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

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Abstract

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 or 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.

Keywords: DNA markers, hybrid rice, *Rf* gene, validation, WA-cms

1. 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 the yield ceiling witnessed in rice. Rice hybrids are cultivated in more than 50% of rice area in China and the technology is being adopted now in India, Vietnam, Philippines, Myanmar and Bangladesh.

Hybrid rice technology offers a potentially viable option for increasing rice yield potential beyond the level of inbred high-yielding varieties by exploiting heterosis, or hybrid vigor, on a commercial scale. Hybrid rice varieties have clearly shown a 1–1.5 t ha^{-1} (20–30%) yield advantage over conventionally bred modern varieties in farmers' fields in China (Lin and Yuan, 1980) and outside China (Yuan and Virmani, 1988; Virmani, 2003). Cytoplasmic male sterility (CMS) combined with a fertility restoration system has been found to be the most efficient genetic tool in commercializing this technology in rice (Lin and Yuan, 1980; Virmani and Wan, 1988). CMS is a maternally inherited trait characterized by the inability of a plant to produce functional pollen that is associated with abnormal open reading frames (ORFs) found in mitochondrial genomes and, in many cases, male fertility can be restored by fertility restorer (Rf) genes associated with nuclear genes encoding pentatricopeptide repeat (PPR) proteins (Chase and Babay-Laughnan, 2004; Hanson and Bentolila, 2004).

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 programmes. However, the markers which have been reported to be linked to the Rf genes have not been validated in alternate populations and the different restorer lines used in India have not been characterized for their allelic status with respect to these markers. 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 or QTLs associated with it.

2. Materials and Methods

2.1. 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 inheritance of fertility restoration and validation of markers, one F_2 mapping population derived from cross IR58025A/IR72 consisting of 250 plants.

2.1.1. Scoring of spikelet fertility

After land preparation, 21-day-old seedlings were transplanted in the field. The panicles of the main and two side tillers from each individual plant constituting the mapping populations were bagged with paper bags before flowering. The seed set in each panicle was counted to calculate the %age of spikelet fertility as given below:

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

The spikelet fertility % age 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 %age, 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).

2.1.1.1. 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_2s were made. These 10 F_2s 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 or 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 PCR protocol involved a total volume of 20 µL containing 20 ng genomic DNA, 0.1 µM of each primer, 2.0 µL of 10× Taq DNA polymerase buffer (100 mM Tris pH 9.0, 500 M KCl), 200 µM of each dNTPs and l U of Taq DNA polymerase. The reaction profile was 5 min at 95 °C, 40 cycles of 30 s at 94 °C, 30 s at 55 °C or 60 °C annealing, 1 min at 72 °C and 10 min at 72 °C for final extension. The PCR products were electrophoresed on Metaphore agarose gel (2.5%) and visualized on Gel Documentation System (Flour ChemTM Alpha Innotech Corporation, San Leandro, USA). 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.

2.1.1.1.1. 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.

2.1.1.1.1.1. 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. All the restorer and maintainer lines were made available from DRR, Hyderabad and were grown at the ARS, experimental farm, Vadgaon Maval during *Kharif*-2012.

3. Results and Discussion

3.1. Inheritance of fertility restoration in F_2 population

The pattern of spikelet fertility (%) of F_2 population derived from IR58025A/IR72 ranged from 0 to >90 % and segregated into two phenotypical classes. Out of 250 F_2 plants, 15 individuals showed complete spikelet sterility (like IR58025A), whereas 4 progenies were found fully fertile (>90%, like IR72). Spikelet fertility in the highest frequency of F_2 individuals (72 plants) ranged between 75-85% spikelet fertility. 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.

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

Sl. No.	Cross combinatons	Gen.	Total no. of plants	Segregation pattern				(FF):(PF+PS):CS	Genetic ratio	χ^2 value	χ^2 table value
				FF	PF	PS	S				
1.	IR58025A/IR72	F2	250	137	67	31	15	137:98:15	9:6:1	0.310	5.991
		BC1	145	37	32	43	33	37:75:33	1:2:1	0.379	5.991

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). 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).

Table 2: Proposed genetic constitution of fully fertile, semi-fertile and complete sterile plants in different crosses 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

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. 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).

3.1.1. 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. ,

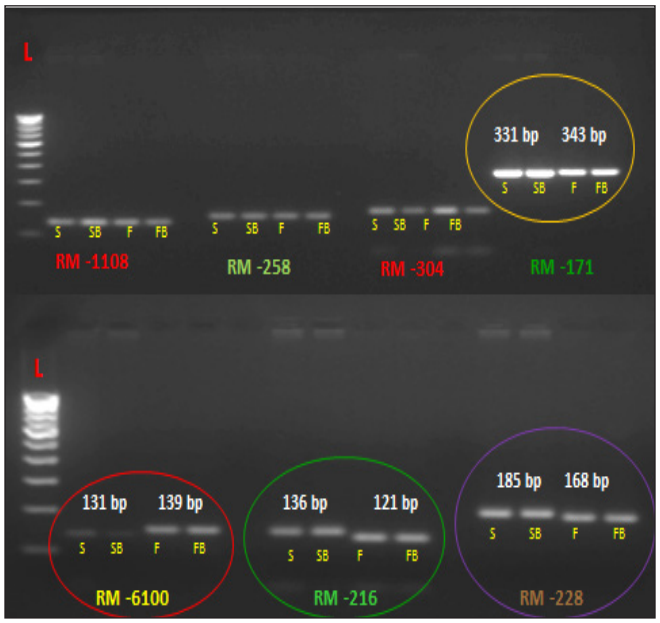
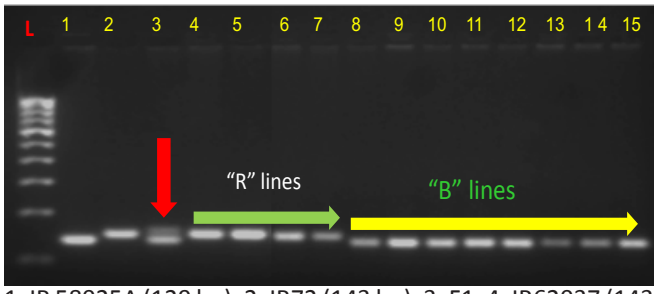


Figure 1: Results of bulk segregant analysis using the sterile parent IR58025A (P1) and the fertile parent IR72 (P2), and their respective bulks (B1 and B2) with SSR marker RM6100; L, 100-bp StepUpTM DNA ladder (Genei, Bangalore, India)

2009; Ngangkham et al. 2010) who have identified the role of the Rf4 locus in fertility restoration and its location on chromosome 10.

3.1.1.1. Validation of RM6100 with a set of maintainer and restorer lines

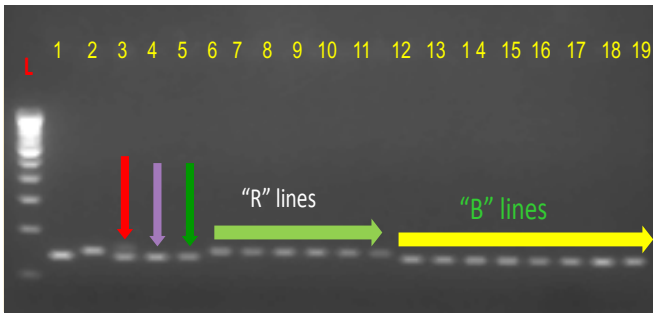
To assess the selection accuracy of RM6100 in marker-aided selection for the trait phenotype, 10 restorer lines and 8 maintainer lines were analyzed. Marker RM6100 clearly differentiated the restorer lines from maintainer lines based on presence of 143bp size amplicon in the restorer line IR72. The rice 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).



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

Figure 2: Validation of Marker RM6100 with R and B lines (set 1)

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 and Table 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).



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

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

Sl. No.	Class of segregation	OP	EP	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		

OP: Observed phenotype; EP: Expected phenotypes (15:1); Figures in the parentheses indicate recombinants

4. Conclusion

The inheritance of fertility restoration in cross IR58025A/IR72 revealed the role of two dominant independent genes and displayed epistasis with incomplete dominance interaction. RM6100 is co-dominant and is capable of discriminating three kinds of genotypes; Rf/Rf, Rf/rf, rf/rf. Hence, this marker is very useful marker for identifying restorers in segregating populations. The SSR marker RM6100 linked to the restorer gene Rf4 may play a crucial role in MAS. The marker is useful for confirmation of hybridity at the seedling stage.

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