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Identification of maintainer lines and validation of SSR markers for development of new rice hybrids for aerobic situation

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ABSTRACT

In order to expand the potential of three line hybrid rice breeding technology for aerobic condition, a study was carried out to identify potential restorer and maintainers suitable for aerobic condition. 72 hybrids were developed using nine newly developed quasi-CMS lines and eight elite lines were evaluated for pollen and spikelet fertility. Results revealed that 62 were found to be completely male sterile and 10 were found to be partial sterile. Among the eight testers used, six acted as maintainers for all the CMS lines and remaining two differed in restorability with respect to CMS lines. Simultaneously validation of SSR markers-linked to Rf locus was carried out on eight testers by comparing with standard maintainer and restorer type marker alleles. In the present study six new maintainer genotypes viz., P23-5-6, P23-5-28, P23-5-236, Rasi, HPR565 and HPR2278 were identified which can be further developed into new CMS lines. Among the seven markers used for validation of Rf locus RM 6100, RM171, RM 1008 and RM6344 were found to be perfectly co-segregating with phenotype. Hence, these are potential markers for selection of maintainer and restorer genotypes for newly developed CMS lines.

Key words: *aerobic rice, maintainers, Rf locus,SSR markers, validation*

Rice is a pioneer food crop in Asia that has high plasticity as it can be cultivated in various ecosystems (Bouman *et al*., 2002). The green revolution enabled the rice production to meet the demands of the increasing population and helped many countries to escape starvation. Currently it is a major staple food providing more than 65% of caloric intake in many developing countries (Sharma *et al*., 2013).The population of the rice consuming countries is increasing at a faster rate, by the year 2025, about 785 million tonnes of rice which is 70 per cent more than the current production will be needed to meet the growing demand (Manomani and Khan, 2003). Along with this, water scarcity is the major problem in the coming days. Hence the second green revolution is possible only if we breed rice for stress conditions like aerobic rice. The exploration of hybrid varieties has potential to overcome the current yield plateaus in rice (Siddiq, 1997). The availability of suitable male sterile lines would render hybridization process easier (Shailaja 1980). In this view the dire need of increasing rice productivity and production encouraged rice scientists to develop and disseminate hybrid rice technology in the aerobic conditions. The use of male sterility system in developing hybrids in crops is possible only when effective maintainers and restorers are identified (Sharma *et al.*, 2012). Therefore, it is necessary to identify maintainers and restorers from the germplasm for development of superior rice hybrids. The establishment of test crosses nursery to identify restorers and maintainers is the first step in the three line heterosis breeding (Akhter *et al.*, 2008). Pollen or spikelet fertility or both have been used as an index to fix the restoration ability of the lines (Sutaryo, 1989; Naresh Babu et al., 2010). Ali and Khan (1996) observed that frequency of the maintainers (63%) was much higher that of restorers among 76 hybrids tested. Similarly Akhter *et al*. (2007); Hussain and Sanghera (2012) and Sabar *et al.*, (2007) found that local germplasm have more frequency of maintainers than

the restorers, whereas Mcwilliam *et al* (1995) found high frequency of restorers (21%) than was the maintainers (11%) from the evaluation of 6000 test crosses in India. However, identification of maintainers and restorers by crossing and evaluation is costly, time consuming, and laborious step. Therefore there is a need to validate molecular markers that are tightly linked to *Rf* genes so that marker-aided selection (MAS) can be used as a tool to identify restorers and maintainers more quickly and more efficiently. Hence the present investigation was undertaken to identify new maintainers/restorers and validation of SSR markers linked to *Rf* locus for identification of restorers and maintainers.

MATERIALS AND METHODS

Experimental materials for the identification of sterility maintainers and fertility restorers comprised of 72 test cross progenies derived from crossing of nine newly developed quasi CMS lines and eight elite lines during kharif 2014. Among nine CMS lines, five (1-5) from IR70369A × MAS 99 cross and other four (6-9) were from KCMS 31A ×0MAS 99 cross (Table 1). Whereas among the testers used four of them were pyramided lines for bacterial blight, water use efficiency and rice tungro tolerance/ resistance viz., P23-5-6, P23-5-28, P23-5-236, P23-5-287 and two were high protein lines viz., HPR565, HPR2278 developed form MAS lab, GKVK, UAS, Bangalore. Remaining two were released varieties *viz.*, Rasi and Paustic-9. The seventy-two test crosses were evaluated for pollen and spikelet fertility during summer, 2015 for identification of fertility restorers and sterility maintainers. Each test cross was raised in single row with two replications following the standard spacing between plant to plant (15 cm) and row to row (20cm) under aerobic condition. Pollen fertility test of test cross F_1 was carried out for their fertility or sterility responses. The spikelets (5 to10) from the just emerged panicle of 3 randomly selected plants were collected in vial containing 70 percent ethanol. With the help of forceps, the anthers from the spikelets were placed on a glass slide containing 2% Acetocarmine strain. Then the anthers were gently crushed by using needle to release the Pollen grains. After removing the debris, a cover slip was put on the slide and observed under microscope. Average of three microscopic fields for each sample was taken to calculate Pollen fertility percent using formula: Pollen fertility (%) = No. of fertile pollen grains /Total no. of pollen grains × 100.

Spikelet fertility test was done on three panicles per plant from five randomly selected plants for each test cross hybrid at maturity. Spikelet fertility of hybrids was assessed by taking the count of well filled and chaffy spikelets in each panicle. Percent spikelet fertility was calculated by using formula:

Spikelet fertility (%) = No. of filled spikelets per panicle / Total no. of spikelet per panicle × 100.

The pollen parents were classified into four categories - Maintainers (M), Partial Maintainers (PM), Partial Restorer (PR) and Restorer (R) according to Virmani *et al.,* 1997 (Table 2).

Seven reported SSR markers *viz.,* RM6100, RM1108, RM6344, RM171, RM490, RM258 and RM1 linked to fertility locus (*Rf*) were used to validate on eight male parents with IR 58025B and KMR-3 as standard for maintainer and restorer allele, respectively. The genomic DNA was isolated using leaf sample of thirty days old seedlings. DNA was prepared as per

Table 1. Nomenclature of CMS lines used in the study

CMS line in BC_3F_1 generation	Symbol used
IR70369A × MAS99	
BC_3F_1-2-1	CMS99-1t
BC_3F_1-5-4	CMS99-2t
BC_3F_1-7-8	CMS99-3t
BC_3F_1-8-5	CMS99-4t
BC_3F_1-10-6	CMS99-5t
KCMS31A × MAS 99	
BC_3F_1-11-6	CMS99-6t
BC_3F_1-12-6	CMS99-7t
BC_3F_1-14-3	CMS99-8t
BC_3F_1-15-7	CMS99-9t

t: tentative

Table 2. Classification of elite lines into maintainers and restorers (Virmani *et al*., 1997)

Pollen fertility range (%)	Category	Spikelet fertility range (%)
0.0-1.00	Maintainers (M)	00.0-5.00
1.1-50.00	Partial maintainer (PM)	05.1-20.00
50.1-80.00	Partial restorer (PR)	20.1-70.00
80.1-100.00	Restorer (R)	70.1-100.00

the modified Cetyl trimethyl ammonium bromide (CTAB) method (Cao and Oard, 1997). Seven reported SSR markers linked to *Rf* locus, with high LOD score markers were used for genotyping of male testers with checks (Table 3). PCR amplication was carried out following the standard procedures.

The bands generated by microsatellite primers were assigned as score M for maintainer type allele and score R for restorer type alleles. The allelic pattern of all seven polymorphic markers on each genotype was compared with pollen fertility and spikelet fertility to know the efficiency of SSR markers.

RESULTS AND DISCUSSION

The hybrids produced by crossing nine CMS lines with eight elite lines behaved differently with regard to pollen fertility (Table 4; Table 5). Based on pollen fertility, 32 hybrids were found to be completely sterile, 34 as partially sterile and 6 as partially fertile, none of them shown complete fertility. Whereas based on spikelet fertility, 62 hybrids were found completely sterile and 10 hybrids showed partial sterile behaviour (Table 6). Sixty two hybrids were found to be completely sterile, 10 hybrids were partial sterility and none of the hybrids were completely fertile based on both pollen fertility and spikelet fertility. Six testers found to be maintainers for all the CMS lines except for line CMS99-8t and P23-5-287 acted as partial maintainers for CMS99-3t, CMS99-7t, CMS99-8t lines and Paustic-9 also acted as partial maintainer for CMS99-2t, CMS99-3t, CMS99-7t, CMS99-8t and CMS99-9t lines. The variations in behaviour of fertility restoration indicate that either the fertility-restoring genes are different or that their penetrance and expressivity varied with the genotypes of the parents or the modifiers of female background. This kind of the differential reaction of the same genotype in restoring the fertility of different CMS lines of same cytoplasmic source was reported by Gannamani (2001), Hariprasanna *et al*. (2005) and Murugan and Ganesan, (2006). Effective maintainers can be used for development of new CMS lines through successive backcross breeding.

The eight male parents were scored using seven SSR markers based on allele pattern of standard checks i.e M (maintainer) for IR58025 type of allele and R (restorer) for KMR-3 type allele (Table 7). Among seven markers used, four markers *viz*., RM171, RM1108, RM6100 and RM6344 showed highly positive association with phenotypic classification of fertility restoration and maintainer type genotype based on pollen fertility and spikelet fertility (Fig.1). Among remaining three markers two were i.e. RM 258, RM 490 showed average association and RM 1 showed poor efficiency in selection of restorer and maintainers as per the phenotypic classifications. The markers RM 6100, RM 1108 and RM 6344 found to be linked to *Rf4* locus first two of which are located on chromosome 10

Table.3 List of SSR markers used for validation of fertility restoration (*Rf*) locus on rice testers

Marker Name	Allele	Chrom. . No	Genetic distance	Sequences (5' – 3')	Reference
RM490	*Rf3*	1	2.8cM	F: ATCTGCACACTGCAAACACCR: AGCAAGCAGTGCTTTCAGAG	Sheeba *et al.* (2009)
RM1	*Rf3*	1	5.6 cM	F: GCGAAAACACAATGCAAAAAR: GCGTTGGTTGGACCTGAC	Ahmadikhah *et al*. (2007)
RM6344	*Rf4*	7	13.3cM	F:ACACGCCATGGATGATGACR: TGGCATCATCACTTCCTCAC	Bazrkar *et al*. (2008)
RM6100	*Rf-4*	10	1.2cM	F: TCCTCTACCAGTACCGCACCR: GCTGGATCACAGATCATTGC	Sheeba *et al.* (2009),
RM258	*Rf6*	10	4.4cM	F: TGCTGTATGTAGCTCGCACCR: TGGCCTTTAAAGCTGTCGC	Majid Sattari. *et al.* (2008), Bazrkar *et al.* (2008)
RM171	*Rf1*	10	3.7cM	F: AACGCGAGGACACGTACTTACR: ACGAGATACGTACGCCTTTG	Sheeba *et al.* (2009), Majid Sattari. *et al.* (2008)
RM1108	*Rf4*	10	1.6cM	F: GCTCGCGAATCAATCCACR: CTGGATCCTGGACAGACGAG	Sheeba *et al.* (2009)

F: Forward primer and R: Reverse primer

Table 4. Classification of crosses using CMS lines (tentative) derived from IR70369A × MAS99 as maintainers and restorers based on pollen fertility and spikelet fertility.

Hybrids	Pollen fertility		Spikelet fertility		Based on Both
	Percent	Class	Percent	Class	
CMS99-1t × P23-5-6	0.00	M	0.00	M	M
CMS99-1t × P23-5-28	0.00	M	0.00	M	M
CMS99-1t × P23-5-236	0.00	M	0.00	M	M
CMS99-1t × P23-5-287	8.90	PM	0.00	M	M
CMS99-1t × RASI	0.00	M	0.00	M	M
CMS99-1t × PAUSTIC-9	9.40	PM	1.35	M	M
CMS99-1t × HPR565	9.30	PM	2.50	M	M
CMS99-1t × HPR2278	8.80	PM	4.12	M	M
CMS99-2t × P23-5-6	0.00	M	0.00	M	M
CMS99-2t × P23-5-28	0.00	M	0.00	M	M
CMS99-2t × P23-5-236	0.00	M	0.00	M	M
CMS99-2t × P23-5-287	21.80	PM	0.00	M	M
CMS99-2t × RASI	0.00	M	0.00	M	M
CMS99-2t × PAUSTIC-9	19.20	PM	7.62	PM	PM
CMS99-2t × HPR565	14.50	PM	0.00	M	M
CMS99-2t × HPR2278	17.80	PM	3.25	M	M
CMS99-3t × P23-5-6	0.00	M	0.00	M	M
CMS99-3t × P23-5-28	0.00	M	2.40	M	M
CMS99-3t × P23-5-236	0.00	M	0.00	M	M
CMS99-3t × P23-5-287	14.60	PM	8.62	PM	PM
CMS99-3t × RASI	0.00	M	2.45	M	M
CMS99-3t × PAUSTIC-9	10.80	PM	5.62	PM	PM
CMS99-3t × HPR565	9.90	PM	4.56	M	PM/M
CMS99-3t × HPR2278	7.50	PM	6.75	M	PM/M
CMS99-4t × P23-5-6	0.00	M	0.00	M	M
CMS99-4t × P23-5-28	0.00	M	0.00	M	M
CMS99-4t × P23-5-236	0.00	M	0.00	M	M
CMS99-4t × P23-5-287	8.30	PM	0.00	M	PM/M
CMS99-4t × RASI	0.00	M	1.80	M	M
CMS99-4t × PAUSTIC-9	8.90	PM	8.51	PM	PM
CMS99-4t × HPR565	6.80	PM	2.68	M	PM/M
CMS99-4t × HPR2278	6.80	PM	2.31	M	PM/M
CMS99-5t × P23-5-6	0.00	M	0.00	M	M
CMS99-5t × P23-5-28	0.00	M	0.00	M	M
CMS99-5t × P23-5-236	0.00	M	0.00	M	M
CMS99-5t × P23-5-287	11.30	PM	2.16	M	PM/M
CMS99-5t × RASI	0.00	M	0.00	M	M
CMS99-5t × PAUSTIC-9	6.90	PM	3.42	M	PM/M
CMS99-5t × HPR565	8.50	PM	2.73	M	PM/M
CMS99-5t × HPR2278	8.50	PM	3.62	M	PM/M

Table 5. Classification of crosses using CMS lines (tentative)derived from KCMS31A × MAS99 as maintainers and restorers based on pollen fertility and spikelet fertility

Hybrids	Pollen fertility		Spikelet fertility		Based on Both
	Percent	Class	Percent	Class	
CMS99-6t × P23-5-6	0.00	M	0.00	M	M
CMS99-6t × P23-5-28	8.00	PM	2.30	M	PM/M
CMS99-6t × P23-5-236	8.70	PM	3.52	M	PM/M
CMS99-6t × P23-5-287	8.70	PM	4.65	M	PM/M
CMS99-6t × RASI	0.00	M	2.13	M	M
CMS99-6t × PAUSTIC-9	9.80	PM	0.00	M	PM/M
CMS99-6t × HPR565	9.10	PM	2.62	M	PM/M
CMS99-6t × P23-5-2278	0.00	M	0.00	M	M
CMS99-7t × P23-5-6	0.00	M	0.00	M	M
CMS99-7t × P23-5-28	7.70	PM	2.42	M	PM/M
CMS99-7t × P23-5-236	7.90	PM	2.15	M	PM/M
CMS99-7t × P23-5-287	23.20	PR	5.56	PM	PR/PM
CMS99-7t × RASI	0.00	M	0.00	M	M
CMS99-7t × PAUSTIC-9	16.60	PM	3.42	M	PM/M
CMS99-7t × HPR565	7.00	PM	4.80	M	PM/M
CMS99-7t × P23-5-2278	0.00	M	0.00	M	M
CMS99-8t × P23-5-6	0.00	M	0.00	M	M
CMS99-8t × P23-5-28	46.50	PR	6.41	PM	PR/PM
CMS99-8t × P23-5-236	50.60	PR	15.3	PM	PR/PM
CMS99-8t × P23-5-287	51.90	PR	18.54	PM	PR/PM
CMS99-8t × RASI	0.00	M	3.20	M	M
CMS99-8t × PAUSTIC-9	65.70	PR	8.56	PM	PR/PM
CMS99-8t × HPR565	17.40	PM	2.24	M	PM/M
CMS99-8t × HPR2278	0.00	M	3.50	M	M
CMS99-9t × P23-5-6	0.00	M	2.45	M	M
CMS99-9t × P23-5-28	9.30	PM	4.56	M	PM/M
CMS99-9t × P23-5-236	9.20	PM	2.84	M	PM/M
CMS99-9t × P23-5-287	8.80	PM	3.45	M	PM/M
CMS99-9t × RASI	0.00	M	3.60	M	M
CMS99-9t × PAUSTIC-9	6.30	PM	7.25	PM	PM/PM
CMS99-9t × HPR565	49.20	PR	2.43	M	PR/M
CMS99-9t × HPR2278	0.00	M	3.58	M	M

as reported by Sheeba *et al*., (2009) and RM 6344 on chromosome7 as reported by Bazrkar *et al*. (2008) respectively. Whereas RM 171 was found to be linked to *Rf1* locus which is located on chromosome 10 (Sheeba *et al.,* 2009; Majid Sattari *et al.,* 2008). These results indicated that *Rf4 and Rf1* locus is the predominant restoration alleles for CMS lines studied. However among these four markers RM1108, RM171, RM6100 showed 100 per cent accuracy for selecting genotypes for fertility restoration. Whereas some of the perfect maintainer found in the study showed banding pattern similar to restorer genotype. This could be attributed to involvement of a different restorer and maintainer gene and effect of modifying genes in the expression of restoration/maintainer phenotype. Therefore, genotypes identified based on markers

Table 6. Classification of rice genotypes into Restorer (R), Maintainers (M), Partial restorers (PR), Partial maintainers (PM) based on pollen and spikelet fertility across nine CMS lines

Testers CMS lines	P23-5-6	P23-5-28	P23-5-236	P23-5-287	RASI	PAUSTIC-9	HPR565	HPR2278
CMS-1t	M	M	M	PM/M	M	PM/M	PM/M	PM/M
CMS-2t	M	M	M	PM/M	M	PM	PM/M	PMM
CMS-3t	M	M	M	PM	M	PM	PM/M	PM/M
CMS-4t	M	M	M	PM/M	M	PM/M	PM/M	PM/M
CMS-5t	M	M	M	PM/M	M	PM/M	PM/M	PM/M
CMS-6t	M	PM/M	PM/M	PM/M	M	PM/M	PM/M	M
CMS-7t	M	PM/M	PM/M	PR/PM	M	PM	PM/M	M
CMS-8t	M	PR/PM	PR/PM	PR/PM	M	PR/PM	PM/M	M
CMS-9t	M	PM/M	PM/M	PM/M	M	PM	PR/M	M

Table 7. Scoring data of male parents (testers) using seven SSR markers associated with Fertility restorer (Rf) locus for maintainer and restorer type allele

Markers Genotypes	RM258	RM1108	RM 1	RM6344	RM171	RM490	RM6100
P-25-5-6	M	M	R	M	M	M	M
P-25-5-28	M	M	R	M	M	R	M
P-25-5-236	R	M	R	R	M	M	M
P-25-5-287	M	M	R	M	M	R	M
Rasi	R	M	R	M	M	M	M
Paustic-9	R	M	M	R	M	R	M
HPR565	M	M	R	M	M	R	M
HPR2278	M	M	M	M	M	M	M

R: Restorer M: Maintainer

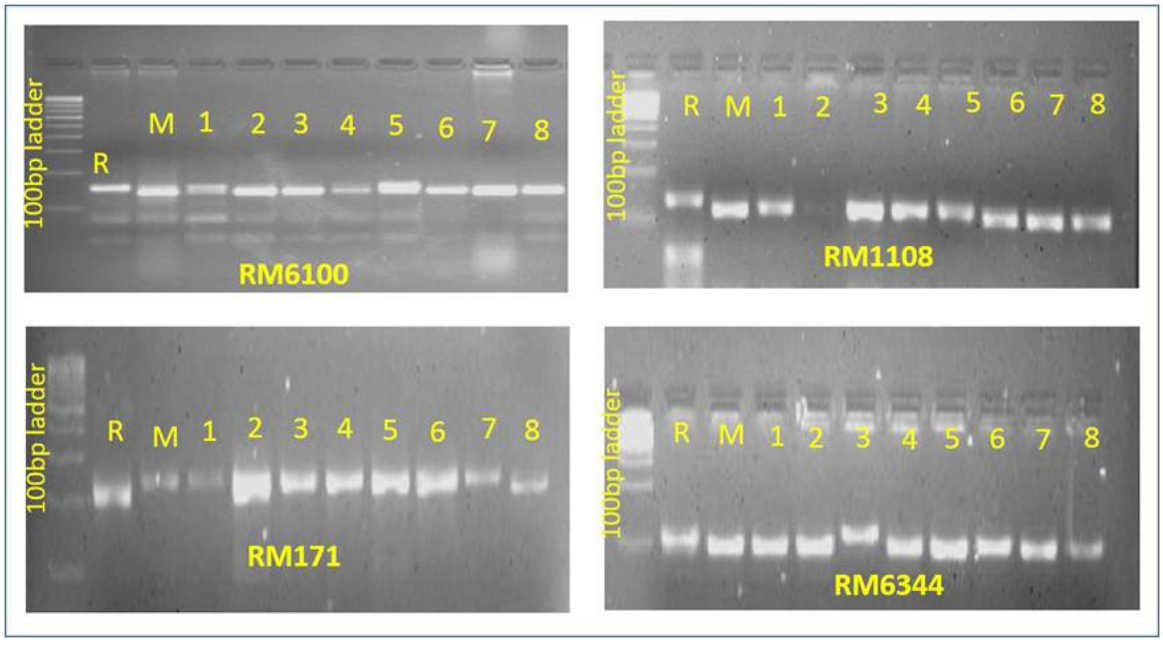

Fig. 1. Screening of polymorphic *Rf* locus specific SSR markers RM6100 (A), RM1108 (B), RM171(C) and RM 6344(D) across the 8 male parents. R: KMR-3, M: IR58025B.1: P-23-5-6, 2:P23-5-28, 3: P23-5-236, 4:P23-5-287, 5: Rasi, 6: Paustic-9, 7: HPR-565, 8: HPR-2278.

should be verified phenotypically through test cross evaluation for the confirmation of restoration/maintainer reaction (Mallikarjun, 2011).The results proved the effectiveness of marker aided selection of restorers and maintainers as phenotypic results confirmed the results of MAS. The results are in line with earlier findings which also reported identification of restorers and maintainers (Veeresha *et al.,* 2013; Naresh babu, 2010 and Naik, 2015).

Among eight testers used six perfect maintainers identified as *viz.*, P23-5-6, P23-5-28, P23-5-236, Rasi, HPR565and HPR 2278.These lines can be developed in to new CMS lines by consecutive backcrosses. Among the *Rf* markers used RM 6100, RM 1108, RM 6344 and RM 171 showed highest accuracy for selecting maintainer and restorer genotypes for these newly developed CMS lines.

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