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Research Article

Validation for the major fertility restorer genes, R*f*3 and R*f*4 of F_4 generation of CBSN 25/ WRM 21-24 and CBSN 25/ WRM 93-20 crosses in rice (*Oryza sativa* L.)

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Abstract
The first step in developing high yielding heterotic hybrids is to find restorers that can effectively restore the fertility of CMS lines. Two distinct and dominant nuclear genes for restoring fertility, R*f*3 and R*f*4, are primarily in charge of the fertility restoration of CMS-WA lines in rice. Identifying fertility restorer lines can be accelerated and made simpler by molecular mapping of R*f*3 and R*f*4. The present investigation was carried out to validate the presence of two fertility restorer genes R*f*3 and R*f*4 in the F_4 generations of two populations *viz*., CBSN 25/WRM 21-24 (86 families) and CBSN 25/WRM 93-20 (79 families) using seven SSR molecular markers. The results revealed that 17 plants in the cross CBSN 25/WRM 21-24 and 47 plants in CBSN 25/WRM 93-20 were found to be double positives for both R*f*3 and R*f*4 genes. Hence, after stabilization these identified plants from both crosses could be used as male parents or restorers in CMS-based hybrid rice breeding programs.

Keywords: Fertility restorer genes, R*f*3 and R*f*4 genes, CMS-WA, hybrid rice, SSRs

INTRODUCTION

The developing countries like India, which has to produce approximately 125 million tonnes of rice by 2030 to feed the increasing population, breeding rice for higher yield continues to be the top goal. Since, rice is a self-pollinating crop, the adoption of male sterility system is essential for economic exploitation of heterosis. One of the realistic, viable, sustainable and environmental friendly ways to break the yield barriers in rice is hybrid rice technology (Sheeba *et al*., 2009). Under identical circumstances, hybrid rice has a yield advantage of roughly 15-20% over the best commercial inbred varieties (Virmani, 1996). In rice, there are three main forms of CMS: Wild-abortive (WA), BaotaiType (BT) and Honglian (HL), whose inheritance patterns and physiological traits have undergone substantial study. The WA type CMS is a sporophytic abortion, which eventually results in the creation of typical abortive pollen, hence failing to produce normal pollen (Sattari *et al*., 2008). Based on cytoplasmic male sterility and fertility restoration systems, the main cause for cytoplasmic male sterility is the malfunction or transformation of the mitochondrial genome prevents it from producing viable pollen (Nematzadeh and Kiani, 2010). Nuclear-encoded genes known as the fertility restorer (R*f*) gene are responsible for restoring pollen fertility. The first step in creating high-yielding heterotic hybrids is to locate restorers that can effectively restore the fertility of the CMS lines. It has already been reported that fertility restoration is controlled by two distinct nuclear genes that

are dominant, with one gene acting more strongly than the other genes. A significant dominant gene regulates WA-cytoplasm fertility restoration was already reported by Huang *et al.* (1986) and Anandakumar and Subramanian (1992). All the identified 17 fertility restoration alleles in rice are dominant except R*f*17. Among them, two genes *viz*., R*f*3 on chromosome 1 and R*f*4 on chromosome 10 are known to regulate the WA cytoplasm's ability to restore fertility (Zhang *et al.* (1997); Yao *et al.* (1997)). The experiment conducted by Nas *et al.* (2003) explained how to identify restorative lines using molecular markers and the PCR-based marker RG140STS had an 83% accuracy rate for locating potential restorers. Molecular mapping of R*f*3 and R*f*4 can speed up and simplify the process of identifying fertility restorer lines (Sattari *et al.* (2007); Sheeba *et al.* (2009)). Jing *et al.* (2001), Ahmadikhah and Karlov (2006), Bazrkar *et al.* (2008), Sheeba *et al.* (2009) and Sattari *et al*. (2008) employed SSR markers to examine genetic diversity and fine mapping of fertility restoration genes. Therefore, the aim of the present research was to use SSR markers to validate the presence of two key fertility restorer genes R*f*3 and R*f*4 in rice.

MATERIALS AND METHODS

The present investigation was carried out with F_4 populations of Restorer x Wild Rice MAGIC lines. The wild rice MAGIC (WRM) lines developed by International Rice Research Institute, Philippines involving AA genome species *viz., O.rufipogon, O.longistaminata, O.nivara, O.barthii, O.meridonalis*and *O.glumaepatula* with two cultivated species, *O. sativa and O.glaberimma.* The two F_4 populations namely CBSN 25/WRM 21- 24 (86 families) and CBSN 25/WRM 93-20 (79 families) were raised in the nursery bed at Department of Rice, Tamil Nadu Agricultural University, Coimbatore during *summer* 2023 season. Twenty-eight days old seedlings of F_4 families were transplanted along with their parental lines at a spacing of 20 x 20 cm in two rows of 3.6 m length/ family in the main field and crop agronomy was taken care of throughout the cropping period.

DNA extraction and PCR: Young disease-free leaf samples were collected from one plant per family, which was randomly chosen for DNA extraction. Genomic DNA was extracted following the Doyle and Doyle (1987) methodology, using a modified CTAB (CetylTrimethyl Ammonium Bromide) method. Utilizing Nanodrop device (GENEVA, Nano), the quantity and quality of DNA were assessed. The concentration of DNA was standardized to 50 ng/µl and stored at -20ºC for further usage. For one reaction (volume 10µl) the cocktail (pcr product) includes 1µl DNA, 3.5µl PCR master mix , 4.5µl sterile water and 1µl Primer (0.5µl forward +0.5 µl reverse primer). The smART Prime 2x PCR master mix was used, which consisted of Taq DNA Polymerase (0.0125 U/µL), Reaction buffer 1mM $Mgcl_2$, 0.1 mM of each dNTPs and 1.25 mL Nuclease free water. PCR amplification (Thermo scientific) was done by initial denaturation at 94°C for 5 minutes followed by 35 cycles of 94°C for 45 seconds, 55°C for 30 seconds, 72°C for 1 minute, extension for around 10 minutes at 72°C, then infinite retrieval at 4°C. Ethidium bromide was added to the gel cast at a concentration of 10µg /10ml, and electrophoresis was performed on a 3% polyacrylamide gel for one hour at 120 volts. Under UV illumination, the gel was seen in the gel documentation unit (Bio-Rad Gel Doc XR imaging system). To confirm the presence of restorer genes, three markers *viz*., RM171, RM258, and RM228 for R*f*4 and four markers namely RM1, RM443, RM10313 and RM10318 for R*f*3 were employed as detailed in **Table 1**.

Table 1. Primer details of R*f*3 and R*f*4 linked markers

S. No.	Primer name		Primer sequences	Gene linked	Chromo-some location	Amplifica-tion product size (bp)	Marker distance (cM)	Annealing temperature (ºC)	References
1	RM1	F	GCGAAAACACAATGCAAAAA	R*f*3	1	113	5.1	55	Alavi *et al.*(2009)
		R	GCGTTGGTTGGACCTGAC						
2	RM443	F	GATGGTTTTCATCGGCTACG	R*f*3	1	124	4.4	55	Bazrkar *et al.* (2008)
		R	AGTCCCAGAATGTCGTTTCG						
3	RM10313	F	ACTTACACAAGGCCGGGAAAGG	R*f*3	1	188	4.2	55	Neeraja *et al.* (2009)
		R	TGGTAGTCGTAACTCTACTCCGATGG						
4	RM10318	F	TGTCTCACACATTGCACACTTACC	R*f*3	1	187	-	55	Shah *et al.* (2012)
		R	GGCCTAACCCAACACATGTCC						
5	RM171	F	AACGCGAGGACACGTACTTAC	R*f*4	10	340	6.2	58	Nematzadeh and Kiani(2010)
		R	ACGAGATACGTACGCCTTTG						
6	RM258	F	TGCTGTATGTAGCTCGCACC	R*f*4	10	148	3.1	55	Nematzadeh and Kiani(2010)
		R	TGGCCTTTAAAGCTGTCGC						
7	RM228	F	CTGGCCATTAGTCCTTGG	R*f*4	10	120	-	55	Ahmadikhah and Karlov(2006)
		R	GCTTGCGGCTCTGCTTAC						

RESULTS AND DISCUSSION

In the current study, two F_4 populations of the crosses CBSN 25/WRM 21-24 and CBSN 25/WRM 93-20 were used for molecular screening of the fertility restorer genes, R*f*3 and R*f*4. Allelic differences, interactions and background factors are impacting fertility more than the presence of R*f*3 and R*f*4 genes on fertility restoration in rice (Cai *et al*., 2013). The effects of these two fertility restorer genes appear to be additive in nature, with R*f*4 having a stronger impact than R*f*3 (Yao *et al*., 1997; Sattari *et al*., 2008). Many R*f*4 markers have been created (Ahmadikhah and Karlov 2006; Ngangkham *et al*., 2010; Balaji *et al.,* 2012), but just a few R*f*3 markers were developed (Nas *et al*., 2003). However, the accuracy of these markers in identifying restorers is restricted since they are all linked markers and not particular for the putative candidate genes underpinning R*f*3 or R*f*4. Many researchers have employed SSR markers associated with fertility restorer genes or gene-based functional markers to screen their breeding materials. However, the marker of choice differs between investigations. In the present investigation four SSR markers *viz.,* RM1, RM443, RM10313, RM10318 for R*f*3 and three markers *viz.,* RM171, RM258, RM228 for R*f*4 were used to confirm the presence of restorer genes in F_4 generation of the crosses CBSN 25/WRM 21-24 and CBSN 25/WRM 93-20 with 86 and 79 plants respectively. The list of families, the chosen plant from each family and their results of molecular markers screening for fertility restorer genes, R*f*3 and R*f*4 are given in **Table 2** and **Table 3.**

Validation of fertility restorer genes in the cross CBSN 25/WRM 21- 24: Four SSR markers were used to validate the presence of the *Rf3* gene. The marker RM1 is found on the long arm of chromosome 1 (5.1cM), and its amplified product size is 113 bp (Alavi *et al.,* 2009). RM1 amplification at 113 bp was observed in 62 plants out of 86 plants screened. Ahmadikhah and Karlov (2006) reported that RM1 is closely linked to the *Rf3* gene which can be used to identify the restorer gene. The marker RM443 resides on chromosome 1 at a genetic distance of 4.4cM, also used to determine the R*f*3 gene (Bazrkar *et al.,* 2008). The presence of R*f*3 was detected in all 86 plants of this cross by amplifying at 124 bp. Likewise plants from each family were screened with another SSR marker RM10313 linked to R*f*3 gene (Neeraja *et al.,* 2009) and 84 plants were found to have R*f*3 by amplifying at 188 bp. Revathi *et al.* (2013) reported that the SSR primers RM10313 associated with R*f*3 gene on chromosome 1 and RM6100 linked to the R*f*4 gene on chromosome 10 were 81% and 86% efficient in identifying restorer lines respectively and Thippeswamy *et al.* (2014) reported two DNA markers in combination (RM10313 and RM6100) showed 100% selection efficiency in identifying restorers. The SSR marker RM10318 situated on chromosome 1 (Shah *et al.,* 2012) indicated that 83 out of the 86 plants had R*f*3 gene amplified at 187 bp (**Fig.1a**). On the whole, 59 plants (49%) could possibly be selected as restorers based on marker banding for all four markers RM1, RM443, RM10313, and RM10318 analysed for R*f*3 gene.

Three SSR markers, RM171, RM258, and RM228 were used to validate the existence of R*f*4 gene. The marker RM171 occupies a position on the long arm of chromosome 10 and whose amplified product size was 340 bp (Nematzadeh and Kiani, 2010), which has been amplified in 81 plants at 340 bp (**Fig 1b**.). Another marker RM258 present on the long arm of chromosome 10 and has an amplicon size of 148 bp (Nematzadeh and Kiani, 2010). It amplified the R*f*4 gene in 42 plants at a size of 148 bp. Nematzadeh and Kiani (2010) reported similar findings and revealed R*f*4 molecular tagging using RM171 and RM258 markers. Marker RM228 has an amplicon size of 120 bp and located on the long arm of chromosome 10 (Ahmadikhah and Karlov, 2006). A similar kind of banding pattern was observed in 47 plants. The R*f*4 gene has been located using three markers namely, RM171, RM258 and RM228 and all three were amplified in 21 plants (18%) out of 86 confirming the probable presence of R*f*4 gene in this population. A total of 17 plants (14%) (3-13,4-6,5-11,7-14,18-24,20-11,21-2,22-12,33-11,36-21,39-24,42-8,63-3,64-9,65-6,66-17, 68-21) out of 86 plants screened of the F_4 generation of the cross CBSN 25/WRM 21-24 were found to be positive for both R*f*3 and R*f*4 genes and the spikelet fertility in those 17 plants found ranging from 77-95%. Similar findings has already been reported by Surender *et al.* (2021), identified 10 potential restorers with 100% efficiency based on molecular screening with SSR primers for both R*f*4 and R*f*3 genes and also Prasanna *et al.* (2022) reported 44 potential restorers (R*f*4 and R*f*3 present) with a hundred percentage efficiency through molecular screening.

Validation of fertility restorer genes in the cross CBSN 25/WRM 93-20: A total of 79 plants each from the families of F_4 population of the cross CBSN 25/WRM 93-20 were screened with the same set of markers that were used in the previous cross to confirm the presence of R*f*3 and R*f*4 restorer genes. The marker RM1 was amplified at 113 bp in 69 plants. Ahmadikhah and Karlov (2006) reported that RM1 is closely linked with R*f*3gene. Whereas all 79 plants were amplified for both markers RM443 and RM10313 (**Fig 1a**.) at 124 bp and 188 bp respectively. Kumar *et al.* (2017) found that iso-cytoplasmic restorer lines expressing solely R*f*4 genes had a high frequency, followed by lines carrying combination of R*f*3 and R*f*4 genes and concluded that R*f*3 had a synergistic effect on fertility restoration. Another marker RM10318 located on chromosome 1 (Shah *et al.,* 2012) had amplification at 187 bp for 77 plants. A total of 68 plants (39%) were observed bands for all the four molecular markers employed namely RM1, RM443, RM10313, RM10318 which confirms the probable existence of the R*f*3 gene in these plants.

Table 2. Molecular marker screening of 86 F_4 families of CBSN 25/WRM 21-24 for fertility restorer genes R*f*3 and R*f*4

S. No	CBSN 25 / WRM 21-24 F_4 families	Plant number	Marker scoring (R*f*3)					Marker scoring (R*f*4)				Rf3 & Rf4
			RM 1	RM 443	RM 10313	RM 10318	TOTAL	RM 171	RM 258	RM 228	TOTAL	
1	1	1-7	1	1	1	1	1	1	1	0	0	0
2	2	2-9	1	1	0	1	0	1	1	0	0	0
3	3	3-13	1	1	1	1	1	1	1	1	1	1
4	4	4-6	1	1	1	1	1	1	1	1	1	1
5	5	5-11	1	1	1	1	1	1	1	1	1	1
6	6	6-20	1	1	1	1	1	0	0	1	0	0
7	7	7-14	1	1	1	1	1	1	1	1	1	1
8	8	8-10	1	1	1	1	1	1	0	1	0	0
9	9	9-3	1	1	1	1	1	0	1	1	0	0
10	10	10-5	1	1	1	1	1	1	0	1	0	0
11	11	11-9	1	1	1	1	1	1	0	0	0	0
12	12	12-6	1	1	1	1	1	1	0	0	0	0
13	13	13-19	1	1	1	1	1	1	0	1	0	0
14	14	14-6	1	1	1	1	1	1	0	1	0	0
15	15	15-1	1	1	1	1	1	1	0	1	0	0
16	16	16-4	1	1	1	1	1	1	0	1	0	0
17	17	17-12	1	1	1	1	1	1	0	1	0	0
18	18	18-24	1	1	1	1	1	1	1	1	1	1
19	19	19-19	1	1	1	1	1	1	1	0	0	0
20	20	20-11	1	1	1	1	1	1	1	1	1	1
21	21	21-2	1	1	1	1	1	1	1	1	1	1
22	22	22-12	1	1	1	1	1	1	1	1	1	1
23	23	23-9	1	1	1	1	1	1	0	0	0	0
24	24	24-4	0	1	1	1	0	1	0	0	0	0
25	25	25-18	0	1	0	1	0	1	1	0	0	0
26	26	26-3	0	1	1	0	0	1	1	0	0	0
27	27	27-8	1	1	1	1	1	1	1	0	0	0
28	28	28-17	1	1	1	1	1	1	1	0	0	0
29	29	29-10	1	1	1	1	1	1	1	0	0	0
30	30	30-18	1	1	1	1	1	1	0	0	0	0
31	31	31-11	1	1	1	1	1	1	1	0	0	0
32	32	32-7	1	1	1	1	1	1	1	0	0	0
33	33	33-11	1	1	1	1	1	1	1	1	1	1
34	34	34-9	1	1	1	1	1	0	1	1	0	0
35	35	35-1	0	1	1	1	0	1	1	1	1	0
36	36	36-21	1	1	1	1	1	1	1	1	1	1
37	37	37-13	0	1	1	1	0	1	0	1	0	0
38	38	38-66	1	1	1	1	1	1	0	1	0	0
39	39	39-24	1	1	1	1	1	1	1	1	1	1
40	40	40-15	1	1	1	1	1	1	1	0	0	0
41	41	41-8	1	1	1	1	1	1	0	1	0	0
42	42	42-8	1	1	1	1	1	1	1	1	1	1
43	43	43-16	1	1	1	0	0	1	0	1	0	0

Table 2. Continued..

S. No	CBSN 25 / WRM 21-24 F_4 families	Plant number	Marker scoring (R*f*3)					Marker scoring (R*f*4)				Rf3 & Rf4
			RM 1	RM 443	RM 10313	RM 10318	TOTAL	RM 171	RM 258	RM 228	TOTAL	
44	44	44-19	0	1	1	1	0	1	0	1	0	0
45	45	45-22	0	1	1	1	0	1	0	1	0	0
46	46	46-10	0	1	1	1	0	1	1	1	1	0
47	47	47-14	0	1	1	1	0	1	0	0	0	0
48	48	48-6	0	1	1	1	0	1	0	0	0	0
49	49	49-2	0	1	1	1	0	1	0	0	0	0
50	50	50-9	0	1	1	1	0	1	0	0	0	0
51	51	51-23	0	1	1	1	0	1	0	0	0	0
52	52	52-12	0	1	1	1	0	1	0	0	0	0
53	53	53-17	0	1	1	1	0	1	0	0	0	0
54	54	54-12	0	1	1	1	0	1	0	0	0	0
55	55	55-8	0	1	1	1	0	1	1	1	1	0
56	56	56-3	0	1	1	1	0	1	0	0	0	0
57	57	57-9	0	1	1	1	0	1	0	1	0	0
58	58	58-20	0	1	1	1	0	1	0	0	0	0
59	59	59-14	0	1	1	1	0	1	0	0	0	0
60	60	60-8	0	1	1	1	0	1	0	0	0	0
61	61	61-5	0	1	1	1	0	0	1	1	0	0
62	62	62-9	0	1	1	1	0	1	0	1	0	0
63	63	63-3	1	1	1	1	1	1	1	1	1	1
64	64	64-9	1	1	1	1	1	1	1	1	1	1
65	65	65-6	1	1	1	1	1	1	1	1	1	1
66	66	66-17	1	1	1	1	1	1	1	1	1	1
67	67	67-13	1	1	1	0	0	1	1	1	1	0
68	68	68-21	1	1	1	1	1	1	1	1	1	1
69	69	69-11	1	1	1	1	1	1	1	0	0	0
70	70	70-8	1	1	1	1	1	1	1	0	0	0
71	71	71-3	1	1	1	1	1	0	0	0	0	0
72	72	72-19	1	1	1	1	1	1	0	0	0	0
73	73	73-4	1	1	1	1	1	1	1	0	0	0
74	74	74-17	1	1	1	1	1	1	1	0	0	0
75	75	75-22	1	1	1	1	1	1	1	0	0	0
76	76	76-5	1	1	1	1	1	1	0	1	0	0
77	77	77-9	1	1	1	1	1	1	0	1	0	0
78	78	78-1	1	1	1	1	1	1	0	1	0	0
79	79	79-25	1	1	1	1	1	1	0	1	0	0
80	80	80-5	1	1	1	1	1	1	0	1	0	0
81	81	81-3	1	1	1	1	1	1	0	1	0	0
82	82	82-14	1	1	1	1	1	1	0	1	0	0
83	83	83-9	1	1	1	1	1	1	1	0	0	0
84	84	84-3	1	1	1	1	1	1	1	0	0	0
85	85	85-2	1	1	1	1	1	1	0	0	0	0
86	86	86-11	1	1	1	1	1	1	0	0	0	0

1 -Indicates amplification product at respective bp for each marker.

0 -Indicates amplification absent.

In this cross the plants with both R*f* genes had the spikelet fertility ranging from 77 – 95% i.e fertile to highly fertile

Table 3. Molecular marker screening of 79 F_4 families of CBSN 25/WRM 93-20 for fertility restorer genes R*f*3 and R*f*4

S. No	CBSN 25 / WRM 93-20 F_4 families	Plant number	Marker scoring (R*f*3)					Marker scoring (R*f*4)				R*f*3 & R*f*4
			RM1	RM443	RM10313	RM10318	TOTAL	RM171	RM258	RM228	TOTAL	
1	1	1-6	0	1	1	1	0	0	0	1	0	0
2	2	2-10	1	1	1	1	1	1	0	1	0	0
3	3	3-7	0	1	1	1	0	1	1	1	1	0
4	4	4-18	0	1	1	1	0	1	1	1	1	0
5	5	5-7	1	1	1	1	1	1	1	0	0	0
6	6	6-23	1	1	1	1	1	0	1	1	0	0
7	7	7-18	1	1	1	1	1	1	1	1	1	1
8	8	8-16	1	1	1	1	1	1	1	1	1	1
9	9	9-7	1	1	1	1	1	1	1	1	1	1
10	10	10-2	1	1	1	1	1	1	1	1	1	1
11	11	11-17	1	1	1	1	1	1	1	1	1	1
12	12	12-6	1	1	1	1	1	1	1	1	1	1
13	13	13-18	1	1	1	1	1	1	1	1	1	1
14	14	14-3	1	1	1	1	1	0	1	1	0	0
15	15	15-8	1	1	1	1	1	1	1	1	1	1
16	16	16-7	1	1	1	1	1	1	0	1	0	0
17	17	17-11	1	1	1	0	0	1	1	1	1	0
18	18	18-7	0	1	1	1	0	1	1	1	1	0
19	19	19-24	1	1	1	1	1	1	1	1	1	1
20	20	20-20	1	1	1	1	1	1	1	1	1	1
21	21	21-7	1	1	1	1	1	1	1	1	1	1
22	22	22-9	1	1	1	1	1	1	1	1	1	1
23	23	23-5	1	1	1	1	1	1	1	1	1	1
24	24	24-1	1	1	1	1	1	1	1	1	1	1
25	25	25-15	1	1	1	1	1	1	1	1	1	1
26	26	26-8	1	1	1	1	1	1	1	1	1	1
27	27	27-11	1	1	1	1	1	1	1	1	1	1
28	28	28-8	1	1	1	1	1	0	1	1	0	0
29	29	29-7	1	1	1	1	1	0	1	1	0	0
30	30	30-1	1	1	1	1	1	1	0	1	0	0
31	31	31-18	0	1	1	1	0	1	0	1	0	0
32	32	32-9	1	1	1	1	1	1	1	1	1	1
33	33	33-22	0	1	1	1	0	1	1	1	1	0
34	34	34-20	1	1	1	1	1	1	1	0	0	0
35	35	35-11	1	1	1	1	1	1	1	1	1	1
36	36	36-16	1	1	1	1	1	1	1	1	1	1
37	37	37-9	1	1	1	1	1	1	1	1	1	1
38	38	38-2	1	1	1	1	1	0	1	1	0	0
39	39	39-7	1	1	1	1	1	0	1	1	0	0
40	40	40-17	0	1	1	1	0	1	1	1	1	0
41	41	41-10	0	1	1	1	0	1	1	1	1	0
42	42	42-2	0	1	1	0	0	1	1	1	1	0
43	43	43-8	1	1	1	1	1	1	1	1	1	1

Table 3. Continued.

S. No	CBSN 25 / WRM 93-20 F_4 families	Plant number	Marker scoring (*Rf*3)					Marker scoring (R*f*4)				R*f*3 & R*f*4
			RM1	RM443	RM10313	RM10318	TOTAL	RM171	RM258	RM228	TOTAL	
44	44	44-11	1	1	1	1	1	1	1	1	1	1
45	45	45-6	1	1	1	1	1	1	1	1	1	1
46	46	46-10	1	1	1	1	1	1	1	1	1	1
47	47	47-4	1	1	1	1	1	1	1	1	1	1
48	48	48-17	1	1	1	1	1	1	1	1	1	1
49	49	49-3	1	1	1	1	1	1	1	1	1	1
50	50	50-9	0	1	1	1	0	1	1	1	1	0
51	51	51-7	1	1	1	1	1	1	1	1	1	1
52	52	52-16	1	1	1	1	1	1	1	1	1	1
53	53	53-7	1	1	1	1	1	1	1	1	1	1
54	54	54-2	1	1	1	1	1	1	0	0	0	0
55	55	55-17	1	1	1	1	1	1	0	1	0	0
56	56	56-8	1	1	1	1	1	1	0	1	0	0
57	57	57-9	1	1	1	1	1	1	0	1	0	0
58	58	58-17	1	1	1	1	1	0	0	1	0	0
59	59	59-4	1	1	1	1	1	1	0	1	0	0
60	60	60-11	1	1	1	1	1	1	0	1	0	0
61	61	61-8	1	1	1	1	1	1	0	1	0	0
62	62	62-10	1	1	1	1	1	1	0	1	0	0
63	63	63-3	1	1	1	1	1	1	0	1	0	0
64	64	64-21	1	1	1	1	1	1	1	1	1	1
65	65	65-25	1	1	1	1	1	1	1	1	1	1
66	66	66-15	1	1	1	1	1	1	1	1	1	1
67	67	67-8	1	1	1	1	1	1	1	1	1	1
68	68	68-17	1	1	1	1	1	1	1	1	1	1
69	69	69-8	1	1	1	1	1	1	1	1	1	1
70	70	70-11	1	1	1	1	1	1	1	1	1	1
71	71	71-3	1	1	1	1	1	1	1	1	1	1
72	72	72-9	1	1	1	1	1	1	1	1	1	1
73	73	73-17	1	1	1	1	1	1	1	1	1	1
74	74	74-19	1	1	1	1	1	1	1	1	1	1
75	75	75-6	1	1	1	1	1	1	1	1	1	1
76	76	76-2	1	1	1	1	1	1	1	1	1	1
77	77	77-15	1	1	1	1	1	1	1	1	1	1
78	78	78-8	1	1	1	1	1	1	1	1	1	1
79	79	79-4	1	1	1	1	1	1	1	1	1	1

1 -Indicates amplification product at respective bp for each marker

0 -Iindicates amplification absent

In this cross the plants with both R*f* genes had the spikelet fertility ranging from 82 – 97% i.e fertile to highly fertile

Three SSR markers namely RM171, RM258 and RM228 were utilized to examine the existence of the R*f*4 gene. The marker RM171 amplified in 71 plants, while marker RM258 amplified in 64 plants at 340 bp and 148 bp respectively. Nematzadeh and Kiani (2010) identified molecular tagging with markers RM171 and RM258 for R*f*4. Whereas the marker RM228 was amplified for 76 plants at 120 bp (**Fig 1b**.). All three of the markers *viz.*, RM171, RM258 and RM228 were amplified in 56 (33%) of the 79 plants screened which indicates the presence

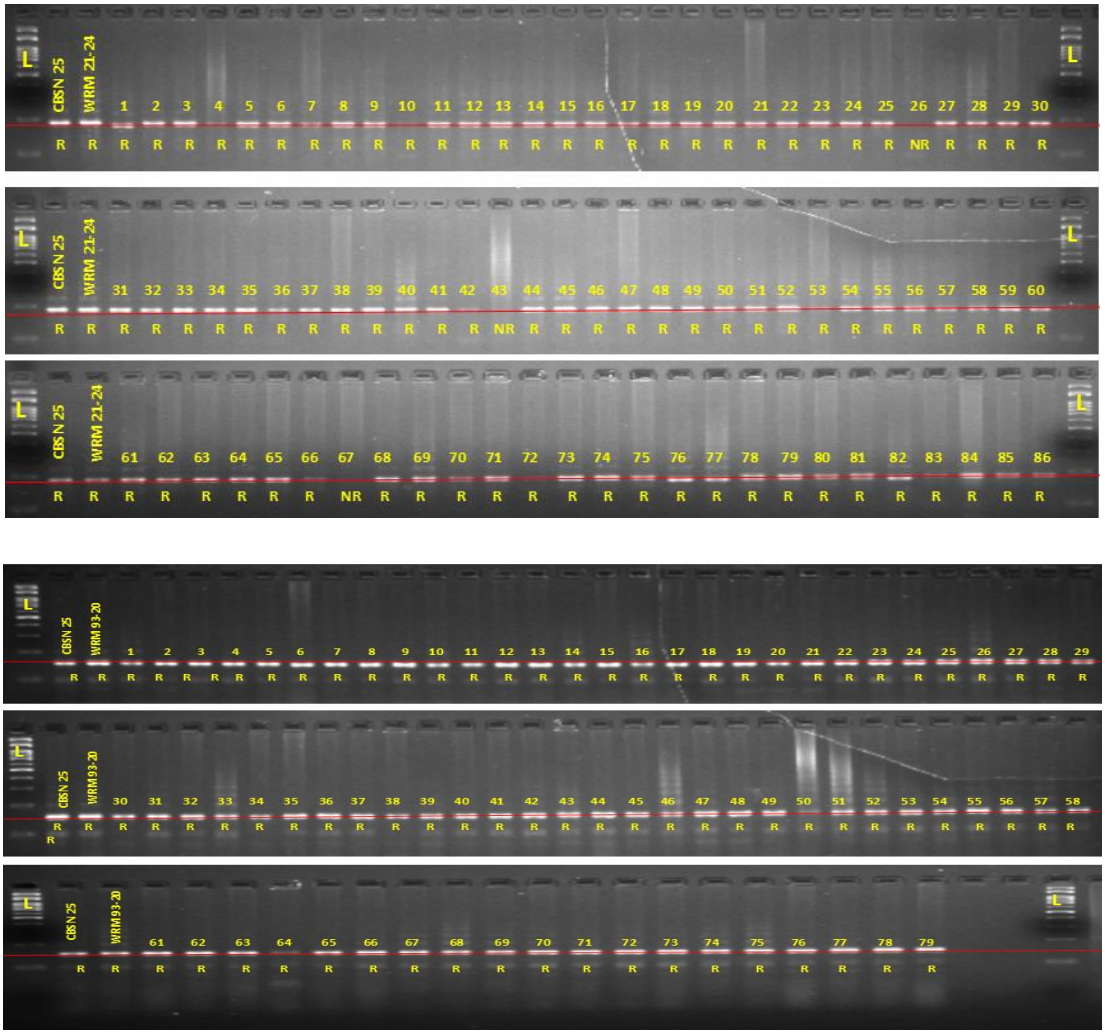

Fig. 1a. RM10318 marker amplification pattern of 86 F_4 families (1-86) of CBSN 25/WRM 21-24 at 187 bp and RM10313 marker amplification pattern of 79 F_4 families (1-79) of CBSN 25/WRM 93-20 at 188 bp. (L represents 100 bp ladder)

of the R*f*4 gene. A total of 47 plants (27%) (7-18,8-16,9-7,10-2,11-17,12-6,13-18,15-8,19-24,20-20,21-7,22-9,23-5,24-1,25-15,26-8,27-11,32-9,35-11,36-16,37-9,43-8,44-11,45-6,46-10,47-4,48-17,49-3,51-7,52-16,53-7,64-21, 65-25, 66-15,67-8,68 -17,69-8,70-11,71-3,72-9,73-17,74-19,75-6,76-2,77-15,78-8,79-4) out of 79 plants chosen from F_4 generation of the cross CBSN 25/WRM 93-20 were found double positives for R*f*3 and R*f*4 genes and these 47 plants had spikelet fertility ranging from 82-97%. This is in accordance with the results of double positives for R*f*3 and R*f*4 genes by Venkanna *et al.* (2022) and suggested that these genotypes may be employed as male parents or restorers in CMS-based hybrid rice breeding programmes. The results obtained on molecular screening are represented in **Fig .2a and Fig .2b.**

In hybrid rice breeding programs, restorer lines with both R*f*3 and R*f*4 fertility restorer genes would be highly beneficial. In the present investigation, a total of seven SSR markers namely RM1, RM443, RM10313, RM10318, RM171, RM258 and RM228 were used to identify the presence of R*f*3 and R*f*4 genes. Seventeen plants (3-13,4-6,5-11,7-14,18-24,20-11,21-2,22-12,33-11,36-21,39-24,42-8,63-3,64-9,65-6,66-17,68-21) among 86 plants screened from the cross CBSN 25/WRM 21-24 were found to be double positives for R*f*3 and R*f*4 genes and the spikelet fertility in these plants ranged from 77-95 % *i.e*. fertile to highly fertile. While, 47 plants (7-18,8-16,9-7,10-2,11-17,12-6,13-18,15-8,19-24,20-20,21-7,22-9,23-5,24-1,25-15,26-8,27-11,32-9,35-11,36-16,37-9,43-8,44-11,45-6,46-10,47-4, 48-17,49-3,51-7,52-16,53-7,64-21,65-25,66-15,67-8,68-17,69-8,70-11,71-3,72-9,73-17,74-19,75-6, 76-2,77-15,78-8,79-4) out of 79 plants screened from the cross CBSN 25/WRM 93-20 of the F_4 generation were found to be double positives for R*f*3 and R*f*4 genes. The range of spikelet fertility for

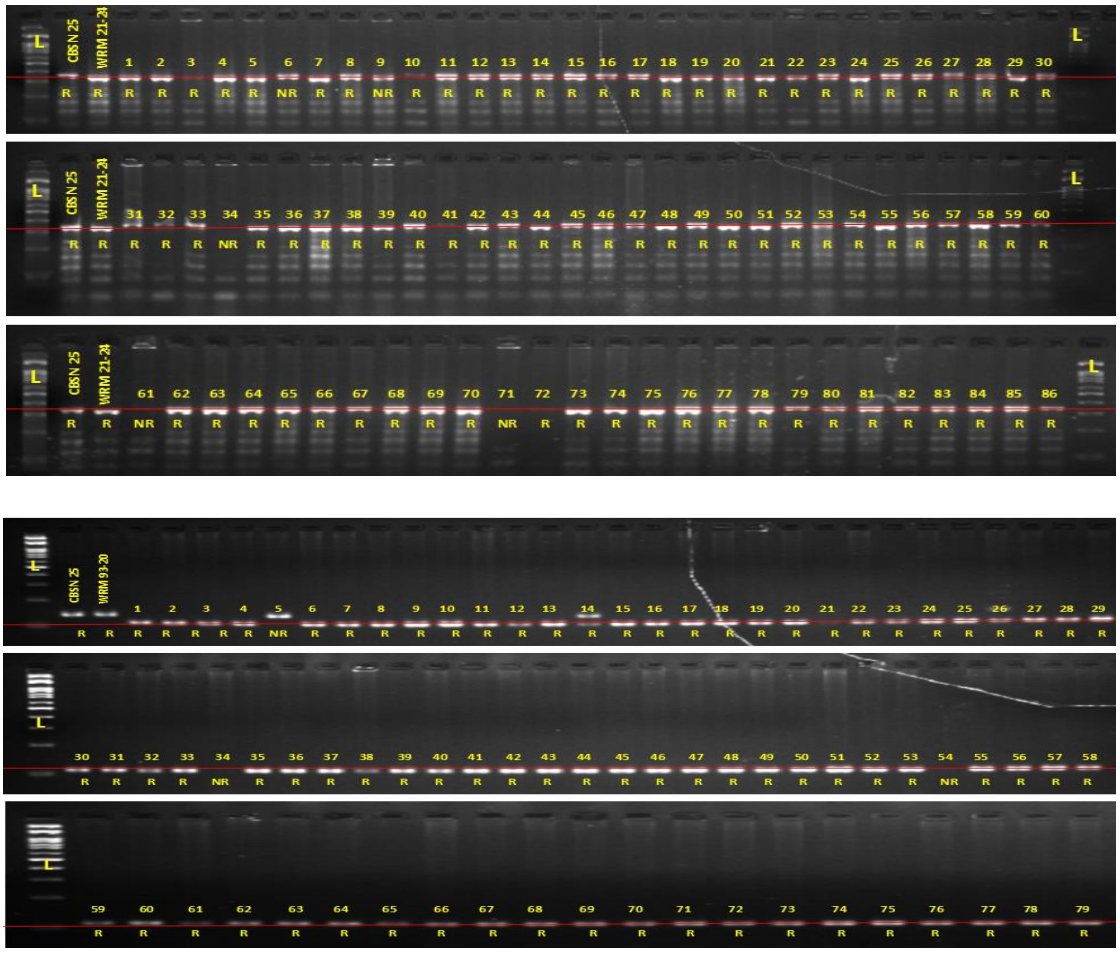

Fig. 1b. RM171 marker amplification pattern of 86 F_4 families (1-86) of CBSN 25/WRM 21-24 at 340 bp and RM228 marker amplification pattern of 79 F_4 families (1-79) of CBSN 25/WRM 93-20 at 120 bp (L represents 100 bp ladder)

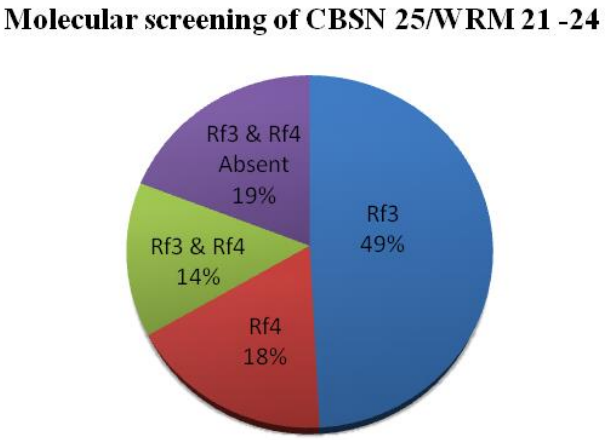

Fig. 2a.Percentage distribution of R*f* genes in 86 plants (from 86 families) of the cross CBSN 25/WRM 21-24 of F_4 population

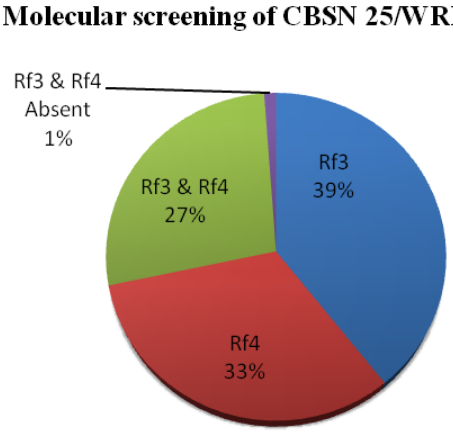

Fig. 2b. Percentage distribution of R*f*genes in 79 plants (from 79 families) of the cross CBSN 25/WRM 93-20 of F_4 population

these 47 plants possessing both R*f*3 and R*f*4 genes was from 82-97 % *i.e.* fertile to highly fertile. After stabilization, the plants identified for the presence of both R*f*3 and R*f*4 genes from the two crosses of the F_4 population can be used as male parents or restorer lines in CMS-based hybrid rice breeding programme to develop superior hybrid rice combinations.

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