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Original Research Article

Genetics of Fertility Restoration and Validation of SSR Marker Associated with it in Rice (*Oryza sativa* L.) Cultivar IR72

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A B S T R A C T

The present study was carried out with the objective to study the inheritance of fertility restoration and to validate the molecular markers, which have been previously reported to be linked to fertility restorer (*Rf*) gene(s) for WA-CMS lines of rice. An F_2 population was developed from a cross between rice (*Oryza sativa* L.) genotypes, IR58025A (sterile parent) and IR72 (fertile parent), to study the inheritance of fertility restoration and to identify the marker associated with fertility restorer genes. Under field conditions, the trait of fertility restoration was observed to be under digenic control as F_2 population segregated in 15:1 ratio for fertile: sterile plants. Out of 12 SSR markers used for Bulk segregant analysis, two SSR markers found to be polymorphic between the parents and the corresponding bulks. One of these SSR markers RM6100 which has been reported to be mapped on chromosome 10 and in close proximity of fertility restorer gene/QTLs in other studies showed expected segregation ratio (15:1) for digenic model in the F_2 population. The accuracy of the marker RM6100 in predicting fertility restoration was validated in 10 restorers and 8 maintainers. RM6100 amplified the *Rf4* linked allele in a majority of the restorers. The closely linked SSR marker RM6100 may be used in marker assisted backcross breeding facilitating the transfer of fertility restoration gene *Rf4* into elite backgrounds with ease.

Introduction

Rice (*Oryza sativa* L.) is the staple food for more than half of the world's population. Hybrid rice technology is considered as one of the promising, practical, sustainable and eco- friendly options to break yield ceiling witnessed in rice. Rice hybrids are cultivated in more than 50% of area in China and the technology is being adopted now in India, Vietnam, Philippines, Myanmar and Bangladesh. The WA-CMS system, discovered in China (Yuan 1977; Lin and Yuan 1980), is the most widely used CMS source, which accounted at one stage for more than 80% of the rice hybrids produced in China and 100% of the hybrids developed outside China. Restorer line plays an important role in successful hybrid rice development. They are detected conventionally through test cross procedure by crossing rice germplasm lines (male parents) with the sterile CMS lines (female parents) and the F_1s are evaluated for the pollen and spikelet fertility. This system of restorer line identification is time consuming and labour intensive. Studies on genetic inheritance of Rf genes of WA-CMS indicated single gene control (Shen *et al.*, 1996, Yao *et al.*, 1997), two independent

genes (Virmani *et al.,* 1986; Govinda Raj and Virmani 1988; Teng and Shen 1994; Bharaj 1995, Tan *et al.,* 1998, Jing *et al.,* 2001, Namatzadeh and Kiani, 2010) and four genes (Zhu *et al.,* 1996). Attempts on DNA marker based linkage mapping analysis revealed chromosomal location of several Rf gene loci: Rf3 on Chromosome 1 (Yao, 1997, Zhuang *et al.,* 2000, He *et al.,* 2002, Ahmadikhah *et al.,* 2007, Sattari *et al.,* 2007), Rf4 on Chromosome 10 (Yao *et al.,*1997, Tan *et al.,*1998, Jing *et al.,* 2001, Zhang *et al.,*2002, Ahmadikhah *et al.,* 2007, Sattari *et al.,* 2007, Sheeba *et al.,* 2009), Rf4 on Chromosome 7 (Bazrkar *et al.,*2008), Rf5 on chromosome 10 (Jing *et al.,* 2001, Ahmadikhah *et al.,* 2007, Rf6 on chromosome 10 (Bazrkar *et al.,* 2008) and Rf7 on chromosome 12 (Bazrkar *et al.,* 2008). These studies have shown several DNA markers closely associated with specific Rf genes that are useful in marker assisted identification of those genes in the rice germplasm and further use in breeding program. The markers linked to the Rf genes could be of significant help in understanding the inheritance of the trait and targeted identification and introgression of Rf genes in breeding program. However, the markers which have been reported to be linked to the Rf genes have not been validated in alternate populations. Therefore, the present investigation was undertaken with the objectives to first study the inheritance of fertility restoration in an F_2 mapping population specifically developed for this purpose and secondly to validate the genes /QTLs associated with it.

Materials and Methods

Plant material

The plant material for the present study included one CMS line IR58025A and its maintainer line IR58025B and these were received from Directorate of rice Research, Hyderabad (India). In addition, 10 restorer lines and 8 maintainers for wild abortive type of cytoplasm collected from Directorate of Rice Research (DRR), Hyderabad, were also used. For studies on genetics of fertility restoration and validation of markers, one F_2 mapping population derived from cross IR58025A/IR72 consisting of 250 plants.

Scoring of spikelet fertility

The seed set in each panicle was counted to calculate the percentage of spikelet fertility as given below:

Spikelet fertility (%) =

$$\frac{\text{Number of fertile spikelets in the panicle (filled)}}{\text{Total number of spikelets in the panicle (filled and unfilled)}} \times 100$$

The spikelet fertility percentage of individual plants was calculated based on the average spikelet fertility of individual panicles selected from each plant. Plants in each population were classified into four classes based on spikelet fertility percentage, namely, fertile (more than 71% spikelet fertility), partially fertile (31–70%), partially sterile (1–30%) and sterile (0%). The spikelet fertility segregation pattern was studied by the fixed ratio χ^2 test as outlined by Gomez and Gomez (1984).

DNA extraction, amplification and bulk segregant analysis

Genomic DNA was extracted from the leaves of both the parents and 165 individual F_2 plants following CTAB method as described by Doyle and Doyle (1990). The quality and quantity of DNA were estimated spectrophotometrically using a NanoDrop (ND-1000, Wilmington, USA). Bulk

segregant analysis (BSA) method as suggested by Michelmore *et al.,* (1991) was used for quick identification of SSR markers associated with fertility restoration. Based on phenotypic observations, two bulks viz., sterile bulk (B1) comprising of five sterile F_2s and fertile bulk (B2) comprising of five resistant F_2 were made. These 10 F_2 were found homozygous when screened with the SSR markers used in the study. A pooled DNA sample was prepared for each bulk by mixing in equal quantity the DNA of five respective component F_2s. The parents and the bulks were screened with 12 SSR primers distributed over three chromosomes of rice genome to determine polymorphism and possible association with fertility restoration. These markers were selected based on previous studies on fertility restoration in rice and from the panel of 50 standard SSR markers reported on the website www.gramene.org. Most of the major fertility restorer genes were reported on chromosomes 1, 7 and 10 of rice genome and the markers associated/linked to these genes reported in the literature were used.

In addition, some markers reported on www.gramene.org available with us were used. The SSR markers found polymorphic among the parents and the bulks were used for F_2 progeny analysis. DNA of 165 F_2 progenies and parents were analyzed to study co segregation of these markers.

Data analysis

The clearly resolved amplicons of SSR were scored manually as homozygote for the allele for sterile parent (0), homozygote for the allele for fertile parent (1) and heterozygote carrying the alleles from both parents (2) in the data sheet. Chi-square (χ^2) test was performed to test the goodness of fit of the F_2 population for the phenotypic and marker data by comparing an observed frequency distribution with an expected one.

Validation of linked molecular markers

To assess the selection accuracy of identified marker after genotyping the plants of F_2 mapping population of cross IR58025A/IR72, marker validation study was conducted. This experiment consisted of 10 known restorers and 8 maintainer lines for wild abortive (WA) type of cytoplasm.

Results and Discussion

Genetics of fertility restoration in F_2 population

Out of 250 F_2 plants, 15 individuals showed complete spikelet sterility (like IR58025A), whereas 4 progenies were found fully fertile (>90%, like IR72). As suggested in earlier reports (Chaudhary *et al.,* 1981; Govinda Raj and Virmani 1988), partially fertile and partially sterile plants were pooled together to make a single category as semi fertile which is then fitted to 9:6:1 and 1:2:1 for F_2 and BC_1 populations respectively.

The observed ratio (137:98:15) of fertile [includes fully fertile (FF); partial fertile (PF); partial sterile (PS)] to completely sterile (CS) individuals did not differ significantly from 9(fertile):6(semi fertile):1(sterile) ratio (χ^2=0.310) revealing the role of two dominant independent genes in the inheritance of fertility restoration and displayed epistasis with incomplete dominance interaction (Table 1). This was confirmed from the segregation behaviour of the test-cross (BC_1) population (1:2:1). It seems that the restoration ability of IR72 is governed by two independent major genes.

This indicated that two dominant genes are responsible for complete fertility, while only one of the either genes conferred partial fertility (semi-epistatic type of gene interaction).

Table.1 Segregation pattern for fertility restoration (spikelet fertility) in different populations (F_2 and BC_1)

Sr. No.	Cross combinatons	Gen.	Total no. of plants	Segregation pattern				(FF):(PF+PS):CS	Genetic ratio	χ^2value	χ^2 table value
				FF	PF	PS	S				
1	**IR58025A/IR72**	**F_2**	**250**	137	67	31	15	137:98:15	**9:6:1**	0.310	5.991
		BC_1	**145**	37	32	43	33	37:75:33	**1:2:1**	0.379	5.991

FF = fully fertile, PF = partially fertile, PS = partially sterile, CS = completely sterile Spikelet fertility reaction: CS = 0 %; PS = 0.1 to 50 %; PF = 50.1 to 75 %; FF = 76 – 100 %
(PF and PS were merged in to one group (semi fertile) to enable Chi-square analysis)

Table.2 Proposed genetic constitution of fully fertile, semi-fertile and complete sterile plants in following cross of rice

Sr. No.	Cross combinations	Segregation pattern		
		FF	SF	CS
1	IR58025A/IR72	9 Rf3— Rf4—	3 Rf3— rf4rf4 3 rf3rf3 Rf4—	1 rf3rf3 rf4rf4

Table.3 Molecular evaluation of F_2 plants of cross (IR58025A/IR72) with SSR marker RM6100

Sr. No.	Class of segregation	Observed phenotype	Expected phenotypes (15:1)	χ^2 value	χ^2 table value at 1 df
1	Fertile including heterozygote	160 (7)	154.68	2.917	**3.84**
3	Sterile	5	10.31		
4	Total	165	165		

Figures in the parentheses indicate recombinants

Fig.1 Results of bulk segregant analysis using the sterile parent IR58025A (P1) and the fertile parent IR72 (P2), and their respective bulks (B1 and B2) with SSR marker RM6100; L, 100-bp StepUpTM DNA ladder (Genei, Bangalore, India)

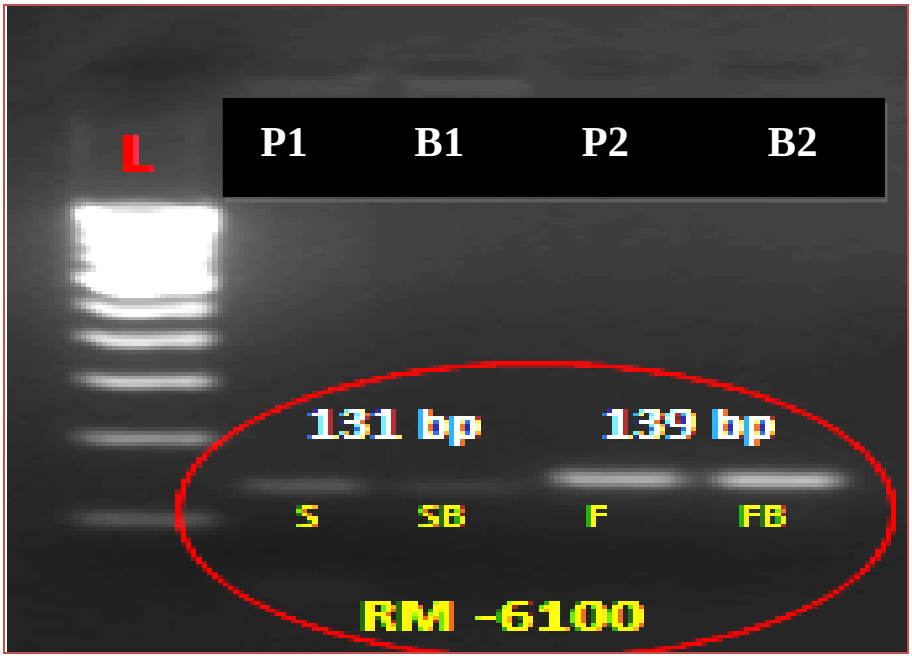
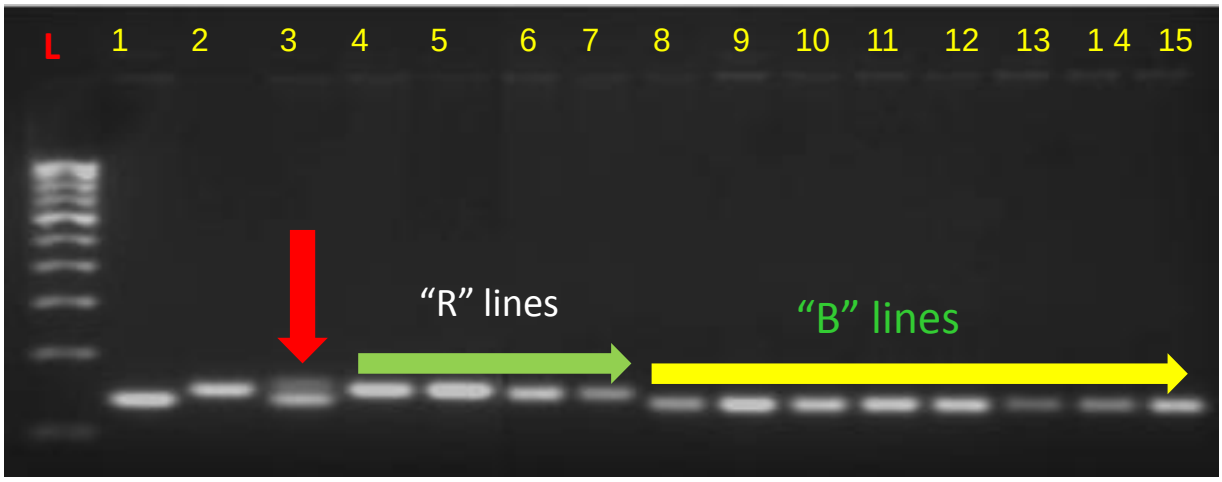
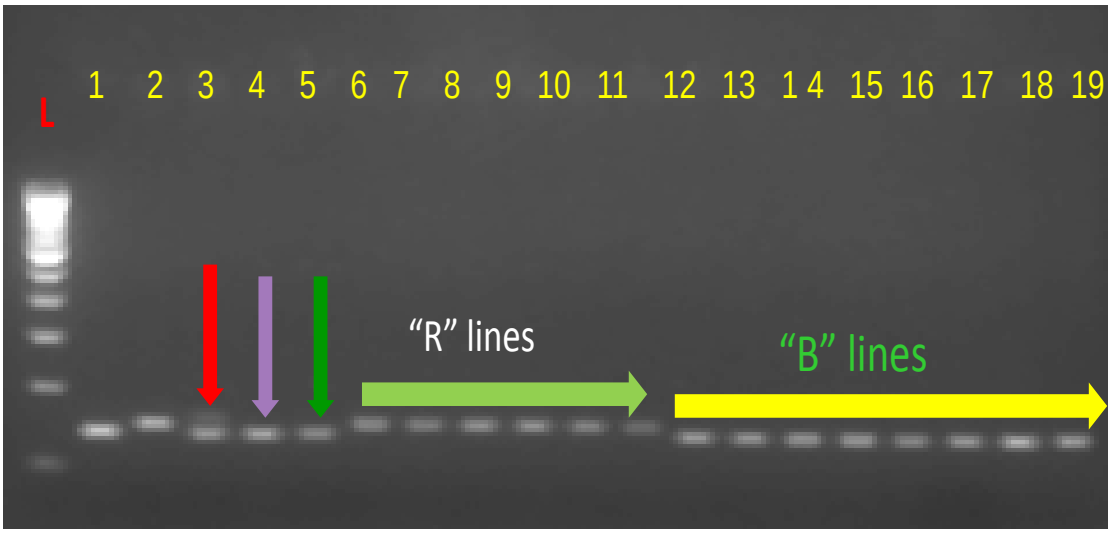


Fig.2 Validation of Marker RM6100 with R and B lines (set 1)



1 IR 58025A (129 bp)
2 IR72 (143 bp)
3 F1
4 IR62037 (143 bp)
5 IR 65483 (143 bp)
6 IR 46 (143 bp)
7 VDN-12-12 (143 bp)
8 IR 58025B(129 bp)
9 IR 62829B
10 IR 68886B
11 IR 69628B
12 IR 68897B
13 IR 68888B
14 DRR2B
15 DRR3B

Fig.3 Validation of Marker RM6100 with R and B lines (Set 2)



1 IR 58025A (129 bp)
2 IR72 (143 bp)
3 F1
4 Indrayani (129 bp)
5 Phule Samrudhi (129 bp)
6 IR 65653 (143 bp)
7 IR 54742
8 IR 69715
9 IR 55838
10 IR 48715
11 IR 65483
12 IR 58025B (129 bp)
13 IR 62829B
14 IR 68886B
15 IR 69628B
16 IR 68897B
17 IR 68888B
18 DRR2B
19 DRR3B

Plants where the recessive gene is allelic for any of the two genes and homozygous or heterozyous for the dominant alleles of the other gene (Rf3_r4r4 and rf3rf3Rf4_) were semi-fertile (Table 2).

The phenotypic data on the spikelet fertility showed that the F_2 population segregated in 15:1 ratio (fertile to sterile). In the cross of IR58025A/IR72 it has been observed that if both genes (Rf3Rf3 Rf4Rf4) are present, plants will be fully fertile like the restorer line, IR72; if only one of the either genes are present (Rf3— rf4rf4 rf3rf3 Rf4—), plants will show partial fertility (semi-epistatic type of interaction) and plants possessing the double recessive genotype (rf3rf3 rf4rf4) are completely sterile like IR58025A (Table 3). The observations of digenic control of fertility restoration in the present study are in agreement with the findings of earlier reports (Young and Virmani, 1984; Virmani *et al.*, 1986). Even though the trait of fertility restoration is controlled by two dominant independent genes, some minor genes and modifiers are also reported to be involved in the expression of the trait (Govinda Raj and Virmani, 1988; Sohu and Phul, 1995).

SSR markers linked to fertility restoration

Among the 12 SSR markers used, marker RM6100 reported polymorphism between two parents IR58025A and IR72 and corresponding bulks indicating its possible association with fertility restoration gene/s

in the mapping population (Figure 1). The F_2 mapping population was genotyped with RM6100 primer to study its possible association with fertility restoration. Segregation study with marker RM6100 recorded a fertile allele of ~143bp amplified in 160 plants, whereas a sterile allele of ~129bp was amplified in 5 plants. Seven F_2 plants exhibited both the alleles (heterozygous). Genetic analysis with chi-square test indicated goodness of fit to the expected ratio of 15:1 for digenic model indicating the association of RM6100 with fertility restorer gene in the present population.

On the basis of observed co-segregation between marker RM6100 and the fertility restoration trait in F_2 generation of cross IR58025A/IR72 at the marker locus, indicated that Rf4 gene carried by IR72 is located on chromosome 10. These results have also been confirmed on the studies of earlier workers (Jing *et al.*, 2001; Mishra *et al.*, 2003; Ahmadikhah and Karlov, 2006; Ahmadikhah *et al.*, 2007; Sheeba *et al.*, 2009) who have identified the role of the Rf4 locus in fertility restoration and its location on chromosome 10.

Validation of RM6100 with a set of maintainer and restorer lines

To assess the selection accuracy of RM6100 in marker-aided selection for the trait phenotype, 10 restorer lines and 8 maintainer lines were analyzed. Marker RM6100 clearly differentiated the restorer lines from maintainer lines based on presence of 143bp size amplicon in the restorer line IR72. The rice genotype VDN-12-12 which was identified as restorer line on the basis of spikelet fertility analysis has the similar band size (143bp) as that of other confirmed restorers, indicating the presence of Rf4 gene in it (Figure 2). The maintainer lines and restorer lines produced bands with band sizes of 129 and 143 bp, respectively. All the restorer lines showed a banding pattern different from maintainer lines except the two rice varieties, Indrayani and Phule Samrudhi, which showed banding pattern identical to the maintainer lines (Figure 3). Therefore, rice varieties Indrayani and Phule Samrudhi do not carry the Rf-4 gene and may be having different set of fertility restorer gene(s).

The genetics of fertility restoration in cross IR58025A/IR72 revealed the role of two dominant independent genes and displayed epistasis with incomplete dominance interaction. RM6100 is a good marker for identification of restorers from germplasm as well as it can be used in hybridity test of F_1 seeds.

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