Mysore J. Agric. Sci., 52 (2) : 323-328, 2018

Survey of SSR Marker Based Polymorphism among Donor (for Blast Resistance) and Two Recipient (Fertility Restorers MSN-36 and KMR-3) Parents and Foreground Selection for Identification of Blast Resistant Genotypes in Rice (*Oryza sativa* L.)

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Abstract

Marker-assisted backcross (MABC) breeding was used to develop blast resistant fertility restorers of rice using SSR markers. MSN-36 and KMR-3 were used as recurrent parents and Tetep as a donor parent with major resistance gene *Pi54* for blast resistance. Twenty-one and twenty-two plants were found to be true F_1's in MSN36×Tetep and KMR3×Tetep, respectively. Out of 60 markers screened, nine markers exhibited polymorphism between donor parent, Tetep and recurrent parents, MSN 36 and KMR3. Four markers were detected as polymorphic between Tetep and KMR3 and three markers were observed as polymorphic between Tetep and MSN36. Out of 40 plants, 16 plants in cross (MSN-36×Tetep)×MSN-36 and 21 out of 45 plants in cross (KMR-3×Tetep)×KMR-3 were found to be gene positive for *Pi54* gene and selected. The selected plants were subjected to further recombinant and background selections at BC_1F_1 generation.

Keywords: Marker-assisted backcross breeding, parental polymorphism, foreground selection, SSR marker

Rice is the staple food for billions of people around the world. The fast growth of the world population demands an increase of 26 per cent in rice production to fulfill the requirement (Khush, 2013). Production and productivity of rice can be significantly increased by hybrid technology. Through good management, a yield advantage of 1.0–1.5 tha^{-1} can be obtained by cultivation of hybrids as compared to the high yielding varieties (Hariprasad *et al.,* 2014). Many of the popular hybrids and their parents are susceptible to various pests and diseases. It is necessary that they should possess resistance or tolerance to major biotic stresses like blast, bacterial blight (BB), stem borer and brown plant hopper etc. for stable performance. Among the biotic stresses, blast caused by *Magnaporthe grisea* (Hebert) Barr. is one of the major diseases, limiting rice production significantly in India (Anon., 2008). Host-plant resistance (HPR) is considered as the most effective strategy for management of blast disease. So far, at least 100 rice blast disease resistance genes (R-genes) have been identified (Sharma *et al.,* 2012; Liu *et al.,* 2013). The identified blast *R* genes were found on all 12 rice chromosomes except 3 and most of them were in a cluster on chromosomes 6, 11 & 12 (Yang *et al.,* 2013).

Among the public bred rice hybrids, KRH-2 and KRH-4 developed by breeders of UAS, Bangalore are becoming increasingly popular across the country. However, susceptibility of these hybrids to blast disease not only resulted in non-realization of their genetic yield potential but also limited their horizontal spread to other farmers' fields. Susceptibility of these hybrids is attributed to the susceptibility of their parents. It will be desirable to introgress at least one resistance conferring dominant gene to their restorer parents (*i.e.,* KMR-3 and MSN-36 of KRH-2 and KRH-4, respectively). The use of resistant version of these restorers helps to develop resistant versions of KRH-2 and KRH-4. Currently, DNA marker technology has significantly contributed to genetic improvement through the selection of desirable traits, such as disease resistance. In marker-assisted backcross (MABC) breeding, molecular markers help monitor the disease resistance genes and to recover maximum of recurrent parent genome. Many rice cultivars resistant to biotic stresses have been developed using MABC and released and are widely adopted by farmers (Xu and Crouch, 2008). Among the blast resistance genes widely deployed in breeding, *Pi54* has been identified as one of the most effective,

broad-spectrum resistance genes in India, which was originally derived from the Vietnamese cultivar, Tetep. It has been reported to be highly effective under Indian conditions as well (Sharma *et al.*, 2010). The dominant gene *Pi54* has been mapped to the long arm of rice chromosome 11 (Sharma *et al.*, 2005). Highly robust, gene-specific markers are available for marker-assisted selection of *Pi54* (Ramkumar *et al.*, 2011). Under these premises, *Pi54* was selected as target blast resistance gene for introgression into MSN-36 and KMR-3, the restorer parents of KRH-4 and KRH-2, respectively in the present study.

Material and Methods

Location and time: For the development of blast resistant fertility restorers with marker assisted backcrossing (MABC) technique, the experiment was initiated in 2017 at Zonal Agricultural Research Station (ZARS), V. C. Farm, Mandya.

Material: MSN-36 and KMR-3, the stable restorer lines of the popular rice hybrids KRH-4 and KRH-2, respectively were used as recurrent parents and Tetep as a donor parent with major resistance gene *Pi54* for blast resistance.

Production of F_1s: Hybridization was carried out between MSN-36 and Tetep and between KMR-3 and Tetep in separate crossing programmes. MSN-36 and KMR-3 were used as female parents while Tetep as male parent in the hybridization programme.

Production of backcross seeds: For producing BC_1F_1 seeds, true F_1s of crosses, MSN36×Tetep and KMR3×Tetep were crossed back to recurrent parents, MSN36 and KMR3, respectively. All BC_1F_1 seeds were used to raise BC_1F_1 plants.

Raising of backcross plants: The mature back crossed seeds were seeded to raise the backcross plants.

Leaf sample collection for DNA extraction: Fresh leaf samples were collected from 25-day old seedlings to extract genomic DNA. The collected leaf samples were then kept in polythene bags and placed in an ice box to carry. Finally, the samples were stored in -20°C freezer.

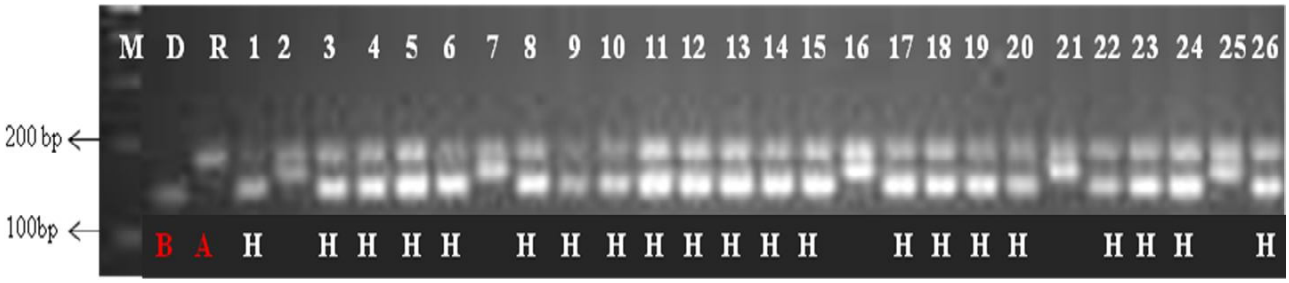
Genomic DNA extraction: Total genomic DNA was extracted from the leaves of each plant using the Cetyl Trimethyl Ammonium Bromide (CTAB) method (Doyle and Doyle, 1987) at Marker Assisted Selection (MAS) Laboratory, Department of Genetics and Plant Breeding, College of Agriculture, GKVK, Bangalore.

PCR analysis for SSR Markers: For PCR amplification, the total volume of the PCR reaction was 10 µl, including 1 µl template DNA, 0.5 µl of forward primer, 0.5 µl of reverse primer, 4 µl master mix (pre-mix containing Taq DNA polymerase, dNTPs, and $MgCl_2$) and 4 µl nuclease free water. This reaction mixture was placed in the PCR plate and run in the DNA thermal cycler. PCR amplification was performed using the touchdown PCR program using the following protocol: Initial denaturation at 94 °C for four minutes, then final denaturation at 94 °C for one minute and annealing at 55 °C for 30s. Polymerization was carried out at 72 °C for two minutes to complete a cycle and the cycle was repeated for 35 times. The final extension was at 72 °C for seven minutes. After PCR, polymorphism in the PCR products were detected by ethidium bromide staining after electrophoresis on three per cent agarose gel using UV transilluminator.

Marker for foreground selection: The robust tightly linked marker RM206F 5'-cccatgcgtttaactattct-3', RM206R 5'-cgttccatcgatccgtatgg-3' linked to the *Pi54* gene at a mapping distance 0.6 cM on chromosome 11 (Sharma *et al.*, 2005) was used for selecting the target gene.

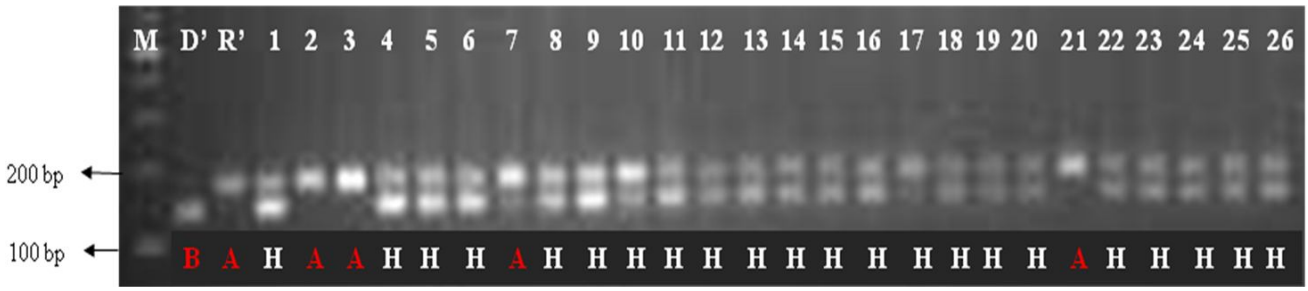
Parental polymorphism survey of SSR markers: In the present study, parental polymorphism was surveyed between Tetep and MSN-36 and also between Tetep and another recurrent parent, KMR-3. Sixty SSR markers were used for parental polymorphism survey which also includes blast resistant *Pi54* gene specific primer pair (RM206).

Confirmation of true hybridity of F_1 material: DNA samples were collected from 26 F_1 plants survived in each cross (*i. e.*, MSN 36×Tetep and KMR-3×Tetep) and PCR was carried out using blast resistant *Pi54* gene specific SSR marker RM 206. PCR amplicons from the 52 F_1 plants were scored as "H" and "A". Score H represented heterozygous alleles



Lane M: 100bp molecular weight ladder; D-Donor parent (Tetep); R-Recurrent parent (MSN-36); Lane 4-29: F_1 progenies of MSN-36 and Tetep; B: homozygous allele of donor parent; A: homozygous allele of recurrent parent; H: heterozygous alleles for donor and recurrent parent.

Fig.1: F_1 confirmation in cross MSN-36×Tetep using SSR marker RM 206.



Lane M: 100bp molecular weight ladder; D'-Donor parent (Tetep); R'-Recurrent parent (KMR-3); Lane 4-29: F_1 progenies of KMR-3 and Tetep; B: homozygous allele of donor parent; A: homozygous allele of recurrent parent; H: heterozygous alleles for donor and recurrent parent.

Fig.2: F_1 confirmation in cross KMR-3×Tetep using SSR marker RM 206.

for donor and recipient parent and "A" represented alleles of recipient parent (Fig. 1 and 2).

Foreground selection at BC_1F_1 generation: In (MSN-36×Tetep)×MSN-36, total of 150 BC_1F_1 seeds were produced from 21 true F_1 plants. From this, 40 plants survived and transplanted to the main field. Foreground selection was performed on these 40 plants using a tightly linked blast resistant marker RM 206 and in (KMR-3×Tetep)×KMR-3 cross, from 22 true F_1 plants, 90 BC_1F_1 seeds were produced of which, forty five seeds germinated and were transplanted to main field. Marker RM 206 was used for foreground selection in these 45 plants. The amplicons similar to MSN-36 or KMR-3 were scored as 'A' which indicated the homozygous allele of the recipient parent for the particular SSR marker. Again, the amplicons similar to Tetep were scored as 'B' which indicated the homozygous allele of the donor parent for the particular SSR marker. However, heterozygous alleles were scored as 'H' having both the amplicons of two parents. Score 'N' indicated the absence of amplicon.

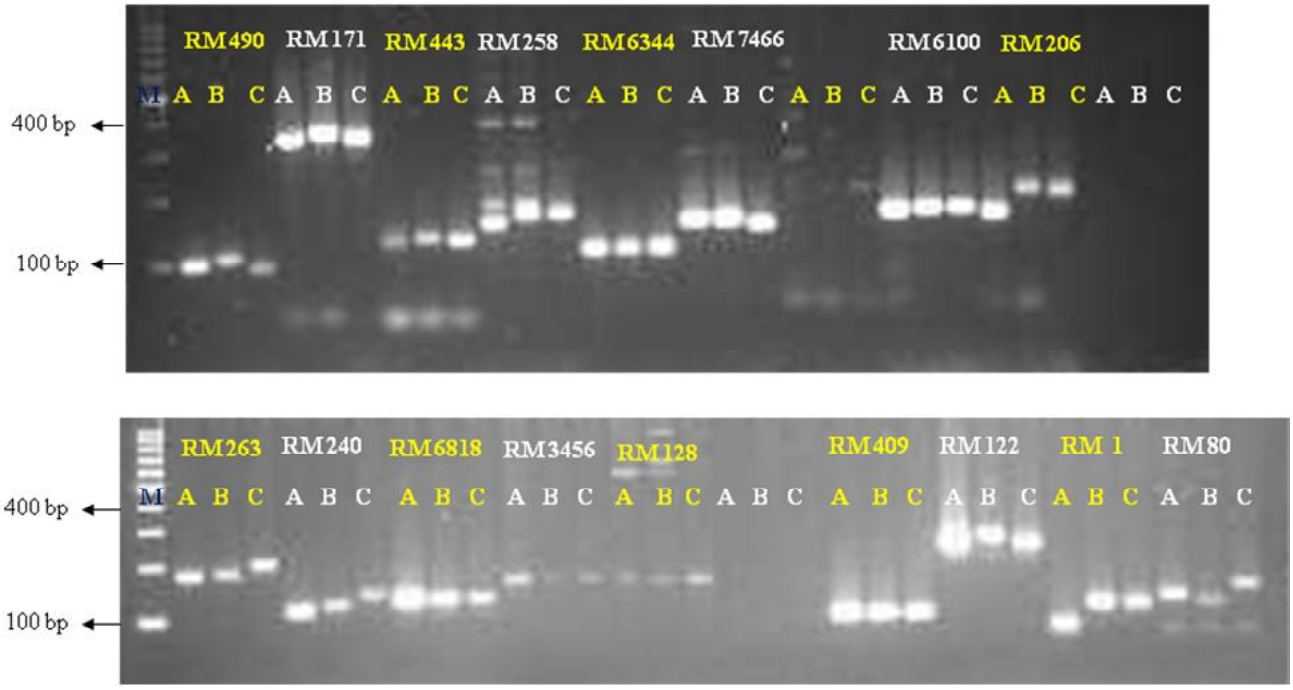
Results and Discussion

Parental polymorphism survey: Out of 60 SSR markers employed to assess parental polymorphism, only 16 markers including blast resistant *Pi54* gene specific primer RM 206 were found to be polymorphic between parents. Out of these 16 primers, nine primers, *viz.*, RM1, RM21, RM22, RM206, RM258, RM3412, RM5626, RM7075 and RM25721 were found to be polymorphic between MSN-36 and Tetep and also between KMR-3 and Tetep. Three primers, *viz.*, RM122, RM490 and RM572 were polymorphic only between MSN-36 and Tetep and four primers, *viz.*, RM80, RM240, RM263 and RM7466 were detected to be polymorphic only between KMR-3 and Tetep (Table I and Fig. 3). In these polymorphic markers, RM206 can be used for the foreground selection and other polymorphic markers can be used in background selection for identification of the plants with maximum percent of recurrent parent genome.

TABLE I

List of polymorphic primers obtained in parental polymorphism survey

Polymorphism	Marker name	Primer forward	Primer reverse	Repeat motif	Anneal temp. (°C)	Product size (bp)
Between	RM1	gcgaaaacacaatgcaaaaa	gcgttggttggacctgac	(GA)26	55	113
MSN-36 and	RM21	acagtattccgtaggcacgg	gctccatgagggtggtagag	(GA)18	55	157
Tetep and	RM22	ggtttgggagcccataatct	ctgggcttctttcactcgtc	(GA)22	55	194
Between	RM206	cccatgcgtttaactattct	cgttccatcgatccgtatgg	(CT)21	55	147
KMR-3 and	RM258	tgctgtatgtagctcgcacc	tggcctttaaagctgtcgc	(GA)21(GGA)3	55	148
Tetep	RM3412	aaagcaggttttcctcctcc	cccatgtgcaatgtgtcttc	(CT)17	55	211
	RM5626	gcagacgagatgagatcg	gtagaggatgggcagcag	(AAG)11	55	188
	RM7075	tatggactggagcaaacctc	ggcacagcaccaatgtctc	(ACAT)13	50	155
	RM25721	gctggaactggatctcgtactgc	gaggacgacgaggatgactacg	(CGT)7	55	90
MSN-36 and	RM122	gagtcgatgtaatgtcatcagtgc	gaaggaggtatcgctttgttggac	(GA)7A(GA)2A(GA)11	55	227
Tetep	RM490	atctgcacactgcaaacacc	agcaagcagtgctttcagag	(CT)13	55	101
	RM572	cggttaatgtcatctgattgg	ttcgagatccaagactgacc	(TC)14	55	159
KMR-3 and	RM80	ttgaaggcgctgaaggag	catcaacctcgtcttcaccg	(TCT)25	55	142
Tetep	RM240	ccttaatgggtagtgtgcac	tgtaaccattccttccatcc	(CT)21	55	132
	RM263	cccaggctagctcatgaacc	gctacgtttgagctaccacg	(CT)34	55	199
	RM7466	cggtctgcctagcttgtctc	accgaacacggaaaagcc	(TAGA)6	55	136



M: 100bp molecular weight ladder; A: Tetep; B: MSN-36; C: KMR-3

Fig.3: Parental polymorphism study for identification of polymorphic markers

Confirmation of true hybridity of F_1 plants: The primer RM206 amplified susceptible allele of 190bp in recipient parents (MSN-36 and KMR-3) and resistant allele of 140bp in donor parent (Tetep) similar to the findings of Sharma *et al.* (2005). Out of 26 plants evaluated, 21 plants amplified alleles specific to both the parents in cross MSN-36×Tetep (Fig.1) and 22 plants were found to be carrying both the parent specific amplicons in cross KMR-3×Tetep (Fig. 2). The plants that amplified alleles specific to both the parents were true F_1s and were selected for further crossing.

Foreground selection at BC_1F_1 generation: In (MSN-36×Tetep)×MSN-36 cross, out of 40 plants, 16 plants were found to be heterozygous for tightly linked marker (score H), 21 plants were found with the locus containing susceptible allele (score A) and three plants score 'N' indicating the absence of amplification. In (KMR-3×Tetep)×KMR-3, twenty one plants were found heterozygous for tightly linked marker RM 206, in 16 plants allele corresponding to susceptible parent was observed and in six plants no band was amplified (Fig. 4). Detected heterozygous plants contain *Pi54* gene, therefore, they were selected and used for further generation advancement.

In a similar study Shankar *et al*. (2018) used Tetep as donor parent and RM206, the *Pi54* gene specific marker for introgression of blast resistance gene into elite rice cultivar Kalanamak and found results akin to above study. Singh *et al.* (2012) employed marker assisted backcross breeding (MABC) approach to incorporate blast resistance gene *Pi54*, from the donor line Tetep into the genetic background of PRR78 (restorer parent of Pusa RH10) to develop Pusa1603. In foreground selection, using gene linked molecular marker RM 206 they could select twelve out of fifty-eight BC_1F_1 plants heterozygous for *Pi54* gene.

In the present study, 16 BC_1F_1 plants from the cross (MSN-36×Tetep)×MSN-36 and 21 plants from (KMR-3×Tetep)×KMR-3 cross that were heterozygous were selected indicating the usefulness of Pi54 specific gene marker RM 206 in efficient foreground selection for breeding blast resistant restore through MABC approach. The selected plants can further be subjected to recombinant and background selections in BC_1F_2.

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(*Received :* May, 2018 *Accepted :* June, 2018**)**