 1 cycle at 95°C for 5 min (initial denaturation), followed by 34 cycles of at 95°C for 30 sec (Denaturation), annealing at 55°C (depending on primers) for 30 sec, extension at 72°C for 1 min, 1 cycle of final extension at 72°C for 10 min, and storage at 4°C.

After completion of PCR, products were run on 3% agarose gel, prepared using 1X TE buffer and ethidium bromide. After the completion of the electrophoresis, the DNA profile was documented using gel documentation unit (UVI Tech Fire reader). Gel pictures were scored on the basis of expected bp for resistant allele, as 1 for presence and 0 for absence of resistant allele.

RESULTS AND DISCUSSION

Improvement of host plant resistance is one of the best methods to protect the yield from *M. oryzae*. Incorporation of major rice blast resistance genes or their variants into elite rice varieties will enhance the host plant resistance and its durability. Genotyping of the accessions with allelic related markers helped to identify major blast resistance genes from different origins. The marker-assisted selection of rice blast resistance genes will help in the breeding program inmulti-diseases resistant rice varieties. The results of genotypic screening of 32 rice varieties (Including checks) for the presence or absence of five major rice blast resistance genes using SSR markers are presented in Table 3 and electrophoresis pattern of each SSR marker linked to blast resistant gene shown in Fig. 1

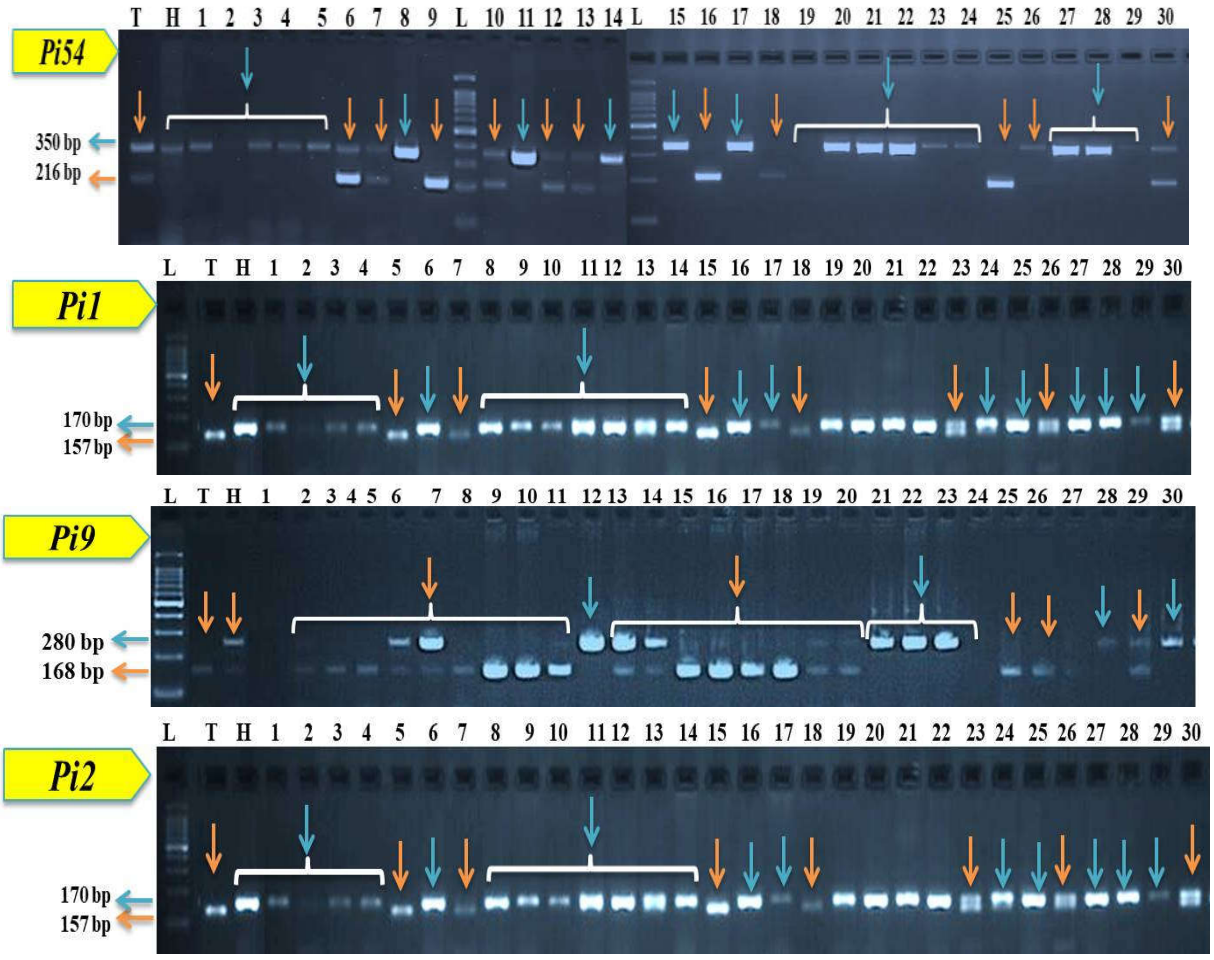

Fig. 1. Agarose gel electrophoretic pattern of 30 rice varieties along with Tetep and HR 12, generated by using SSR markers PIKHMAS (*Pi54/Pikh*) RM 224 (*Pi1*), AP56595 (*Pi2*) and NMSM Pi-9-1(*Pi9*), where L is 100 bp DNA size marker, T is Tetep, H is HR 12 and numbers 1-30 represent rice varieties as described in Table 1.

Estimation of PCR results for five blast resistance genes *viz.Pi54, Pi1*, *Pi2*,*Pi9* and *Pik*were determined by visualization of amplicons on near 216 bp, 157 bp, 288 bp, 168 bp and 300 bp of positive fragments, respectively. The genetic frequencies of the five major rice blast resistance genes were ranged from 25% to 90.6%. Similar results were obtained by Yan *et al*. (2017), screened 32 rice accessions and got genetic frequencies between of 9.4% to 100.0% using 11 SSR markers for blast resistance genes, Singh *et al.* (2015), published the genetic frequencies of *Piz-5*,*Pi-9*, *Pi-tp*(t), *Pi-1*, *Pi5*(t), *Pi-33*, *Pi-b*, *Pi-27*(t), *Pik-h* and *Pi-ta* in 192 rice accessions ranged from 19.79% to 54.69%, and only 17 accessions harbored 7–8 blast resistance genes, Kim *et al*. (2010), in 84 rice accessions possessed more than three positive bands of the eight rice blast resistance genes and Imam *et al*. (2014), reported the genetic frequency of *Pi-z*, *Piz-t*, *Pi-k*, *Pik-p*, *Pik-h*, *Pi-ta*/*Pi-ta2*, *pi-ta*, *Pi-9* and *Pi-b* ranged from 6% to 97% in the select set of rice germplasms. Interestingly, in this study, out of the 32 accessions, one TRV, Mugadsuganda positive for 5 (*Pi54/Pikh, Pi1, Pi2, Pi9 and Pik*) blast resistance genes, 9 (Raichursanna, Doddabatta, Doddabaikalu, Gulwadisannakki, Jenugudu, Kannur, NeergulaBatta, Manjupani and Kagi sale 2) were positive for 4, 10 were positive for 3 resistance genes and four TRVs like, Naweli (*Pi2*), ThornadaBatta (*Pi2*), Adribatta (*Pi2*) and Mullubatta (*Pi9*) had only one blast resistance gene. Relatively, more blast R genes were detected in the rice varieties.

The SSR marker AP 56595 is linked to *Pi2* on chromosome no. 6 shows positive allele near 288 in 29 varieties with highest genetic frequency of 90.6% i.e. *Pi2* gene distributed abundantly in the rice varieties used in this study followed by Marker NMSM Pi-9-1 linked to Pi9 on chromosome no.6 shows positive allele near 168 bp in 26 varieties with genetic frequency of 81.25%. Marker K-2167 linked to gene Pikh on chromosome no. 11, revealed presence of 300 bp fragment in 18 rice varieties with genetic frequency of 56.25%, marker PikhMAS is linked to blast resistance gene Pi54/Pikhon chromosome no. 11, revealed the presence of a 216 bp fragment specific for *Pi54/Pikh* in 13 rice varieties and shows genetic frequency of 40.65%. The blast resistance gene *Pi1* linked by marker RM 224 on chromosome no. 11was least distributed in the rice varieties used in this study with genetic frequency of 25%. Similar results were obtained by Singh et al. (2015), screened 119 rice accessions with 10 SSR markers.

This study illustrated the utility of SSR markers to identify TRVs 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

These findings imply that the main reason for high and broad spectrum blast resistance of these TRVs was the numerous blast resistance genes they harbored. As a result, these varieties can be used as sources of resistance genes in designing future breeding programs, and there is good possibility of obtaining enhanced resistance through gene pyramiding by marker-assisted selection.

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Table 1: List of varieties and checks used in this study

Sl. No.	Varieties Names	Sl. No.	Varieties Names
1	Naweli	17	DoddaAlur
2	Ambemohar	18	NeergulaBatta
3	Navara	19	Duddoge
4	BidagiKannapa	20	Kari doodi
5	RaichurSanna	21	Torandabatta
6	MugadSuganda	22	AdriBatta
7	DappaneyaBiliJaddu	23	Basumathi
8	DoddaBaikalu	24	MulluBatta
9	GulwadiSannakki	25	Intansel
10	DoddaBatta	26	ManjuPani
11	Balaji	27	BiliMunduga
12	Annaporna	28	Rat Bat
13	Jenugudu	29	Kagi Sale 1
14	MuttinaSanna	30	Kagi Sale 2
15	BiganMunji	31	Tetep
16	Kannur	32	HR 12

Table 2 List of SSR markers, primers and their sequences used in molecular validation of blast resistance genes

Sl. No.	Marker	Gene	Forward Primer	Reverse Primer	Chr. No.	Resistant allele size (bp)	Susceptible allele size (bp)
1	PIKH MAS	*Pi54*	CAATCTCCAAAGTTTTCAGG	GCTTCAATCACTGCTAGACC	11	216	350
2	RM 224	*Pi1*	ATCGATCGATCTTCACGAGG	TGCTATAAAAGGCATTCGGG	11	157	170
3	AP56595	*Pi2*	CTCCTTCAGCTGCTCCTC	TGATGACTTCCAAACGGTAG	6	288	310
4	NMSM Pi-9-1	*Pi9*	CGAGAAGGACATCTGGTACG	GAGATGCTTGGATTTAGAAGAC	6	168	280
5	K-2167	*Pik*	CGTGCTGTCGCCTGAATCTG	CACGAACAAGAGTGTGTCGG	11	300	619

Table 3: Scores of genotypic screening of 32 rice varieties with 5 SSR markers for blast resistance genes

Sl. No.	Varieties Names	Name of the marker and genes					No. of Genes present
		Pi54 (PIKHMAS)	***Pi1 (RM 224)***	***Pi2 (*AP56595*)***	***Pi9* (NMSM Pi-9-1)**	***Pik (*K-2167*)***	
1	Naweli	0	0	1	0	0	1
2	Ambemohar	0	0	1	1	0	2
3	Navara	0	0	1	1	0	2
4	BidagiKannapa	0	0	1	1	0	2
5	RaichurSanna	0	1	1	1	1	4
6	DoddaBatta	1	0	1	1	1	4
7	MugadSuganda	1	1	1	1	1	5
8	DappaneyaBiliJaddu	0	0	1	1	1	3
9	DoddaBaikalu	1	0	1	1	1	4
10	GulwadiSannakki	1	0	1	1	1	4
11	Balaji	0	0	1	1	1	3
12	Annaporna	1	0	1	0	1	3
13	Jenugudu	1	0	1	1	1	4
14	MuttinaSanna	1	0	1	1	0	3
15	BaiganMunji	0	1	1	1	0	3
16	Kannur	1	0	1	1	1	4

17	DoddaAlur	0	0	1	1	1	3
18	NeergulaBatta	1	1	1	1	0	4
19	Duddoge	0	0	1	1	0	2
20	Kari doodi	0	0	1	1	0	2
21	TorandaBatta	0	0	1	0	0	1
22	AdriBatta	0	0	1	0	1	1
23	Basumathi	0	1	1	0	0	2
24	MulluBatta	0	0	0	1	0	1
25	Intansel	1	0	1	1	1	3
26	ManjuPani	1	1	0	1	1	4
27	BiliMunduga	0	0	1	1	1	3
28	Rat Bat	0	0	1	0	1	2
29	Kagi Sale 1	0	0	1	1	1	3
30	Kagi Sale 2	1	1	1	0	1	4
31	Tetep	1	1	0	1	0	3
32	HR 12	0	0	1	1	0	2
	Genetic Frequency (%)	**40.65**	**25**	**90.6**	**81.25**	**56.25**	

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

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