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Identification of Suitable Restorers for WA-CMS Lines of Rice (*Oryza sativa* L.) through Conventional and Molecular Breeding Methods

Anirban Nath[1], Disharee Nath[1], Chand Kumar Santra[2], Tapash Dasgupta[3] 10.18805/ag.D-5089

ABSTRACT

The current study extensively evaluates 51 genotypes for their fertility restoration potential using test crosses with five WA (wild abortive) Cytoplasmic Male Sterile lines namely IR58025A, IR6897A, IR79156A, IR80559A and APMS6A. Also the genotypes were screened using SSR markers RM6100 and RM10313, tightly linked with the fertility restorer genes *Rf*4 and *Rf*3 respectively. The two way approach helped in identifying potential restorers for five WA-CMS lines and also detected the presence of dominant *Rf* genes in their genetic background. The R-lines identified can be safely presumed to be strong restorers for consecutive A-lines they were crossed with. The study also identified a potential maintainer CN1039-9 for the A-line IR58025A. The maintainer line identified can be later exploited for developing new CMS lines.

Key words: Marker assisted selection, Restorer, Testcross, WA-CMS.

INTRODUCTION

The three line system of hybrid rice development using the WA (Wild Abortive) cytoplasmic male sterility has been considered as one of the most effective method of generating heterotic hybrid combinations. However, identification of suitable restorer lines still remains a challenge for the system. The traditional process of identifying restorer lines involve 'test crossing' of random genotypes with available A-lines followed by evaluation of F_1 hybrids in the consecutive seasons. According to Virmani (1997), lines restoring spikelet fertility up to 80% and higher can be designated as a potential restorer for the corresponding CMS lines. Although, the traditional process of test crossing has been widely used, yet the method can be quite tedious owing to the time, labour and resources required for the program. Moreover, the trait spikelet fertility percentage which is used as a parameter for identifying potential restorer lines can be highly influenced by environmental factors that may mask the true restoration potentials of the R-lines being tested. The modern approach for restorer identification involves the utilization of tightly linked molecular markers for fertility restorer genes *Rf4* and *Rf3*. Studies related to the nature of gene action concerning the fertility restorer genes suggested different interactions like recessive epistasis (Govindaraj and Virmani, 1988), semi-epistasis (Pradhan and Jachuck, 1999), epistatic gene interaction with incomplete dominance (Sarkar *et al.* 2002), etc. On the contrary, researchers like Young and Virmani, (1984) and Virmani *et al.* (1986) suggested that fertility restoration is predominantly governed by two independently expressed dominant genes. The two fertility restorer genes were finally mapped and designated as *Rf4* and *Rf3* located on chromosome 10 and 1 respectively by the investigations of Yao *et al.* (1997), Zhang *et al.* (1997), Ahmadikhah and Karlov (2006), Ahmadikhah and Alavi (2009), etc. The mapping of the fertility restorer genes helped in identifying tightly linked markers, among which the SSR primers RM6100 and RM10313 have been widely utilized for the Marker assisted selections of restorer lines in hybrid rice breeding programs. Singh *et al*. (2005) reported that the SSR marker RM6100 is tightly linked to the *Rf4* gene being located at a distance of 1.2cM on the long arm of chromosome 10. Whereas the marker RM10313 has been reported to be linked to the *Rf3* gene identified by Neeraja (2008) at a distance of 4.2 cM from the gene located on the short arm of chromosome 1. Sheeba *et al*. (2009) reported that RM6100 was significantly associated with the trait spikelet fertility % with selection accuracy of 94.87%. Singh *et al*. (2005) reported that the selection accuracy of

[1]Department of Genetics and Plant Breeding, Institute of Agricultural Science, University of Calcutta. 51/2, Hazra Road, Kolkata-700 019, West Bengal, India.
[2]Rice Research Station, Govt. of West Bengal, Chinsurah-712 102, West Bengal, India.
[3]School of Agriculture and Rural Development, Ramakrishna Mission Vivekananda University, Narendrapur, Kolkata-700 103, West Bengal, India.

Corresponding Author: Anirban Nath, Department of Genetics and Plant Breeding, Institute of Agricultural Science, University of Calcutta. 51/2, Hazra Road, Kolkata-700 019, West Bengal, India. Email: anirbannathkol@gmail.com

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the marker was 97%. In the case of RM10313, the marker showed a selection accuracy of 80 to 85% in the works of Prasad *et al*. (2017). Both the markers in combination have been used for the identification of restorer lines by Singh *et al*. (2014). Although the marker assisted selection of restorer lines is a less tedious option compared to test crosses, yet deviations from the expected results are often observed due to the influence of environmental interactions coupled with the sterile cytoplasm of the A-lines as indicated by Virmani and Edwards (1983). On the background of the above mentioned facts, the current analysis identifies potential restorers for five WA-CMS lines using a combination of molecular marker assisted approach along with the traditional test crosses. A combination of the two approaches can take into account both the genotypic and phenotypic facets associated with the restoration potential of the genotypes being investigated.

MATERIALS AND METHODS

For test crossing, five WA-CMS lines (Table 1A) along with 51 genotypes to be tested (Table 1B) were staggered sown on the second week of June 2015 at the Rice Research Station, Chinsurah, West Bengal, India. Twenty one days old seedlings were transplanted at the rate of single seedling/ hill. Test crosses were performed and F_1 hybrids were evaluated in the following *Aman* season. Spikelet fertility % of the F_1 hybrids were calculated using the formula:

$$\text{Spikelet fertility \%} = \frac{\text{number of fertile spikelet}}{\text{Total number of spikelet}} \times 100$$

As per Virmani, (1997) F_1 hybrids with ≥80% spikelet fertility were designated as restorers. Hybrids which exhibited whitish colour of anthers and pollens were separately evaluated. Pollens from the suspected F_1s were observed using IKI (Iodine potassium iodide) solution as stain. Test crosses exhibiting 99% or above pollen sterility% were considered to be maintainer reactions. Pollen sterility% was calculated using the formula:

$$\text{Pollen sterility \%} = \frac{\text{number of sterile pollens}}{\text{Total number of pollens}} \times 100$$

Marker assisted screening of genotypes for the detection of fertility restorer genes *Rf4* and *Rf3*.

DNA isolation

Fresh Leaf samples from 51 genotypes were collected and leaves were grounded using liquid nitrogen. The powdered leaf samples were allowed to thaw in 2µL Eppendorf tubes and DNA was extracted using PROMEGA Plant DNA Minikit.

PCR and gel electrophoresis

A reaction solution of 25 µl comprising of Primers (Table 2), dNTPs, reaction buffer, $MgCl_2$ and TaqDNA polymerase were added to the template DNA. Amplification was performed using Eppendorf (Germany) thermo cycler. According to the cycle profile initial denaturation was performed at 94°C for 5 minutes, followed by 36 cycles of 1 minute with a denaturation at 94°C. Next, 2 minutes of annealing was done at temperatures varying according to the specific primer profile. Finally 2 minute extension followed by another 5 minute extension at 72°C was allowed. PCR products were subjected to electrophoresis in 3% Agarose gel using 1X TAE buffer at 120 volts. DNA bands were visualized under UV light using the Gel Documentation Unit (UVP, UK). Banding patterns recorded using the two markers are discussed in Fig 1 and 2.

RESULTS AND DISCUSSION

The test crosses conducted between five WA-CMS lines and fifty-one genotypes helped in identifying potential R-lines (Table 3) that can be used in future breeding programs aimed at developing heterotic hybrid rice. Test crosses with IR58025A showed thirty-two fertile reactions among which nineteen crosses exhibited 90% or above spikelet fertility. A possible maintainer reaction was identified in a cross with CN1039-9 (Table 5). The top five crosses with IR58025A, exhibiting maximum spikelet fertility in the hybrids included IR58025A x KMR3 (96.3), IR58025A x CNR 47 (94.78), IR58025A x IR 40750R (94.5), IR58025A x IR 71604-4-1-4-4-4-2-2-2R (94.3) and IR58025A x CNR 45 (94.28). Similar results involving IR58025A as the female parent were reported earlier by Kumar *et al*. (2017). Among the test crosses with IR69897A, thirty fertile reactions were observed with fifteen crosses exhibiting spikelet fertility of 90% and above. The top five best performing crosses in terms of spikelet fertility % included IR69897A x KMR 3 (95.46), IR69897A x CNR 55-3 (94.63), IR69897A x CNR 55-15 (94.48), IR69897A x CNR 93 (94.28) and IR69897A x BR 1356 Sel 2R (94.03). Test crosses with IR79156A revealed twenty-five restorer reactions, among which fourteen crosses exhibited 90% or above spikelet fertility. The top five best performing crosses with the highest spikelet fertility % included IR79156A x CNR 45 (96.21), IR79156A x DR 714-1-2R (96.1), IR79156A x CNR 55-6 (94.81), IR79156A x CNR 47 (94.31) and IR79156A x CNR 33 (93.71). Kumar *et al.* (2017) and Ponnuswamy *et al.* (2020) earlier reported strong fertility restoration (with 90% and above spikelet fertility) in the crosses involving IR79156A as the female parent. Test crosses with IR80559A revealed twenty-seven fertile reactions among which nine reactions exhibited spikelet fertility of ≥90%. The top five crosses exhibiting highest spikelet fertility included IR80559A x CNR 93 (96.85), IR80559A x NDR 97(93.05), IR80559A x CNR 98 (92.15), IR80559A x BR 827-35 (91.55) and IR80559A x MTU 9992 (91.49). Test crosses performed with APMS6A were evaluated. Twenty four fertile reactions were identified comprising of nine crosses that exhibited 90% or above spikelet fertility. The top five crosses with APMS6A showing maximum fertility restoration included crosses like APMS6A x CNR 33 (96.05), APMS6A x BR 1356 Sel 2R (94.2), APMS6A x CNR 45 (94.08), APMS6A x CNR 47 (93.98) and APMS6A x Ajaya (93.98). Spikelet fertility of ≥90 per cent in

test crosses using APMS6A as the female parent has been earlier reported by Revathi *et al*. (2013).

Subsequently, the test crosses were followed by marker-assisted genotyping using SSRs linked to dominant *Rf* genes (Table 4). Based on the earlier reports of Singh *et al*. (2014) and Ramalingam *et al*. (2017), a 175 bp dominant allele for the gene *Rf4* was detected using the marker RM6100. Out of fifty-one genotypes screened, thirty-three exhibited the presence of a 175 bp restorer allele suggesting the presence of the dominant *Rf4* gene. In the case of *Rf3*, earlier investigations by Sing *et al.* (2014) and Thippani *et al.* (2017) indicated that the RM10313 marker

Table 1A and B: Lists of genotypes used for the current experiment.

Table 1A: WA-CMS lines used for test crossing.

A- Lines	Names of A- lines used	Origin
1.	IR58025A	International Rice Research Institute, Philippines
2.	IR69897A	International Rice Research Institute, Philippines
3.	IR79156A	International Rice Research Institute, Philippines
4.	IR80559A	International Rice Research Institute, Philippines
5.	APMS6A	Andhra Pradesh Rice Research Institute, Maruteru, India

Table 1B: Germplasms used for restorer line identification.

1	CNR 33	18	IR 6876-1	35	Sabarmati
2	CNR 45	19	BR 827-35	36	Divya
3	CNR 47	20	BR 1356	37	PNR519
4	CNR 55-3	21	BR 1356 Sel 2R	38	Bj-1
5	CNR 55-6	22	NDR 97	39	Rasi
6	CNR 55-10	23	MTU 9992	40	Basmati 370
7	CNR 55-15	24	Ajaya	41	Kasturi
8	CNR 57	25	DR 714-1-2R	42	Gajapati
9	CNR 77	26	Salivahana	43	Anjali
10	CNR 93	27	IR66	44	Kavya
11	CNR 98	28	Badshabhog	45	Mahalaxmi
12	CNR 102	29	Radhunipagal	46	Shahbhagidhan
13	KMR 3	30	Thakurbhog	47	IR64
14	IR 10198R	31	Akashyadhan	48	MTU1010
15	IR 40750R	32	Surekha	49	Puja
16	IR 71604-4-1-4-4-4-2-2-2R	33	Rajendra	50	CN 1039-9
17	IR 34686-1R	34	Vandana	51	PNR 546

Table 2: Details of two SSR markers used for screening of genotypes in terms of fertility restorer genes *Rf4* and *Rf3*.

Primer	Chr. no.	*Rf* gene	Forward Sequence	Reverse sequence
RM6100	10	*Rf 4*	TTCCCTGCAAGATTCTAGCTACACC	TGTTCGTCGACCAAGAACTCAGG
RM10313	1	*Rf 3*	ACTTACACAAGGCCGGGAAAGG	TGGTAGTGGTAACTCTACCGATGG

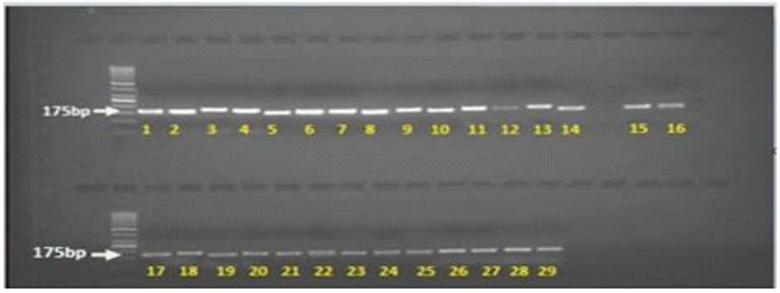

Fig 1: Amplification pattern of restorer lines tested with RM6100 (*Rf4*). Genotypes in lane numbers 1 to 29 corresponds to [1. CNR 33, 2. CNR 45, 3. CNR 47, 4. CNR 55-3, 5. CNR 55-6, 6. CNR 55-10, 7. CNR 55-15, 8. CNR 57, 9. CNR 77, 10. CNR 93, 11.CNR 98, 12.CNR 102,13.KMR 3, 14.IR 10198R, 15. IR 40750R, 16. IR 71604-4-1-4-4-4-2-2-2R,17. IR 34686-1R, 18. IR 6876-1, 19. BR 827-35, 20. BR 1356, 21. BR 1356 Sel 2R, 22. NDR 97, 23. MTU 9992, 24. Ajaya, 25. DR 714-1-2R, 26. Salivahana, 27. Kasturi, 28. Gajapati, 29. Anjali].

Table 3: Restorer lines identified from test crosses with five elite WA-CMS lines.

CMS	lines	Restorers identified with spikelet fertility% in F_1 ≥80% and ≥90%
IR58025A	≥90%	1) CNR 33, 2) CNR 45, 3) CNR 47, 4) CNR 55-3, 5) CNR 55-6, 6) CNR 55-10, 7) CNR 55-15, 8) CNR 57, 9) CNR 77, 10) CNR 93, 11) CNR 98,12) KMR 3, 13) IR 10198R, 14) IR 40750R, 15) IR 71604-4-1-4-4-4-2-2-2R, 16) IR 34686-1R, 17) BR 1356, 18) MTU 9992, 19) Ajaya.
	≥80%	1) CNR 102, 2) IR 6876-1, 3) BR 827-35, 4) BR 1356 Sel 2R, 5) NDR 97, 6)Salivahana, 7) IR66, 8) Vandana, 9) Bj-1, 10) Basmati 370, 11) Gajapati, 12) MTU1010, 13) Puja.
IR6897A	≥90%	1) CNR 33, 2) CNR 55-3, 3) CNR 55-10, 4) CNR 55-15, 5) CNR 57, 6) CNR 77, 8) CNR 93, 9) CNR 98, 10) KMR 3, 11) IR 10198R,12) BR 827-35, 13)BR 1356, 14) BR 1356 Sel 2R 15) MTU 9992,16) Ajaya.
	≥80%	1) CNR 45, 2) CNR 47, 3) CNR 102, 4) IR 40750R, 5) IR 71604-4-1-4-4-4-2-2-2R, 6) IR 34686-1R, 7) IR 6876-1, 8) NDR 97, 9) DR 714-1-2R, 10) Salivahana, 11) IR66, 12) Vandana, 13) Bj-1, 14) MTU1010, 15) Puja.
IR79156A	≥90%	1) CNR 33, 2) CNR 45, 3) CNR 47, 4) CNR 55-3, 5) CNR 55-6, 6) CNR 55-15, 7) CNR 57, 8) CNR 77, 9) IR 40750R, 10) IR 34686-1R, 11) IR 6876-1, 12) NDR 9723, 13) Ajaya, 14) DR 714-1-2R.
	≥80%	1) CNR 55-10, 2) CNR 93, 3) CNR 98, 4) CNR 102, 5) KMR 3, 6) IR 10198R, 7) IR 71604-4-1-4-4-4-2-2-2R, 8) BR 827-35, 9) BR 1356, 10) MTU 9992, 11) Bj-1.
IR80559A	≥90%	1) CNR 55-3, 2) CNR 77, 3) CNR 93, 4) CNR 98, 5) IR 10198R, 6) IR 71604-4-1-4-4-4-2-2-2R, 7) BR 827-35, 8) NDR 97, 9) MTU 9992.
	≥80%	1) CNR 45, 2) CNR 47, 3) CNR 102, 4) KMR 3, 5) IR 40750R, 6) IR 34686-1R, 7) IR 6876-1, 8) BR 1356, 9) BR 1356 Sel 2R, 10)Ajaya, 11) Akashyadhan, 12) Vandana, 13) Bj-1, 14) Gajapati.
APMS6A	≥90%	1) CNR 33, 2) CNR 45, 3) CNR 47, 4) CNR 55-10, 5) BR 1356, 6) BR 1356 Sel 2R, 7) NDR 97, 8) MTU 9992, 9) Ajaya.
	≥80%	1) CNR 55-3, 2) CNR 55-6, 3) CNR 55-15, 4) CNR 57, 5) CNR 77,6) CNR 93, 7) CNR 98, 8)CNR 102, 9) KMR 3,10) IR 10198R, 11) IR 40750R, 12) IR 71604-4-1-4-4-4-2-2-2R, 13) IR 6876-1, 14) BR 827-35, 15) DR 714-1-2R.

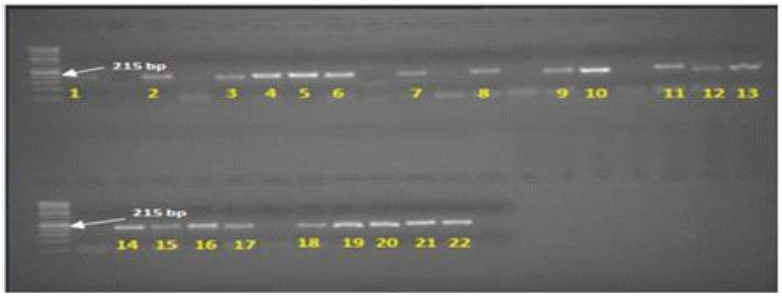

Fig 2: Amplification pattern of restorer lines tested with RM10313 (*Rf3*). Genotypes in lane numbers 1 to 22 corresponds to [1.CNR 33, 2.CNR 45, 3.CNR 47, 4.CNR 55-3, 5. CNR 55-6, 6. CNR 55-10, 7. CNR 55-15, 8. CNR 57, 9. CNR 7710. CNR 93, 11.CNR 98, 12.CNR 102, 13.KMR 3, 14.IR 10198R, 15. IR 71604-4-1-4-4-4-2-2-2R, 16. IR 34686-1R, 17. IR 6876-1, 18. BR 1356, 19. BR 1356 Sel 2R, 20. NDR 97, 21. MTU 9992, 22. Ajaya.].

amplifies a 215bp dominant restorer allele. In the current study, thirty of the 51 genotypes screened showed the presence of the dominant *Rf3* gene represented by the 215 bp allele. Overall the experiment revealed that 27 out of 51 genotypes exhibited the presence of dominant alleles for both the fertility restorer genes.

Based on the test crosses and the marker assisted screening, genotypes exhibiting 90% and above spikelet fertility along with the presence of dominant fertility restorer genes can be directly used for hybrid rice development. The experiment also identified a potential maintainer for IR58025A. The pollen sterility% in the F_1s obtained from the crosses with CN1039-9 was separately analyzed. The evaluation revealed that the genotype CN1039-9 produced a sterile reaction with all the A-lines except in crosses with IR80559A (Table 5). Highest pollen sterility % was observed in the cross IR58025A X CN 1039-9 (99.3%) suggesting that the genotype is a possible maintainer for IR58025A. Thus the genotype can be used as a recurrent parent in a CMS line development program using IR58025A.

In most of the cases, the results from the marker assisted screening of the genotypes complemented the results observed in the test crosses. Few exceptions were recorded in which the presence of dominant *Rf* genes did not show significant influence on the fertility restoration resulting in cases where partial restoration of spikelet fertility were observed. Few such examples included Akashyadhan and Anjali which possessed the dominant *Rf4Rf3* combination, yet the fertility restoration was partial among the test crosses. Likewise, in case of Kasturi

Table 4: Genotypic screening of fifty one varieties for determining presence or absence of desired alleles for the two fertility restorer genes *Rf4* and *Rf3*.

Genotypes	RM6100	RM10313	*Rf* gene combinations
CNR 33	Rf4	Rf3	Rf4/Rf3
CNR 45	Rf4	Rf3	Rf4/Rf3
CNR 47	Rf4	Rf3	Rf4/Rf3
CNR 55-3	Rf4	Rf3	Rf4/Rf3
CNR 55-6	Rf4	Rf3	Rf4/Rf3
CNR 55-10	Rf4	Rf3	Rf4/Rf3
CNR 55-15	Rf4	Rf3	Rf4/Rf3
CNR 57	Rf4	Rf3	Rf4/Rf3
CNR 77	Rf4	Rf3	Rf4/Rf3
CNR 93	Rf4	Rf3	Rf4/Rf3
CNR 98	Rf4	Rf3	Rf4/Rf3
CNR 102	Rf4	Rf3	Rf4/Rf3
KMR 3	Rf4	Rf3	Rf4/Rf3
IR 10198R	Rf4	Rf3	Rf4/Rf3
IR 40750R	Rf4	No	Rf4
IR 71604-4-1-4-4-4-2-2-2R	Rf4	Rf3	Rf4/Rf3
IR 34686-1R	Rf4	Rf3	Rf4/Rf3
IR 6876-1	Rf4	Rf3	Rf4/Rf3
BR 827-35	Rf4	No	Rf4
BR 1356	Rf4	Rf3	Rf4/Rf3
BR 1356 Sel 2R	Rf4	Rf3	Rf4/Rf3
NDR 97	Rf4	Rf3	Rf4/Rf3
MTU 9992	Rf4	Rf3	Rf4/Rf3
Ajaya	Rf4	Rf3	Rf4/Rf3
DR 714-1-2R	Rf4	No	Rf4
Salivahana	Rf4	Rf3	Rf4/Rf3
IR66	No	No	No
Badshabhog	No	No	No
Radhunipagal	No	No	No
Thakurbhog	No	No	No
Akashyadhan	Rf4	Rf3	Rf4/Rf3
Surekha	No	Rf3	Rf3
Rajendra	No	Rf3	Rf3
Vandana	Rf4	Rf3	Rf4/Rf3
Sabarmati	No	No	No
Divya	No	No	No
PNR519	No	No	No
Bj-1	Rf4	No	Rf4
Rasi	No	No	No
Basmati 370	Rf4	No	Rf4
Kasturi	Rf4	No	Rf4
Gajapati	Rf4	Rf3	Rf4/Rf3
Anjali	Rf4	Rf3	Rf4/Rf3
Kavya	No	No	No
Mahalaxmi	No	No	No
Shahbhagidhan	No	No	No
IR64	No	No	No
MTU1010	No	No	No
Puja	No	Rf3	Rf3
CN 1039-9	No	No	No
PNR 546	No	No	No

Table 5: Results of the test crosses involving CN 1039-9 and five A-lines.

Test cross	Pollen sterility %	Category
IR58025A X CN 1039-9*	99.3	STERILE
IR6897A X CN 1039-9	92.4	STERILE
IR79156A X CN 1039-9	95.6	STERILE
IR80559A X CN 1039-9	90.2	PARTIALLY STERILE/ STERILE
APMS6A X CN 1039-9	94.8	STERILE

*strong maintainer reaction.

dominant *Rf4* was detected, yet a partial fertility restoration was observed among the hybrids. Such infrequent cases where the presence of dominant *Rf* genes failed to produce the desired spikelet fertility in hybrids was previously reported by Singh *et al*. (2014). A plausible explanation for such deviation can be attributed to modifying factors (minor genes) which, according to Allard (1960) tends to diminish the full expression of major genes. The findings of the Marker Assisted Screening also suggest that, in most cases, the presence of dominant *Rf4* allele was necessary for high spikelet fertility (above 80 per cent). Presence of *Rf3* alone could only result in partial or poor restoration, as in the case of Rajendra and Surekha, which showed below 80 per cent spikelet fertility when test crossed with most of the A-lines. Thus, the observation is consistent with the theory that *Rf4* has a stronger influence on the restoration of spikelet fertility and its presence has a significant impact on the strength of the restorer line. Young and Virmani (1984); Virmani *et al.* (1986) suggested similar findings.

CONCLUSION

Thus the overall experiment discerned key restorer lines complementing five WA-CMS lines and the nature of *Rf* genes they possess. Germplasms with strong restoration potential along with dominant *Rf* genes identified from the current experiment can be safely presumed to be R-lines for the A-lines they were crossed with. These lines can be readily exploited for developing heterotic hybrid rice combinations. Weak or partial restorers cannot be readily used in hybrid rice development. Such lines can be further improved by pyramiding of strong fertility restorer genes by restorer x partial restorer crosses in future breeding programs.

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