Identification of Good Restorer Lines Through Molecular Confirmation of *Rf3* and *Rf4* Genes in Rice Hybrids

Abstract

Pollen fertility can be restored by nuclear-encoded genes known as fertility restorer (*Rf)* genes. The initial stage in creating high-yielding heterotic hybrids involves the identification of restorers capable of effectively restoring the fertility of CMS lines. In the case of CMS-WA lines, fertility restoration is primarily governed by two separate and dominant nuclear fertility-restoring genes, namely *Rf3* and *Rf4*. In this study, we aimed to investigate the molecular conformation of *Rf3* and *Rf4* genes in a set of restorer lines and hybrids in rice. Two functional markers, RM SF21-5 for *Rf3* and RMS-PRR-9-1 for *Rf4*, were employed to identify the allelic status of the fertility restorer genes. Results revealed that among the 42 rice entries screened, Four rice restorer lines and four hybrids were found to possess both *Rf3* and *Rf4* genes, making them valuable for hybrid breeding programs. five rice hybrids lacked both *Rf3* and *Rf4* genes. The five hybrids which are lacked restorer genes were unsuitable for such breeding programs. These findings establish *Rf3*and *Rf4* as major fertility-restoring genes in rice, consistently contributing to complete fertility restoration. The genotyping results provide valuable insights for selecting appropriate parental lines and restorers in hybrid rice breeding programs. The presence or absence of these fertility-restoring genes plays a crucial role in developing high-yielding and productive rice varieties through effective breeding strategies.

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Key words: Rice, *Rf3*, *Rf4*, Fertility restoration, male sterility

INTRODUCTION

Rice (*Oryza sativa*) is a crucial crop worldwide, providing sustenance to a significant portion of the global population. it is the most widely consumed food grain globally, and its consumption is projected to rise by 3% to reach 108 million tonnes, as per the USDA's report in 2021 (1). The total global production of rice for the period 2021-2022 is estimated to be approximately 515.05 million tonnes, with India contributing 126,500 metric tonnes in the same year (source: https://www.worldagriculturalproduction.com/crops/rice.aspx).

A Hybrid rice breeding programs have greatly contributed to achieving high-yielding and superior quality rice varieties. These programs rely on the exploitation of cytoplasmic male sterility (CMS) systems in combination with restorer genes to restore male fertility, enabling successful hybridization. Identification of restorer lines (that restore the fertility of CMS lines) is the foremost step for superior-yielding heterotic rice hybrids (2). Among the restorer genes, *Rf3* and *Rf4* play pivotal roles in the restoration of male fertility in CMS lines, making them essential components of hybrid rice breeding.

Understanding the molecular conformation of *Rf3* and *Rf4* genes in restorer lines is of paramount importance to ensure their efficacy in hybrid rice production. Molecular markers have emerged as powerful tools to aid in the identification and characterization of these genes, facilitating efficient selection of superior restorer lines by breeders.

Several studies have been conducted to unravel the molecular basis and genetic variation of *Rf3* and *Rf4* genes in rice. In rice, there have been a total of 17 alleles identified for fertility restoration. All of these alleles, except *rf17*, are dominant in rice. At least two specific genes, *Rf3* (located on chromosome 1) and *Rf4* (located on chromosome 10), are known to be responsible for controlling fertility restoration of WA cytoplasm in rice. (3,4). Pranathi (5) developed gene-based functional markers, namely RMS-PPR9-1 for *Rf4* and RMS SF21-5 for *Rf3*. These markers serve as valuable tools for identifying and studying the *Rf4* and *Rf3* genes. For instance, Li *et al*. (6) conducted a comprehensive analysis of the genetic diversity and molecular characterization of *Rf3* and *Rf4* genes in a diverse panel of restorer lines. Their study revealed multiple allelic variants of *Rf3* and *Rf4* genes, emphasizing the significance of considering allelic diversity for effective hybrid rice breeding. Furthermore, the development of advanced molecular techniques, such as polymerase chain reaction (PCR) and DNA sequencing, has facilitated the creation of specific markers for detecting the presence of *Rf3* and *Rf4* genes in rice lines. Zhou (7) successfully developed allele-specific PCR markers for *Rf3* and *Rf4* genes, allowing rapid and accurate screening of restorers in breeding programs. In addition to genetic diversity, the molecular conformation of *Rf*3 and *Rf4* genes has also been linked to functional characteristics. Liu (8) conducted a study focusing on the molecular structure and function of the *Rf3* gene in rice. Their research revealed that *Rf3* encodes a protein with a pentatricopeptide repeat (PPR) domain, which plays a critical role in RNA binding and processing.

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The main aim of this study is to identify good restorer lines which are having high restoring ability by confirming *Rf3* and *Rf4* genes through molecular studies.

Material and Methods:

Plant Material

The present study was conducted at Regional Agricultural Research Station, Warangal, Professor Jayashankar Telangana State Agricultural University (PJTSAU) during *rabi, 2023* with an objective of molecular confirmation of Restores lines for presence of two major fertility restorer genes i.e. *Rf3* and *Rf4* through functional markers (Table 1)

Table.1. List of markers used in the present study for screening of fertility restorer genes

S. No	Primer	Sequence		Chromosome	Annealing temperature ^{0}C	Reference
1.	RM SF 21-5 (*Rf3*)	F	GAGTTGGGGGTCGAGAAATC	10	55^{0}C	Pranathi *et al*., (2016)
		R	CGTACGTGCGGCTAGGATCAA			
2.	RMS PRR 9-1 (*Rf4*)	F	GAGTTTTGAATAGATTTACGTGTGGA	1	55^{0}C	Pranathi *et al*., (2016)
		R	AGTGTCCAGATTCGTAGTAATGC			

Marker assisted selection for fertility restorer genes

DNA was isolated from the 10 restorer lines and 30 rice hybrids along with two checks by following the protocol of Zheng *et al*., (9) and potential restorer KMR3R is used as a reference to compare the positive alleles. The PCR based SSR marker RM SF21-5 and RMS PRR 9-1 were used to identify the allelic status with respect to two major fertility restorer genes i.e. R*f3* and *Rf4*. PCR was performed using 1 U of Taq DNA polymerase (Fermentas, Lithuania) and 1x PCR buffer (Genei, India) in 10-µl reaction volume with a thermal profile of 94 ^{o}C for 5 min (initial denaturation), followed by 35 cycles of denaturation at 94^{o}C for 30s, annealing at 55^{o}C for 30s, extension at 72^{o}C for 1 min and a final extension of 7min at 72^{o}C. The amplified product of *Rf3* and *Rf4* were electrophoretically resolved on a 3.5% Seakem LE® agarose gel (Lonza, USA) containing 0.5 mg/ml of ethidium bromide in 0.5x TBE buffer and visualized under UV. The primer RM-SF21-5 for *Rf3* produced a positive band at 172bp indicating the presence of the restorer allele, while a negative band appeared at 127bp indicating the absence of the restorer allele with the parent KMR3R. On the other hand, the functional marker RM SF21-5 for *Rf3* showed positive alleles at 172bp and negative alleles at 127bp.

Results and Discussion

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A total of 30 rice hybrids and 10 restorer lines including two checks were screened to determine the presence or absence of fertility restoration genes, namely *Rf3* and *Rf4*. The screening process involved the use of specific primers i, e. RM-SF-21-5 for *Rf3* and RMS-PRR-9-1 for *Rf4*.The results representing the screening of *Rf3* and *Rf4* are depicted in table 2.

Table 2: Details of rice hybrids and their genotyping results by using functional markers for the present investigation

S. No.	Name of the rice entries	*Rf3* gene: RM SF 21-5 marker	*Rf4* gene: RMS PRR-9-1marker
PARENT	KMR3R	RR	RR
RESTORER LINES			
1.	JGL24355	rr	RR
2.	JGL24502	RR	rr
3.	JGL24440	rr	RR
4.	JGL35149	rr	RR
5.	JGL36147	RR	RR
6.	JGL36172	RR	RR
7.	JGL38156	RR	rr
8.	JGL36199	RR	rr
9.	WGL1272	RR	RR
10.	JR 70	RR	RR
HYBRIDS			
11.	CMS59AXJGL24355	rr	RR
12.	CMS59AXJGL24502	rr	rr
13.	CMS59AXJGL24440	rr	Rr
14.	CMS59AXJGL35149	rr	rr
15.	CMS59AXJGL36147	RR	RR
16.	CMS59AXJGL36172	RR	RR
17.	CMS59AXJGL38156	RR	rr
18.	CMS59AXJGL36199	rr	RR
19.	CMS59AXWGL1272	rr	RR
20.	CMS59AXJR70	rr	RR
21.	CMS52XJGL24355	rr	rr
22.	CMS52XJGL24502	Rr	rr
23.	CMS52XJGL24440	rr	Rr
24.	CMS52XJGL35149	rr	rr
25.	CMS52XJGL36147	rr	RR
26.	CMS52XJGL36172	RR	RR
27.	CMS52XJGL38156	RR	Rr
28.	CMS52XJGL36199	RR	rr
29.	CMS52XWGL1272	rr	rr
30.	CMS52XJR70	RR	RR
31.	CMS64XJGL24355	rr	RR
32.	CMS64XJGL24502	rr	RR
33.	CMS64XJGL24440	Rr	RR
34.	CMS64XJGL35149	Rr	Rr
35.	CMS64XJGL36147	rr	Rr
36.	CMS64XJGL36172	rr	RR
37.	CMS64XJGL38156	RR	rr
38.	CMS64XJGL36199	RR	rr

39.	**CMS64XWGL1272**	**rr**	**rr**
40.	**CMS64XJR70**	**RR**	**rr**
CHECKS			
41.	**US312**	**rr**	**RR**
42.	**KRH4**	**rr**	**RR**

Molecular confirmation of *Rf3* gene by using RMS-SF-21-5 functional Marker:

The 30 rice hybrids and 10 restorers including two checks were screened for the presence of *Rf3* gene by using RM SF21-5 functional marker, The results revealed that seven restorers(JGL24502,JGL36147,JGL36172,JGL38156,JGL36199,WGL1272,JR70)and10hybrids(CMS59AXJGL36147,CMS59AXJGL36172,CMS59AXJGL38156,CMS52AXJGL36172,CMS52AXJGL38156,CMS52AXJGL36199,CMS52AXJR70,CMS64XJGL38156, CMS64AXJGL36199, CMS64AXJR70) showed the prescence of *Rf3* with similar banding with KMR-3.

On the other hand, 3 restorer lines (JGL24355, JGL24440, JGL35149), 17 hybrids (CMS59AXJGL24355,CMS59AXJGL24502,CMS59AXJGL24440,CMS59AXJGL35149,CMS59AXJGL36199,CMS59AXWGL1272,CMS59AXJR70,CMS52AXJGL24355, CMS52AXJGL24440,CMS52AXJGL35149,CMS52AXJGL36147,CMS52AXWGL1272,CMS64AXJGL24355,CMS64AXJGL24502,CMS64AXJGL36147,CMS64AXJGL36172,CMS64AXWGL1272) and 2 checks (US312,KRH4) showed the abscence of *Rf3* gene. Moreover, three rice hybrids were found to exhibit heterozygosity for both alleles. These hybrids were identified as CMS52AXJGL24502, CMS64AXJGL24440, and CMS64AXJGL35149.

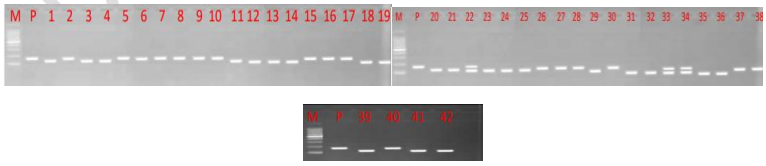

Fig 1: Molecular confirmation of Rf3 gene by using RM SF21-5functional Marker

The Lane number M represents 100bp ladder, P represents positive control (KMR-3R), while the numbers from 1 to 42written on the top of gels represents list of rice entries used for present study and details were given in Table 1.

Molecular confirmation of *Rf4* gene by using RMS PRR 9-1 functional Marker:

The 30 rice hybrids 10 restorer lines including two checks, when screened for the presence of *Rf4* gene by using RMS PRR 9-1 functional marker, The results revealed that thereare7restorerlines(JGL24440,JGL35149,JGL36147,JGL36172,JGL24355,WGL1272,JR70)13hybrids(CMS59AXJG24355,CMS59AXJGL36147,CMS59AXJGL36172,CMS59AXJGL36199,CMS59AXWGL1272,CMS59AXJR70,CMS52AXJGL36147,CMS52AXJGL36172,CMS52AXJR70,CMS64AXJGL24355,CMS64AXJGL24502,CMS64AXJGL24440,CMS64AXJGL36172)and2checks(US312,KRH4) showed the prescence of *Rf4* with similar banding with KMR-3.

Out of the 42 rice entries screened, four rice restorer lines (JGL36147, JGL36172, WGL1272,JR70)andfourhybrids(CMS59AXJGL36147,CMS59AXJGL36172,CMS52AXJR70, CMS52AXJGL36172) were found to carry both major fertility restorer genes, *Rf3* and *Rf4*. Conversely, we also observed five rice hybrids (CMS59AXJGL24502, CMS59AXJGL35149,CMS52AXJGL24355,CMS52AXJGL35149,andCMS52AXWGL1272) that lacked both major fertility restorer genes, *Rf3* and *Rf4*.

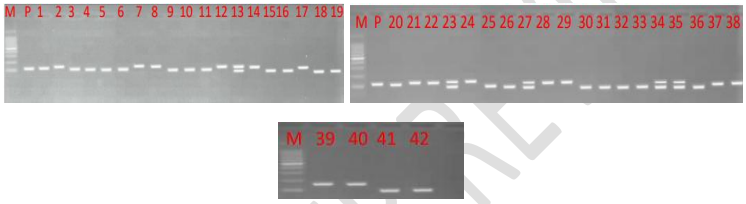

Fig 2: Molecular confirmation of Rf4 gene by using RMS PRR9-1 functional Marker

The Lane number M represents 100bp ladder, P represents positive control (KMR-3R), while the numbers from 1 to 42 written on the top of gels represents list of rice entries used for present study and details were given in Table 2.

Researchers have extensively studied and confirmed the effectiveness of *Rf3* and *Rf4* markers in fertility restoration through various studies. For instance, Shidenur (10) conducted screening on 310 NPT lines to assess fertility restoration using DRRM- Rf3- 5, DRRM- Rf3- 10, and functional markers RMS- SF21- 5, RM6100, and RMS- PPR9- 1. Similarly, Pranathi (5) undertook screening to distinguish between 120 restorers and 44 non-restorers based on fertility restoring ability. They also developed functional markers for *Rf3* and *Rf4* to aid in their research. Venkanna (2) conducted a study focusing on the fertility restoration of CMS-WA lines, which is mainly governed by two independent and dominant nuclear fertility restoring genes, *Rf3* and *Rf4*. In their study, they aimed to genotype 25 rice genotypes to determine the presence of *Rf3* and *Rf4* genes using functional markers.

Conclusion

Based on the genotyping results for fertility restoration, it was observed that the four rice restorer lines (JGL36147, JGL36172, WGL1272, JR70) and four hybrids (CMS59AXJGL36147,CMS59AXJGL36172, CMS52AXJGL36172,CMS52AXJR70) possess both *Rf3* and *Rf4* genes. Among ten restorer lines used in this study, three

restorer lines have capability of restoring fertility in rice hybrids. Hence these three restorer lines can be used in future hybrid breeding programmes.

Future scope:

The future scope of this study lies in expanding the investigation to a larger and more diverse set of rice genotypes to further validate the role of *Rf3* and *Rf4* genes in fertility restoration. Additionally, exploring other potential restorer genes and incorporating advanced genomic technologies can enhance the precision and efficiency of hybrid rice breeding programs.

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