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Molecular Mapping of Fertility Restorer Genes Controlling WA Cytoplasmic Male Sterility in Rice using SSR Markers

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Abstract

Cytoplasmic male sterility (CMS) based on wild-abortive (WA) cytoplasm has been extensively utilized in hybrid rice breeding programs. The investigation was carried out during the *Kharif* seasons of 2020 and 2021 at the Indira Gandhi Krishi Vishwavidyalaya, Raipur, Chhattisgarh. The experimental material comprised three CMS lines IR58025A, CRMS31A, and CRMS32A along with twenty aromatic traditional rice varieties used as testers. A total of 60 crosses were generated using a line x tester mating design to assess the inheritance pattern of fertility restorer (*Rf*) genes in aromatic rice hybrids. The inheritance analysis was performed on the BC_1F_2 population derived from the effective restorer cross IR58025A x Indrabhes Dubraj. Pollen and spikelet fertility segregation followed Mendelian segregation ratios, confirming a dominant monogenic gene action with a 3:1 segregation pattern. For molecular mapping, SSR markers were employed in the BC_1F_2 generation of the IR58025A x Indrabhes Dubraj cross. Genetic linkage analysis revealed that 14 SSR markers on chromosome 1 were associated with the Rf3 gene, one SSR marker on chromosome 2 was linked to the *Rf2* gene, and five SSR markers on chromosome 10 were associated with the *Rf4* gene. Among these, *Rf3* and *Rf4* were identified as the major fertility restoration loci, located on chromosomes 1 and 10, respectively.

Key words : Hybrid Rice, *Rf* genes, molecular mapping, SSR markers.

Cytoplasmic male sterility (CMS) in plants results from lesion or rearrangement in the mitochondrial genome, resulting in the inability of plants to produce functional pollen (Jing et al., 2001). Since nuclear genes are essential for restoring pollen fertility, CMS has become a valuable biological tool in hybrid seeds production, particularly through the widely adopted three line system. This system involves a CMS line, maintainer line and a restorer line carrying the restorer of fertility (*Rf*) gene to restore fertility is essential for the development of hybrid varieties. In rice, there are several CMS/*Rf* systems have been identified, including BT-, HL- and wild abortive (WA)-CMS (Virmani and Wan 1988). Among them, WA-type CMS, derived from the cytoplasm of wild rice, is the most widely used in indica hybrid seed production. WA type sterility is expressed sporophytically, making it highly suitable for large scale hybrid breeding.

To date, six restorers of fertility (*Rf*) genes in rice have been discovered. "These genes encode pentatricopeptide repeat (PPR) proteins, which are RNA binding proteins and involved in post-transcriptional mRNA process in cell organelles. *Rf*1a and *Rf*1b restore fertility in CMS-BT while in CMS-HL, partial fertility restoration (about 50%) can be restored by either *Rf5* or *Rf*6; together, they restore 75% fertility in the F_1 generation. Further analysis by sequencing and cloning concluded that *Rf*1 and *Rf*5 are identical and are located on chromosome 10". *Rf*3 and *Rf*4 restores fertility in CMS-WA and are located in chromosome 1 and 10 respectively with the latter recently cloned. CMS-LD fertility can be restored by *Rf*1 or *Rf*2 while CMS-CW can only be restored by a single gene *Rf*17 (Huang et. al., 2014).

"Molecular markers have become indispensible tool for accelerating the introgression of *Rf* genes and quantitative trait loci (QTL) into elite cultivars or breeding line through backcrossing. Markers linked to the gene can be used to select plants possessing the desired trait, and markers throughout the genome can be used to select plants that are genetically similar to the recurrent parent" (Young and Tanksley 1989, Hospital et al. 1992). Searching for restorer genes is a good approach where phenotyping is very time-consuming and requires the determination of spikelet sterility in testcross progeny (Yao et al. 1997).

Material and Methods

The investigation was carried out during two consecutive seasons *viz*., *Kharif*-2019 and *Kharif*-2020 at the Indira Gandhi Krishi Vishwavidyalaya, Raipur, Chhattisgarh.

During *kharif* 2019, twenty genotypes as testers including traditional varieties/land races (Table 1) were selected on the basis of their quality and aroma and three lines used for crossing programme to generating F_1 crosses. Crosses were made in Line x Tester fashion to developed 60 F_1 hybrids. In *kharif* 2020 all the crosses, parents and standard checks were sown at a standard spacing of 20 x 20 cm in a randomized block design with three replications.

Table 1. Details of lines and testers used in the present study

Female parent (Lines)	Cytoplasmic source	Source
IR 58025A	Wild Abortive	IRRI, Manila, Philippines
CRMS31A	Kalinga	NRRI, Cuttack, Orissa
CRMS32A	Kalinga	NRRI, Cuttack, Orissa
Male Parent (Testers)		
Male parent	**Male parent**	**Male parent**
Kalajeera	Kalikamod	Tarunbhog
Tilkasturi	Ramjeera	Nagri Dubraj
Dhaniya Phool	Dhaniya Dhan	Jao Phool
Kasturibhog	Basabhog	Dagri Kajar
Indrabhes Dubraj	Samundchini	Tulsi Bhog
Chinnor	Kalimuch	Barhasal
Tulsimogra	Jawaphool	

A set of hybrids was generated using CMS lines in a line x tester mating design as proposed by Kempthorne (1957). In order to enhance seed setting, the spikelets of CMS plant that were expected to flower the next day were clipped one third at the top. The clipped panicles were completely covered with butter paper bags to prevent contamination by foreign pollen grains. On the next morning, around 8:30 am, panicles from the male parents (testers) were collected during anthesis time and were kept in pollination room at temperature of 400C under high humidity in glass bottles. After the completion of anthesis the pollen grains were carefully dusted over clipped spikelets. The pollinated panicles were immediately bagged again and labelled properly mentioning the date and cross combination. In this manner, 60 crosses were successfully attempted for the study. The bagged panicles were harvested and separated from mother plants after 30-35 days of hybridization. The hybrid seeds of each cross were collected separately and sun dried. The seeds were treated with Bavestin as a precautionary measure against fungal attack before storing in paper bags.

Development of mapping population : After the hybridization programme we were selected some special genotypes for the development of mapping population BC_1F_1 and BC_1F_2 because selected genotypes have capacity to restore fertility in the hybrids.

Collection of samples and DNA Extraction : Green young and healthy leaves from BC_1F_2 plants were collected on the basis of fertility. The collected samples were placed in cooling pads to transfer and then stored at -20°C. Total rice genomic DNA was extracted from young succulent disease and insect free seedlings by CTAB method. Protocol of CTAB method given by (Doyle and Doyle (1987).

Quantification and Dilution of DNA : The DNA samples were quantified by Thermo Scientific NANODROP 2000C Spectrophotometer. After that genomic DNA was diluted suitably for proper amplification. DNA was diluted in such a way that the diluted samples contained about 20 ng μl^{-1} of DNA. Dilution was carried out according to the formula:

$$\text{Dilution} = \frac{\text{Required conc. of DNA (ng } \mu l^{-1}\text{) x Total volume required } (\mu l)}{\text{Available conc.of crude DNA (ng } \mu l^{-1}\text{)}}$$

The diluted DNA was subsequently used for PCR amplification.

Selection of molecular markers : Seventy three SSR markers (located on chromosome 1 and 10 which are known to be linked to *Rf* genes) were used for the detection of parental polymorphism. These markers are cited in various literatures for the work on molecular mapping of *Rf* genes (Anis G. B, 2019).

PCR amplification : PCR analysis was done using a set of random SSR (single sequence repeat) markers for molecular mapping of *Rf* gene with selected population. About 2 µl of diluted template DNA of each genotype was dispensed in the bottom of PCR plates. Separate cocktail was prepared in an eppendor*f* tube; 18 µl of cocktail was added to each tube to make final volume 20 µl.

Visualization of amplified products in Polyacrylamide gel electrophoresis : Five percent polyacrylamide gel (vertical) was used for better separation and visualization of PCR amplified microsatellite products, since polyacrylamide gels have better resolution for amplified products. Gels were casted in CBS-SCIENTIFIC electrophoresis unit. Glass plates were prepared before making the gel solution. Both glass plates (outer and inner notched glass plates) were cleaned thoroughly with warm water, detergent and then with deionised water.

Result and Discussion

Population development and fertility scoring : Among 60 test crosses evaluated, the cross IR58025A X Indrabhes Dubraj was identified as a superior combination based on its higher fertility restoration ability. This cross was advanced to develop the back cross population BC_1F_1. BC_1F_1 population was further used to raise BC_1F_2 population (Fig. A) and used for mapping of *Rf* genes. Based on segregation pattern of population it was expected to yield dominant monogenic gene action (3:1).

Around 72 plant population were developed in BC_1F_1 generation among these about 39 fertile plants were selected and 33 sterile plants were rejected on the basis of pollen fertility and spikelet fertility (pollen fertility >85% and spikelet fertility <10%). All the 39 fertile plants (having both homogenous and heterogeneous state of *Rf* gene) were used to grow BC_1F_2 population.508 plants were obtained in BC_1F_2 population. Out of these 508 plants; 150 plants were fertile (pollen fertility > 85%), 247 plants were found to be partial fertile/partial sterile and 111 plants were fully sterile (on the basis of pollen fertility).

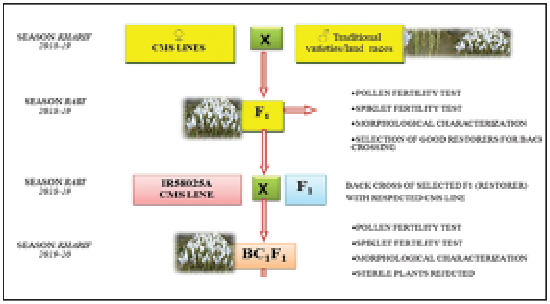

Fig. A. Procedure for development of BC_1F_2 population

Fully sterile plants (111) were rejected and out of remaining 397 plants (i.e. fully fertile and partial fertile) the highly fertile (pollen fertility >85%) 70 plants were selected for further molecular analysis.

Parental polymorphism : Seventy three SSR markers (located on chromosome 1 and 10 which are known to be linked to *Rf* genes) were used for the detection of parental polymorphism. These markers are cited in various literatures for the work on molecular mapping of *Rf* genes (Anis G. B, 2019). Out of 73 SSR markers, 20 markers viz., RM258, RM171, RM6100, RM228, RM263, RM1108, RM302, RM6470, RM1141, RM6887, RM3403, RM6515, RM6236, RM1247, RM6117, RM5548, RM7536, RM7180, RM148 and RM243 presented in Table-4.20 were found to be polymorphic among the parents IR58025A and Indrabhes Dubraj. (Fig. B).

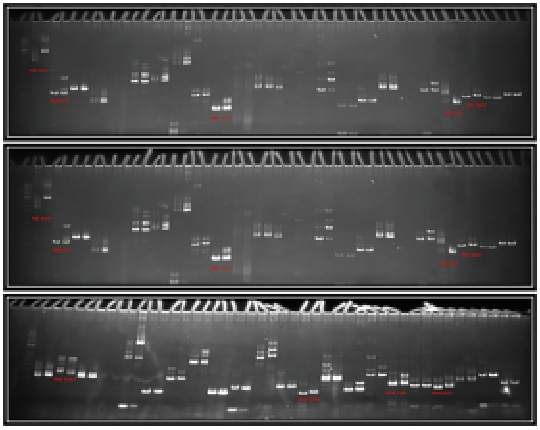

Fig. B. Parental polymorphism based on selected SSR markers

Screening of population with polymorphic markers : The 20 SSR markers viz., RM258, RM171, RM6100, RM228, RM263, RM1108, RM302, RM6470, RM1141, RM6887, RM3403, RM6515, RM6236, RM1247, RM6117, RM5548, RM7536, RM7180, RM148 and RM243 polymorphic with the parents of IR58025A x Indrabhes Dubraj of BC_1F_2 population i.e. fully fertile plants has been screened across the population for detection of *Rf* gene in the population with fertile plants (Fig. B). Two types of bands were obtained in the entire population (Fig. C). Some bands were similar to P_1 (fertile parent Indrabhes Dubraj) and some were heterozygous for both the parents P_1 and P_2 (sterile parent IR58025A CMS line).

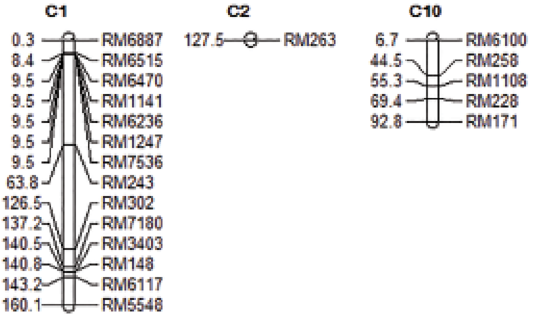

Fig. C. Map positions of all the markers on chromosome

Mapping of *Rf* genes with polymorphic markers : Searching for restorer genes is a good approach when phenotyping is very time-consuming and requires the determination of spikelet sterility in testcross progeny. To establish more precisely the genetical and physical maps of the *Rf* gene, high-resolution mapping of this locus was carried out using simple sequence repeat (SSR) markers in BC_1F_2 population. The genetic linkage analysis indicated that 14 SSR markers (RM302, RM6470, RM1141, RM6887, RM3403, RM6515, RM6236, RM1247, RM6117, RM5548, RM7536, RM7180, RM148 and RM243) on chromosome 1 were linked with the *Rf*3 gene. One SSR marker *viz*., RM-263 on chromosome 2 was linked to *Rf*2 gene and 5 SSR markers RM258, RM171, RM6100, RM228, and RM1108 on chromosome 10 were linked with the *Rf*4 gene. The major loci restoring the fertility have designated as *Rf*3 and *Rf*4 genes have been mapped on chromosome 1 and 10 and primers data were presented on Table 2. Map position of all the markers is shown in Fig. C.

Molecular markers are particularly useful for accelerating the process of introducing a gene or quantitative trait loci (QTL) into an elite cultivar or breeding line via backcrossing. Markers linked to the gene can be used to select plants possessing the desired trait, and markers

Table 2. SSR primers used for developing genotypic data

Markers	Sequence	Chromosome	Map distance	Linked gene
RM 258	F - TGCTGTATGTAGCTCGCACC R - TGGCCTTTAAAGCTGTCGC	10	44.5cM	*Rf*4
RM 171	F - AACGCGAGGACACGTACTTAC R - ACGAGATACGTACGCCTTTG	10	92.8cM	*Rf*4
RM-6100	F - TCCTCTACCAGTACCGCACC R - GCTGGATCACAGATCATTGC	10	6.7cM	*Rf*4
RM-228	F - CTGGCCATTAGTCCTTGG R - GCTTGCGGCTCTGCTTAC	10	69.4cM	*Rf*4
RM-263	F - CCCAGGCTAGCTCATGAACC R - GCTACGTTTGAGCTACCACG	2	127.5cM	*Rf*2
RM-1108	F - GCTCGCGAATCAATCCAC R - CTGGATCCTGGACAGACGAG	10	55.3cM	*Rf*4
RM-302	F - TCATGTCATCTACCATCACAC R - ATGGAGAAGATGGAATACTTGC	1	126.5cM	*Rf*3
RM-6470	F - ACCTTTCCCATGGTGGAATC R - TTATACCTTCGACGGGAACG	1	9.5cM	*Rf*3
RM-1141	F - TGCATTGCAGAGAGCTCTTG R - CAGGGCTTTGTAAGAGGTGC	1	9.5cM	*Rf*3
RM-6887	F - CCTTGTATCAGATGCCAGGG R - GAGAGCAGGAGTGATTTGCC	1	0.3cM	*Rf*3
RM-3403	F - AACGACTGCTCCCTCTTCAG R - AGCTTGCAAGGCATTAGCTC	1	140.5cM	*Rf*3
RM-6515	F - GCTCGGCTAGTGACGATTTC R - GTGGTAGGCGACATAGCTCC	1	8.4cM	*Rf*3
RM-6236	F - AACCCACTATCAGTTCACAAGC R - GAGATGGAGAGAGAAAGATTGG	1	9.5cM	*Rf*3
RM-1247	F - GACGTCTCATCCTCCATGGTTCC R - TTGCGATGCCGAGGAGATAAGG	1	9.5cM	*Rf*3
RM-6117	F - AGCACAAACCTAGCGGAGTG R - ACCTGAGCATGGATACTCGG	1	143.2cM	*Rf*3
RM-5548	F - GGTGCAGAGTGATGCAATTC R - AACATTAGGGATGAGGCTGG	1	160.1cM	*Rf*3
RM-7536	F - TGCTAGGGCATCTAAACAGG R - ATACGTGTAGGCAGCACTAGTG	1	9.5cM	*Rf*3
RM-7180	F - GTGTTTATAGGGGTGCCACG R - TGTTGGTGGTGCAGGTAAAG	1	137.2cM	*Rf*3
RM-148	F - ATACAACATTAGGGATGAGGCTGG R - TCCTTAAAGGTGGTGCAATGCGAG	1	140.75cM	*Rf*3
RM-243	F - GATCTGCAGACTGCAGTTGC R - AGCTGCAACGATGTTGTCC	1	63.8cM	*Rf*3

throughout the genome can be used to select plants that are genetically similar to the recurrent parent (Young and Tanksley,1989; Hospital et al., 1992; Semgan et al., 2006). High yield potential of CMS derived F_1 hybrids depends upon their high pollen fertility and spikelet fertility, which determined by the number and mode of action of restorer genes present in their restorer parent. Knowledge of the genetic control of male fertility restoration is also useful to transfer fertility restoring genes to promising breeding lines and undertake improved restorer breeding programme. It may also help in efficient transfer of restorer genes in other agronomically desirable genotypes (Sohu and Phul, 1995).

Ultimately, identification of closely linked markers would help in map-based cloning of fertility restorer genes to understand the molecular and biochemical basis of fertility restoration and the possible role of *Rf* genes in other biological processes. Similar results were reported by Yao et al. (1997), Tan et al. (1998), Jing et al. (2001), Hospital (2001), Liu et al. (2004), Bazrkar et al. (2008), Sheeba et al. (2009), Shah et al. (2012), Balaji et al. (2012), Namaky et al. (2016) and Anis (2019).

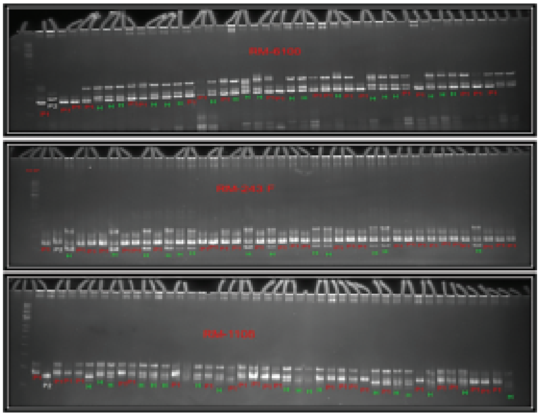

Fig. D. Population polymorphism for different SSR markers used in study

Conclusion

Inheritance study of BC1F2 with one of the effective restorers (Indrabhes Dubraj) reveals that cross IR58025A X Indrabhes Dubraj were governed with dominant monogenic gene action (3:1). Molecular studies revealed that the major loci restoring the fertility have designated as *Rf*3 and *Rf*4 and have been mapped on chromosome 1 and 10.The genetic linkage analysis indicated that 14 SSR markers on chromosome 1 was linked with the *Rf*3 gene. Five SSR markers on chromosome 10 were linked with the *Rf*4 gene and One SSR marker on chromosome 2 was linked to *Rf*2 gene.

Authors' contribution

Conceptualization of research (AKT, DS); Designing of the experiments (AKT, DS); Contribution of experimental materials (AKT, DS); Execution of field/lab experiments and data collection (AKT, SU); Analysis of data and interpretation (AKT, JKT, SU); Preparation of the manuscript (AKT, JKT, SU).

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