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Short Communication

Screening and validation of fertility restoration genes (*Rf*) in wild abortive CMS system of rice (*Oryza sativa* L.) using microsatellite markers

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Abstract

The present study was carried out with the objective of validating linked SSRs markers to *Rf* genes in diverse rice breeding lines. SSR markers enabled to determine the chromosomal locations of the two *Rf* genes (*Rf3* and *Rf4*) in the wild-abortive cytoplasmic male sterility (WA-CMS) system. Forty breeding lines with no prior information on fertility restoration were screened with the help of molecular markers linked to major fertility genes *Rf3* and *Rf4.* The breeding lines were crossed with two WA-CMS lines (IR79156A and Pusa 6A) and the 80 F_1s were evaluated for pollen and spikelet fertility to identify restorers and maintainers. Four SSRs reported to be linked to *Rf* genes (*Rf3* and *Rf4*) were used to detect the allelic status in 40 breeding lines. The results revealed that out of 40, eight lines found to be common effective restorers for both the CMS lines (IR 79156A and Pusa6A). Two markers (RM171 and RM6100) proved to be associated with *Rf4* genes. Results demonstrated that these markers could be used to screen large number of restorers and non-restorer lines in hybrid rice breeding programs in short time.

Key words: WA-CMS, fertility restoration genes, rice, simple sequence repeat

Hybrid rice technology is one of the most important and practically feasible technologies to enhance the rice productivity. Cytoplasmic-genetic Male Sterility (CMS) combined with a fertility restoration system has been found to be the most efficient genetic tool to exploit hybrid vigor on a commercial scale in rice (Lin and Yuan 1980; Virmani and Wan 1988). Wild abortive (WA) type of CMS being used in nearly 90% of the rice hybrids in China and 100% of the hybrids developed outside China (Sattari et al. 2008). Recently, microsatellite or simple sequence repeat (SSR) markers have been used in hybrid rice program for tagging of fertility restorer genes and classification of male sterile and male fertile lines. Two major fertility restoration genes, *Rf3* and *Rf4*, are required for the production of viable pollen in WA-CMS type of CMS. These genes have been mapped on chromosomes 1 and 10, respectively (Yao et al. 1997; Zhang et al. 1997; Zhang et al. 2002). Bazarkar et al. (2008) found that SSRs markers, RM443 and RM315 are flanking the *Rf3* gene at a distance of 4.4cM and 20.7cM on chromosome 1, respectively. The marker RM6100 located on chromosome 10 mapped 7cM away from *Rf4*gene (Yao et al. 1997; Zhang et al. 1997; Zhang et al. 2002; Revathi et al. 2013; Raafat El-Namaky et al. 2016). The markers RM171 and RM6100 may facilitate MAS selection of WA-CMS based restorer lines in large breeding sets, thus avoiding routine testcrossing in a hybrid rice breeding program (Sheeba et al. 2009; Nematzadeh and Kiani 2010; Kiani 2015). The objective of this research was therefore to screen for fertility restoration genes by using SSR markers in diverse breeding lines.

A total of 40 breeding lines and cultivars without prior information about fertility restoration status were collected from different universities (Table 1). TheWA-CMS lines, IR79156A and Pusa 6A were obtained from

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Barwale Foundation, Hyderabad and IARI, New Delhi, respectively. Both the WA-CMS lines crossed to all 40 breeding lines to produce 80 F_1 hybrids during 2012 *kharif* season at the Agricultural Research Farm, Banaras Hindu University, Varanasi, Uttar Pradesh. The F_1 progenies were raised during *rabi* (off-season) 2012-13 at National Rice Research Institute (NRRI) Cuttack, Odisha to study their pollen and spikelet fertility status. Both, pollen and spikelet fertility were used as the main criteria for the evaluation of fertile and sterile plants. The fertility and sterility of pollen and spikelet was recorded according to Virmani et al. (1997). To determine pollen fertility, the mature anthers were harvested and the pollen grains were stained with 1% iodine potassium iodide (I_2-KI) solution. The numbers of dark blue (with stain) and clear pollen grains (without stain) in each sample were counted using an optical microscope. The spikelet fertility was measured using the method described by (Virmani et al. 1997; Li et al. 2005).

Screening of restoration ability

A total of four SSRs namely, RM315, RM443, RM171, and RM6100 were used to detect fertility restoration genes (*Rf3* and *Rf4*) in two WA-CMS lines (IR 79156A and Pusa6A) and 40 lines or cultivars. To extract DNA from each lines, leaves were harvested from three week old plants. The DNA extraction and purification was done using CTAB protocol of Murray and Thompson (1980). The PCR was performed following standard procedures. The gels were stained with 0.05% ethidium bromide solution and photographed with Alpha Imager, USA gel documentation system. The markers size of amplified product RM315 (175bp non-restorer), RM443 (160bp non-restorer), RM171 (320bp restorer, 330bp non-restorer) and RM6100 (185bp restorer, 175bp non-restorer).

The 80 F_1s obtained from 42 parental lines (40 normal and 2 CMS) ranged from completely fertile (22) to completely sterile (12) (Table 1). The remaining 46 hybrids expressed varying degrees of fertility, 21 being partial restorer and 25 as partial maintainers. Eight parental lines *viz*., IET21519, IET22218, IET22228, IET22202, IET21542, Sarju-52, BPT5204 and MTU7029 produced fertile F_1s with either of the WA-CMS lines. On the other hand four genotypes (IET22237, NDR-97, Nagina-22 and Karhani) produced completely sterile hybrids when crossed with anyWA-CMS lines. The 8 restorer lines common for both the CMS lines can be effectively utilized in hybrid rice program.

Screening of all 40 breeding lines with four SSR primers revealed that markers RM171 and RM6100 were associated with fertility restorer genes (*Rf4*) located on chromosome 10 in our breeding lines. These two markers were able to identify above-mentioned common fertility restorer lines (IET21519, IET22218, IET22228, IET22202, IET21542, Sarju-52, BPT5204 and MTU7029) based on *Rf4* allele. Both the CMS lines along with 8 common restorers and their combinations (16 F_1s) were tested with SSRs for fertility restoration (Figs. 1 and 2). We found that all

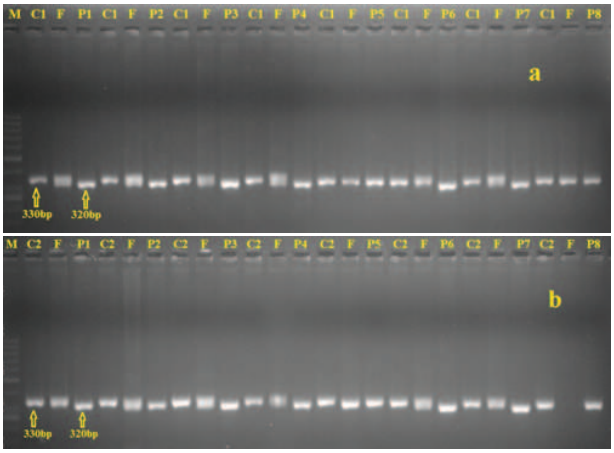

Fig. 1. (a & b). Amplification with RM171 marker linked to *Rf4* gene in heterotic combinations and breeding linesM-50bp Ladder, C1-IR 79156A and C2-Pusa 6A CMS Lines, F-Hybrid Progeny, P1-IET 21519, P2-IET-22218, P3-IET-22228, P4-IET-22202, P5-IET-21542, P6-Sarju-52, P7-BPT-5204, P8-MTU 7029-Breeding Lines

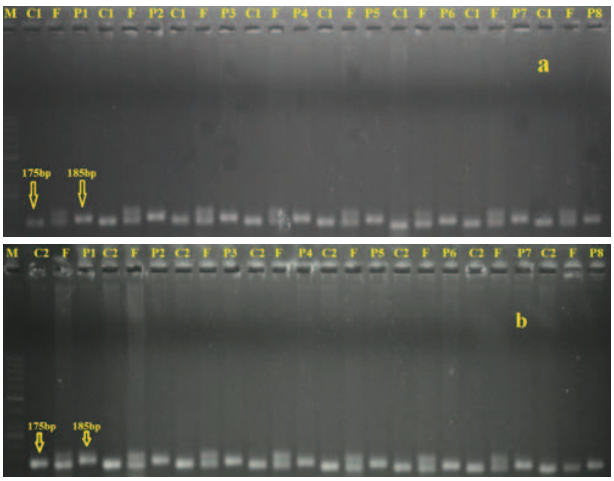

Fig. 2. (a & b). Amplification pattern of SSR marker RM6100 linked with *Rf4* in heterotic combination and breeding lines. M-50bp Ladder, C1-IR 79156A and C2-Pusa 6A CMS Lines, F-Hybrid Progeny, P1-IET 21519, P2-IET-22218, P3-IET-22228, P4-IET-22202, P5-IET-21542, P6-Sarju-52, P7-BPT-5204, P8-MTU 7029-Breeding Lines

Table 1. Per cent pollen and spikelet fertility of 40 genotypes involving two cytoplasmic male sterile lines and fertility classification at NRRI, Cuttack

S.N.	Genotypes	Source of seeds	NRRI Cuttack 2012- 2013					
			IR 79156A		Pusa 6A		IR 79156A	Pusa 6A
			PF	SF	PF	SF		
1.	IET-21519	BHU, Varanasi	85.71	84.41	84.73	77.57	R	R
2.	IET-22218	BHU, Varanasi	86.77	83.9	87.08	82.74	R	R
3.	IET-22228	BHU, Varanasi	84.60	76.41	83.78	76.70	R	R
4.	IET-22202	BHU, Varanasi	84.66	79.04	85.96	79.72	R	R
5.	IET-21542	BHU, Varanasi	87.07	82.12	91.38	83.70	R	R
6.	Sarjoo-52	BHU, Varanasi	87.48	79.21	91.11	84.06	R	R
7.	BPT-5204	BHU, Varanasi	91.44	82.90	82.35	78.48	R	R
8.	MTU-7029	BHU, Varanasi	85.64	78.59	86.34	85.60	R	R
9.	IET-20524	BHU, Varanasi	84.43	81.86	67.04	62.15	R	PR
10.	Krhani	BHU, Varanasi	83.28	79.55	68.20	58.47	R	PR
11.	HUR-8-1	BHU, Varanasi	84.63	85.86	21.12	16.17	R	PM
12.	IET-20924	BHU, Varanasi	68.81	65.88	32.18	25.87	PR	PM
13.	Danteswari	BHU, Varanasi	62.91	56.28	46.63	50.45	PR	PM
14.	IET-22225	BHU, Varanasi	78.10	76.88	64.17	50.71	PR	PR
15.	HUR-3022	BHU, Varanasi	68.01	65.33	64.15	49.57	PR	PR
16.	Adam Chini	BHU, Varanasi	73.46	66.90	70.66	59.29	PR	PR
17.	RPBIO-226	BHU, Varanasi	66.31	65.60	57.90	51.70	PR	PR
18.	Type-3	BHU, Varanasi	72.27	66.16	87.14	80.48	PR	R
19.	Pant Dhan-4	BHU, Varanasi	42.93	39.87	0.00	0.00	PM	M
20.	Vandana	NDUAT, Faizabad	43.22	32.22	0.00	0.00	PM	M
21.	IET-20935	NDUAT, Faizabad	42.88	33.23	16.17	7.54	PM	PM
22.	IET-20556	NDUAT, Faizabad	16.96	12.58	24.10	10.61	PM	PM
23.	Rajendra Kasturi	BHU, Varanasi	48.00	37.36	36.95	31.73	PM	PM
24.	Pant Sugandh Dhan-17	IARI, New Delhi	29.10	18.89	28.41	17.24	PM	PM
25.	HUR-5-2	GBPUAT, Pantnagar	33.13	26.40	49.83	34.87	PM	PM
26.	IET-22251	GBPUAT, Pantnagar	52.33	43.33	72.17	65.62	PM	PR
27.	IET-21528	GBPUAT, Pantnagar	46.56	43.90	63.98	53.87	PM	PR
28.	Vardhan	BHU, Varanasi	43.33	33.76	71.00	64.96	PM	PR
29.	Akshaya Dhan	BHU, Varanasi	33.80	25.06	52.71	51.97	PM	PR
30.	HUBR-2-1	BHU, Varanasi	44.46	37.30	66.46	54.56	PM	PR
31.	Pant Dhan-12	BHU, Varanasi	25.11	12.60	71.21	62.81	PM	PR
32.	Anjali	BHU, Varanasi	47.08	43.72	60.37	54.31	PM	PR
33.	NDR-359	BHU, Varanasi	34.86	27.10	92.34	84.93	PM	R
34.	IDR-763	BHU, Varanasi	47.41	35.56	85.50	78.73	PM	R
35.	IET-22237	BHU, Varanasi	0.00	0.00	0.00	0.00	M	M
36.	NDR-97	BHU, Varanasi	0.00	0.00	0.00	0.00	M	M
37.	Khutadhan	BHU, Varanasi	0.00	0.00	0.00	0.00	M	M
38.	Nagina-22	BHU, Varanasi	0.00	0.00	0.00	0.00	M	M
39.	HUR-105	BHU, Varanasi	2.60	0.79	26.20	10.71	M	PM
40.	Pusa Basmati-1460	BHU, Varanasi	0.00	0.00	63.99	58.58	M	PR

PF = Pollen fertility (%); SF = Spikelet fertility M = Maintainer; R = Restorer; PM = Partial Maintainer; PR = Partial Restorer

the fertility restorer for both the CMS carrying *Rf4* allele. The 16 F_1s exhibited more than 82% and 76% pollen fertility and spikelet fertility, respectively (Table 2) confirming the presence of *Rf4* allele.

There are numerous reports about the fertility restorer genes in the F_1s of CMS lines crossed with germplasm and breeding lines. In general, approximately 20% F_1 hybrids are fertile while 10% are completely sterile (Akhter et al. 2008). The present

Table 2. Means of spikelet and pollen fertility of F_1 hybrids (only full restorers) in rice

Cross combination (F_1)	Pollen fertility (%)	Spikelet fertility (%)
IR 79156A x IET 21519	85.7	84.4
IR 79156A x IET 22218	86.8	83.9
IR 79156A x IET 22228	84.6	76.4
IR 79156A x IET 22202	84.7	79
IR 79156A x IET 21542	87.1	82.1
IR 79156A x Sarjoo-52	87.5	79.2
IR 79156A x BPT 5204	91.4	82.9
IR 79156A x MTU-7029	85.6	78.6
Pusa 6A x IET 21519	84.7	77.6
Pusa 6A x IET 22218	87.1	82.7
Pusa 6A x IET 22228	83.8	76.7
Pusa 6A x IET 22202	86	79.7
Pusa 6A x IET 21542	91.4	83.7
Pusa 6A x Sarjoo-52	91.1	84.1
Pusa 6A x BPT 5204	82.4	78.5
Pusa 6A x MTU-7029	86.3	85.6
Grand Mean	86.6	80.9
SE (m)	0.8	1.0
CD (5%)	1.6	2.1
CD (1%)	2.3	3.0

results are also in the agreement of the previous studies. The selected eight lines used as male parents crossed with both WA-CMS lines (IR79156A and Pusa 6A), showed stability for fertility restoration. The other 32 lines produces F_1s with varying degree of fertility e.g., from completely fertile to completely sterile including partial sterile and partial fertile lines. The lack of complete fertility or sterility might be due to gene × gene (epistasis) or G×E interactions (Malarvizhi et al. 2003). The lines (IET22237, NDR-97, Nagina-22 and Karhani) showed complete sterility in cross with any of two WA-CMS lines. It indicates that these lines lacking for any fertility restoration gene which may be used as breeding lines to develop source of CMS in to different genetic backgrounds.

Based on the fertility restoration ability of lines with both WA-CMS sources, the 8 parental lines and their 16 hybrids were screened with SSRs for *Rf* genes. Out of four SSRs reportedly linked to *Rf* gene (Sheeba et al. 2009; Bazrkar et al. 2008), only two markers, namely RM171 and RM6100 showed polymorphism between CMS and fertility restorer lines. Therefore, these markers can not only be used to identify the fertility restorer gene but also to test the purity of hybrids (Kiani 2015). Polymorphism for the markers RM315 and RM443 linked to *Rf3* gene was not observed in the studied material possibly due to the presence of a different set of fertility restorer genes. For example, Mishra et al. (2003) identified *Rf*-(u1), a new fertility restoration gene for WA-CMS system, which has been identified in Basmati type restorer line, PRR78R and mapped on chromosome 10. The other probable reason could be due to a recombination event occurred between the marker and the *Rf3* gene during the development as the markers RM315 and RM443 are not closely linked to *Rf3* being at a distance of 20.7cM and 4.4cM, respectively (Bazrkar et al. 2008). On the other hand, the markers RM171 and RM6100 mapped at 3.2cM and 1.2cM, respectively from *Rf4* gene (Ahmadikhah and Karlov 2006; Sheeba et al. 2009). The lack of *Rf3* gene and presence of *Rf4* gene in our material indicates that *Rf4* is a major fertility restoration gene. This is in accordance with the studies of Suresh et al. (2012) who reported that *Rf4* is a major locus for fertility restoration for WA-CMS in most of the cases and *Rf3* in some cases. Some studies have reported that the two fertility restorer genes are additive in their inheritance and the effect of *Rf4* appeared to be larger than that of *Rf3* (Yao et al. 1997; Sattari et al. 2008; Cai et al. 2013). Similar findings in respect of a major locus *Rf4* for fertility restoration of the WA-CMS on the long arm of rice chromosome 10 have been reported earlier by several researchers (Zhang et al. 2002; Ahmadikhah and Karlov 2006; Sattari et al. 2007; Sheeba et al. 2009; Revathi et al. 2013; Cai and Zhang 2013; Kiani 2015; El-Namaky et al. 2016). Interestingly, all fully fertile F_1s showed presence of *Rf4* gene specific amplification, which indicated that the reported markers are closely linked to the fertility restoration gene. There are some combinations where a line restored fertility with WA-CMS line (IR79156A) while some remained partial restorer or partial maintainer. This could be explained due to the presence of epistatic interactions (Govindaraj and Virmani 1988).

Authors' contribution

Conceptualization of research (PKB, SKS); Designing of the experiments (PKB, SKS); Contribution of experimental materials (PKB, SKS); Execution of field/ lab experiments and data collection (PKB, SKS); Analysis of data and interpretation (UK, PKB, SKS); Preparation of manuscript (UK, PKB, SKS).

Declaration

The authors declare no conflict of interest.

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