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

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**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 *Rf4* and *Rf6* 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 F₁ 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), Ahmadikah and Karlov (2006), Ahmadikah 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
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 F₁ hybrids were evaluated in the following Aman season. Spikelet fertility % of the F₁ 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) F₁ hybrids with ≥80% spikelet fertility were designated as restorers. Hybrids which exhibited whitish colour of anthers and pollen were separately evaluated. Pollens from the suspected F₁s 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 ground 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, MgCl₂ 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 (UVF, 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 71604-4-1-4-4-2-2-2R (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 CNR 45 (96.1), IR79156A x IR 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 observed 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 (94.42), 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...
Identification of Suitable Restorers for WA-CMS Lines of Rice (*Oryza sativa* L.) through Conventional and Molecular... 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 Sing *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 Thippa *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 | B-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 71604-4-1-4-4-2-2-2R | 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       | *Rf4*     | TTTCTTGGCAAGATCTAGCTACACC | TGGTGTGCACCAAGAACTCAGG |
| RM10313| 1        | *Rf3*     | ACTTACACACAGGCCGAAAGG | TGGTAGTGGTAACTCAGCAGG |

**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 71604-4-1-4-4-2-2-2R, 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]
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### Table 3: Restorer lines identified from test crosses with five elite WA-CMS lines.

| CMS lines | Restorers identified with spikelet fertility% in F₁ ≥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, |
| IR6897A   | ≥80% 1) CNR 102, 2) IR 6876-1, 3) BR 827-35, 4) BR 1356 Sel 2R, 5) NDR 97, 6) Salivahana, 7) IR 66, 8) Vandana, 9) Bji-1, 10) Basmati 370, 11) Gajapati, 12) MTU1010, 13) 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) CNR 93, 10) KMR 3, 11) IR 10198R, 12) BR 827-35, 13) BR 1356 Sel 2R, 14) MTU 9992, 15) Ajaya |
| IR80559A  | ≥80% 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, 15) BR 1356 Sel 2R, 16) MTU 9992, 17) Bji-1 |
| APMS6A    | ≥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) CNR 93, 10) KMR 3, 11) IR 10198R, 12) IR 40750R, 13) IR 71604-4-1-4-4-4-2-2-2R, 14) IR 6876-1, 15) BR 1356 Sel 2R, 16) MTU 9992, 17) MTU 1010, 18) Ajaya |

**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 77, 10. CNR 93, 11. KMR 3, 12. IR 10198R, 13. IR 71604-4-1-4-4-4-2-2-2R, 14. IR 34686-1R, 15. IR 6876-1, 16. BR 827-35, 17. BR 1356, 18. MTU 9992, 19. IR 40750R, 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 215bp 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 F₁s 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 71604-1-4-1-4-1-2-2-2R | 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              |
| Aajya     | 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 |
| APMSS6A 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.

ACKNOWLEDGEMENT
The first author is immensely grateful to Rice Research Station, Chinsurah, Hooghly, West Bengal 712103 under Department of Agriculture, Govt. of West Bengal for providing all the infrastructural support for carrying out the experiments.

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