# Molecular Screening for Fertility Restorer Genes *Rf3* and *Rf4* of WA -CMS and Evaluation of $F_1$ hybrids in Rice (*O. sativa* L.)

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**Abstract**

**In rice WA (Wild Abortive) CMS system is commercially used for hybrid seed production. In WA-CMS fertility restoration is governed by two independent and dominant genes namely *Rf4* and *Rf3*. Conventionally, the process of screening for the trait of fertility restoration is by tedious testcross progeny evaluation. In this study, earlier reported SSR markers RM6100 and RM 10313 linked to *Rf4* and *Rf3,* respectively have been utilized to screen one hundred breeding lines and identified that 61 lines to carry both *Rf3* and *Rf4* genes and these lines can be utilized in hybrid rice breeding as restorers. A set of eighteen restorer lines with different combination of *Rf4* and *Rf3* were selected for crossing with five CMS lines *viz.,* APMS6A, Pusa 5A, IR58025, IR68897, IR79156 and IR68888 and seventy test cross progenies were evaluated for their fertility restoration based on pollen and spikelet fertility. The hybrids *viz.,* APMS6A X GQ-86, IR 79156A X IR-55778R, APMS 6A X VG-269 and IR 68888A X BR-827-35 were observed to have more than 90% spikelet fertility. In this study observed that restoration ability varied with different CMS lines hence CMS lines also playing major role achieving higher heterosis**.

**Key words**: Hybrid Rice, Molecular markers, Fertility restoration, *Rf4*, *Rf3*.

**R**ice is a staple food for more than half of the world's population. Hybrid rice have clearly shown a standard heterosis of 15–20% in commercial cultivation mainly in the *indica* genotypes (Hussain *et al.,* 2010). The magnitude of heterosis depends on the choice of appropriate parental lines. Rice being self pollinated crop, use of male sterility system is a prerequisite for commercial exploitation of heterosis in rice.

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The WA cytoplasm is the most widely used since it is a most stable system and the pollen sterility is almost nearly complete (Shinjyo and Omura 1966). Pollen abortion in WA-CMS is sporophytic, forming typical abortive pollen (Huang *et al*., 2003). CGMS system/ Three-line system has been widely used for developing rice hybrids. This system involves a CMS or 'A' line, a maintainer or 'B' line and a restorer or 'R' line. Since three lines are required for the production of a hybrid, this is popularly called as three line system. Cytoplasmic male sterility (CMS) is a maternally inherited trait that results in inability of the plant to produce fertile pollen. Pollen fertility is restored by nuclear-encoded genes called fertility restorer (*Rf)* gene. For developing high yielding heterotic hybrids, the first step is to identify restorers that can efficiently restore the fertility of CMS lines.

Earlier investigations confirmed that fertility restoration is governed by two independent dominant nuclear genes with one gene being stronger in action than the other (Young and Virmani 1984; Virmani *et al*., 1986). Different studies also indicated different types of gene interaction like recessive epistasis, (Govinda Raj and Virmani 1988) semi-epistasis (Pradhan and Jachuck 1999), epistasis with incomplete dominance (Govindaraj and Virmani 1988; Sarkar *et al*., 2002), epistasis with complete dominance (Sohu and Phul 1995) or no interaction (Li and Yuan 1986). Huang *et al*. (1986), Anandakumar and Subramaniam (1992) reported that a major dominant gene controls fertility restoration of WA-cytoplasm. However most of the genetic studies of fertility restoration for the WA CMS system have suggested that fertility restoration is governed by two genes namely *Rf4* and *Rf3* have been mapped to chromosomes 10 and 1 respectively (Yao *et al*., 1997; Zhang *et al.,* 1997; Ahmadikhah and Karlov 2006; Ahmadikhah and Alavi 2009). The use of molecular markers linked to *Rf* genes can enhance the selection efficiency, save time and avoid the complications associated with phenotype-based screening. The genetic linkage analysis indicated that the SSR markers RM6100 reported by Singh *et al.* (2005), on the long arm of chromosome 10, linked with the *Rf4* gene at distance of 1.2 cM and RM10313 reported by Neeraja (2008), on the short arm chromosome 1, linked with *Rf3* gene at a distance of 4.2 cM have been utilized to screen one hundred breeding lines for the identification of restorers. Among these breeding lines eighteen lines have been selected for test crossing to study the relative

role of *Rf3* and *Rf4* genes in fertility restoration of WA-CMS system.

## Materials and Methods

### Plant material

The leaf samples of one hundred breeding lines were collected from 15-20 days old seedlings grown at Directorate of Rice Research, Rajendranagar, Hyderabad, during early hours (8am to 9am) and stored at -20$^0$C for DNA isolation.

### Molecular analysis

DNA was isolated from young leaves by CTAB method reported by Dellaporta *et al*. (1983). With respect to the SSR markers, polymerase chain reaction was carried out using 15–20 ng of template DNA, 250 µM of dNTPs (Eppendorf, USA), 5 pmoles of each F and R primer, 1 unit of Taq DNA polymerase (Bangalore Genei, India), 1X PCR reaction buffer (Bangalore Genie, India) in a total volume of 10 µl. The cycling conditions were an initial denaturation at 94$^0$C for 5 min followed by 35 cycles of PCR amplification under the following parameters: 30 s at 94$^0$C, 30 s at 55$^0$C, and 1 min at 72$^0$C, followed by a final extension at 72$^0$C for 7 min. The sequences for the SSR primers are presented in (Table 1). Amplified PCR products were resolved in 3% agarose gel, stained with ethidium bromide and visualized under UV light using the Alpha Imager® 1220 gel documentation system (Alpha Innotech Corporation San Leandro, CA, USA).

**Spikelet fertility was calculated by:**

$$\text{Spikelet fertility}\,\% = \frac{\text{Number of fertile spikelets in the panicle}}{\text{Total number of spikelet in the panicle}} \text{ X } 100$$

## Results and Discussion

In rice, after the deployment of semi- dwarf varieties, hybrid rice technology has been the major strategy for raising further the ceiling of genetic yield. In hybrid seed production using three line system, the combination of a CMS line, a maintainer line and a restorer line carrying the fertility restorer gene (*Rf*) to restore fertility is indispensable for the development of hybrids (Virmani *et al*., 2003). Wild abortive (WA) type cytoplasmic male sterility (CMS) is commercially used for production of hybrid seeds in Asia.

### Screening for fertility restorer genes *Rf4* and *Rf3*

The one hundred breeding lines have been screened for the presence of fertility restorer gene *Rf4* (Table 2) located on chromosome 10, with the help of SSR marker RM6100

reported by Singh *et al.* (2005). Figure 1 shows the amplification pattern of *Rf4* gene. Out of one hundred, seventy lines showed the presence of *Rf4* by amplifying 175- bp size fragment and twenty three lines showed the absence of *Rf4* by amplifying 165-bp size and seven showed the heterozygous amplification pattern. Based on these results we can confirm that out of one hundred breeding lines seventy are restorers, twenty three are non- restorers and seven lines may be partial restorers. In same way breeding lines were screened with the help of SSR marker RM10313 linked to *Rf3* gene reported by Neeraja (2008). Out of one hundred screened, seventy seven showed the presence of *Rf3* by amplifying 215- bp size fragment and twenty three showed the absence by amplifying 200- bp product size (Figure 2). Based on molecular screening results we can assume that out of one hundred breeding lines, seventy seven are restorers, twenty three are non- restorers and the identified restorer lines could be effectively utilized in hybrid rice breeding program.

**Evaluation of rice hybrids**

To confirm the fertility restoration of identified restorer lines, eighteen lines with different combinations of *Rf4*, *Rf3* and without *Rf* genes were selected for test crossing with known five CMS lines (Table 3) and seventy $F_1$ hybrids were produced. Of the seventy hybrids with or without fertility restorer genes *Rf4* & *Rf3*, ten hybrids were identified to have more than 90% spikelet fertility. The results of $F_1$ spikelet fertility is presented in Table 4. The $F_1$ hybrids which are identified as restorer with high spikelet fertility (>90%) are APMS6A x VG 269, Pusa5A x BR-827-35, IR 79156 A x (IR55778R, KMR 3R and GQ 86) and partial restorers (< 70%) are IR 68897A x $KMR_3R$ and IR 79156A x IBL 57 and partial sterile (<50%) are IR 68897A x C-20R and IR 68897A x EPLT 109 and maintainers (<20%) are IR 68897 A x GQ 37-1 & Pusa 5A x BR 827-35. But presence or absence the *Rf* genes under study were not showing a significant influence on spikelet fertility of the $F_1$ hybrids. According to Govind *et al*. (1988) the fertility restoration is governed by two independent and dominant genes, and one of the genes appeared to be stronger in action than the other. Cai *et al.* (2013) studied allelic differentiations and effects of the *Rf3* and *Rf4* genes on fertility restoration in rice and explained allelic differences, interactions and background effects are influencing the fertility than presence of these genes. These

two fertility restorer genes are additive in their inheritance and the effect of *Rf4* appeared to be larger than that of *Rf3* (Yao *et al*. 1997, Zhuang *et al*. 2001, Sattari *et al*. 2008).

The mode of action of the two genes varied in different CMS/restorer combinations. The present study also confirms that mode of action of fertility restorer genes are different in different CMS/restorer combination. Although all the CMS lines derived from WA source, restorer lines performance varied with the different CMS lines hence CMS diversification may have direct influence on improving grain yield heterosis.

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**Table 1: Primer sequence**

| Name of the Primer | Gene tagged | Primer Sequence (5' - 3') | Amplification Product Size (bp) | AT(° C) |
|---|---|---|---|---|
| RM 6100 | *Rf4* | F:TTCCCTGCAAGATTCTAGCTACACC<br>R:TGTTCGTCGACCAAGAACTCAGG | 175 Restorer<br>165 Non Restorer | 55 |
| RM 10313 | *Rf3* | F: ACTTACACAAGGCCGGGAAAGG<br>R: TGGTAGTGGTAACTCTACCGATGG | 215 Restorer<br>200 Non restorer | 55 |

**Table 2: Screening results of *Rf4* and *Rf3***

| S. No. | Genotype | RM 6100 | RM 10313 | *Rf3&Rf4* |
|---|---|---|---|---|
| 1. | BCW-56 | *Rf4* | *Rf3* | *Rf3/Rf4* |
| 2. | EPLT-109 | *Rf4* | *Rf3* | *Rf3/Rf4* |
| 3. | EPLT-104 | *Rf4* | *Rf3* | *Rf3/Rf4* |
| 4. | RPHR-612-1 | *Rf4* | *Rf3* | *Rf3/Rf4* |
| 5. | RPHR-111-3 | *Rf4* | *Rf3* | *Rf3/Rf4* |
| 6. | RPHR-1096 | *Rf4* | No | *Rf4* |
| 7. | KMR-3 | *Rf4* | *Rf3* | *Rf3/Rf4* |
| 8. | RPHR-619-2 | *Rf4* | *Rf3* | *Rf3/Rf4* |
| 9. | RPHR-1009 | *Rf4* | *Rf3* | *Rf3/Rf4* |
| 10. | RPHR-1004 | H | *Rf3* | *Rf3* |
| 11. | RPHR-1005 | *Rf4* | *Rf3* | *Rf3/Rf4* |
| 12. | SC5 2-2-1 | No | *Rf3* | *Rf3* |
| 13. | GQ-37-1 | No | *Rf3* | *Rf3* |
| 14. | RPHR-611-1 | *Rf4* | *Rf3* | *Rf3/Rf4* |
| 15. | SALIVAHANA | *Rf4* | *Rf3* | *Rf3/Rf4* |
| 16. | RPHR-1124 | No | *Rf3* | *Rf3* |
| 17. | SC5 22-2-3-1 | No | *Rf3* | *Rf3* |
| 18. | GQ-102 | No | *Rf3* | *Rf3* |
| 19. | GQ-70 | *Rf4* | *Rf3* | *Rf3/Rf4* |
| 20. | GQ-58 | *Rf4* | *Rf3* | *Rf3/Rf4* |
| 21. | GQ-54 | *Rf4* | *Rf3* | *Rf3/Rf4* |
| 22. | RPHR-998 | *Rf4* | *Rf3* | *Rf3/Rf4* |

| | | | | |
|---|---|---|---|---|
| 23. | GQ-64-1 | *Rf4* | *Rf3* | *Rf3/Rf4* |
| 24. | IRCD 16-9-2-1 | *Rf4* | *Rf3* | *Rf3/Rf4* |
| 25. | IRCD 16-1-4-2-1 | *Rf4* | *Rf3* | *Rf3/Rf4* |
| 26. | DR 714-1-2R | *Rf4* | No | *Rf4* |
| 27. | RPHR-945-1-2 | No | *Rf3* | *Rf3* |
| 28. | SG22-289-3 | *Rf4* | *Rf3* | *Rf3/Rf4* |
| 29. | IBL-52-1 | No | *Rf3* | *Rf3* |
| 30. | VG-13 | No | No | No |
| 31. | VG-58 | *Rf4* | No | *Rf4* |
| 32. | VG-175 | No | No | No |
| 33. | VG-269 | No | No | No |
| 34. | VG-294 | No | No | No |
| 35. | IR-40750R | No | No | No |
| 36. | MTU-9992 | No | *Rf3* | *Rf3* |
| 37. | C-20R | *Rf4* | No | *Rf4* |
| 38. | UPRI-92-133 | *Rf4* | No | *Rf4* |
| 39. | BR-827-35 | No | No | No |
| 40. | IR-66 | *Rf4* | No | *Rf4* |
| 41. | NDR-3026 | *Rf4* | *Rf3* | *Rf3/Rf4* |
| 42. | AJAYA-R | *Rf4* | *Rf3* | *Rf3/Rf4* |
| 43. | PNR-3158 | No | No | No |
| 44. | SC5 9-3 | No | No | No |
| 45. | TCP-3699 | *Rf4* | *Rf3* | *Rf3/Rf4* |
| 46. | IR-55178R | *Rf4* | *Rf3* | *Rf3/Rf4* |
| 47. | SG-27-105 | *Rf4* | *Rf3* | *Rf3/Rf4* |
| 48. | SG-27-131 | *Rf4* | *Rf3* | *Rf3/Rf4* |
| 49. | SG-27-175 | *Rf4* | *Rf3* | *Rf3/Rf4* |
| 50. | SG-27-177 | *Rf4* | *Rf3* | *Rf3/Rf4* |
| 51. | RPHR-255 | *Rf4* | *Rf3* | *Rf3/Rf4* |
| 52. | IBL-57 | *Rf4* | *Rf3* | *Rf3/Rf4* |
| 53. | RPHR-517 | *Rf4* | *Rf3* | *Rf3/Rf4* |
| 54. | SG-17-118-3 | *Rf4* | *Rf3* | *Rf3/Rf4* |

| | | | | |
|---|---|---|---|---|
| 55. | RPHR-118 | *Rf4* | No | *Rf4* |
| 56. | GQ-25 | *Rf4* | *Rf3* | *Rf3/Rf4* |
| 57. | GQ-25-74 | *Rf4* | *Rf3* | *Rf3/Rf4* |
| 58. | RPHR-124 | *Rf4* | *Rf3* | *Rf3/Rf4* |
| 59. | SG-26-120 | *Rf4* | *Rf3* | *Rf3/Rf4* |
| 60. | SG-22-23-1 | *Rf4* | *Rf3* | *Rf3/Rf4* |
| 61. | NRI-38P2 | *Rf4* | *Rf3* | *Rf3/Rf4* |
| 62. | RPHR-972P1 | *Rf4* | *Rf3* | *Rf3/Rf4* |
| 63. | SHRABANI | *Rf4* | *Rf3* | *Rf3/Rf4* |
| 64. | SC5 28-4-1-1 | No | *Rf3* | *Rf3* |
| 65. | RPHR-628-2 | No | *Rf3* | *Rf3* |
| 66. | PNR-2-49 | No | *Rf3* | *Rf3* |
| 67. | RPHR-695-1 | No | *Rf3* | *Rf3* |
| 68. | TG-70P1 | No | *Rf3* | *Rf3* |
| 69. | TG-64P4 | *Rf4* | *Rf3* | *Rf3/Rf4* |
| 70. | TG-23P4 | *Rf4* | *Rf3* | *Rf3/Rf4* |
| 71. | B-95-12 | *Rf4* | *Rf3* | *Rf3/Rf4* |
| 72. | 376 | *Rf4* | *Rf3* | *Rf3/Rf4* |
| 73. | 524-2 | *Rf4* | *Rf3* | *Rf3/Rf4* |
| 74. | 541-2 | *Rf4* | *Rf3* | *Rf3/Rf4* |
| 75. | 1163 | H | *Rf3* | *Rf3* |
| 76. | BR-22 | *Rf4* | *Rf3* | *Rf3/Rf4* |
| 77. | SN-199 | *Rf4* | *Rf3* | *Rf3/Rf4* |
| 78. | SN-230 | *Rf4* | *Rf3* | *Rf3/Rf4* |
| 79. | SN-234 | *Rf4* | *Rf3* | *Rf3/Rf4* |
| 80. | SN-241 | No | *Rf3* | *Rf3* |
| 81. | SN-247 | *Rf4* | *Rf3* | *Rf3/Rf4* |
| 82. | SN-257 | *Rf4* | *Rf3* | *Rf3/Rf4* |
| 83. | R-42 | *Rf4* | *Rf3* | *Rf3/Rf4* |
| 84. | R-43 | *Rf4* | *Rf3* | *Rf3/Rf4* |
| 85. | R-57 | *Rf4* | *Rf3* | *Rf3/Rf4* |
| 86. | AYT-1(APO) | *Rf4* | *Rf3* | *Rf3/Rf4* |

| | | | | |
|---|---|---|---|---|
| 87. | AYT-3(IR-72667-16-1-B-B-3 | H | *Rf3* | *Rf3,Rf4(H)* |
| 88. | IR-78877-181-B-1-2 | H | No | *Rf4(H)* |
| 89. | IR-79956-B-60-2-3 | H | No | *Rf4(H)* |
| 90. | CR-691-58 | No | No | *NO* |
| 91. | IRRI-7 | *Rf4* | *Rf3* | *Rf3/Rf4* |
| 92. | IRRI-10 | *Rf4* | *Rf3* | *Rf3/Rf4* |
| 93. | IRRI-37 | *Rf4* | *No* | *Rf4* |
| 94. | VIBHAV | *Rf4* | *Rf3* | *Rf3/Rf4* |
| 95. | VIKRAMARYA | H | No | *Rf4(H)* |
| 96. | PHALYUNA | *Rf4* | No | *Rf4* |
| 97. | ADHITYA | H | No | *Rf4(H)* |
| 98. | IET-19367 | *Rf4* | *Rf3* | *Rf3/Rf4* |
| 99. | AJAYA | *Rf4* | *No* | *Rf4* |
| 100. | R-38 | *Rf4* | *Rf3* | *Rf3/Rf4* |

**Table 4: Spikelet fertility of hybrid**

| S. No. | Hybrid and *Rf* gene | SPF (%) |
|---|---|---|
| 1 | APMS6A/VG-269 (no *Rf* gene) | 91.0 |
| 2 | APMS6A/IR66 (*Rf4*) | 73.7 |
| 3 | PUSA5A/RPHR-1124 (*Rf3*) | 79.5 |
| 4 | PUSA5A/VG-269 (no *Rf* gene) | 66.1 |
| 5 | PUSA5A/EPLT-109 (*Rf3* & *Rf4*) | 50.0 |
| 6 | PUSA5A/GQ-86 (*Rf3* & *Rf4*) | 90.8 |
| 7 | PUSA5A/IBL-57 (*Rf3* & *Rf4*) | 64.5 |
| 8 | IR58025A/SG27-105 (*Rf3* & *Rf4*) | 77.4 |
| 9 | IR58025A/GQ-70 (*Rf3* & *Rf4*) | 63.7 |
| 10 | IR58025A/BR827-35 (no *Rf* gene) | 70.8 |
| 11 | IR68897A/KMR-3 (*Rf3* & *Rf4*) | 58.0 |
| 12 | IR68897A/C-20R (*Rf4*) | 42.7 |
| 13 | IR68888A/BR827-35 (no *Rf* gene) | 92.1 |
| 14 | IR79156A/GQ37-1(*Rf3*) | 90.9 |
| 15 | IR79156A/IR55778R (*Rf3* & *Rf4*) | 92.8 |
| 16 | IR79156A/RPHR1096 (*Rf4*) | 62.3 |
| 17 | IR79156A/KMR-3 (*Rf3* & *Rf4*) | 90.3 |
| 18 | IR79156A/GQ86 (*Rf3* & *Rf4*) | 73.9 |
| 19 | IR79156A/C-20R (*Rf4*) | 42.7 |
| 20 | IR79156A/IBL-57( *Rf3* & *Rf4*) | 59.9 |

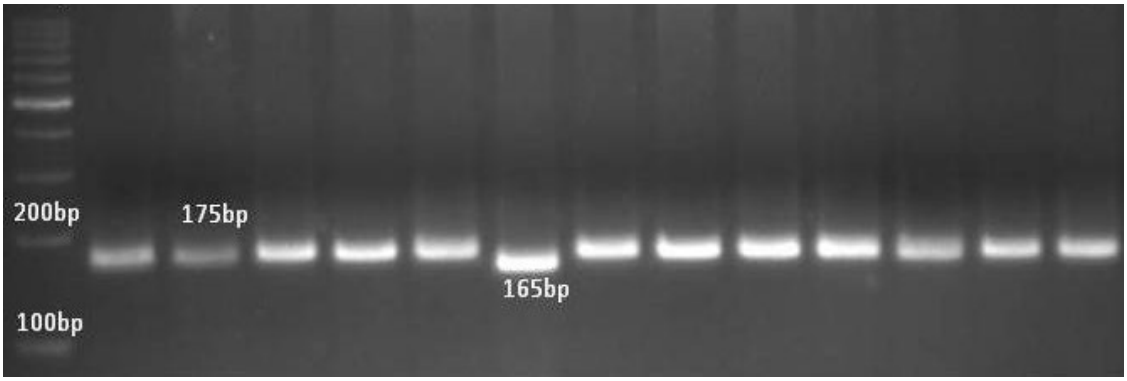


**Figure 1: Screening of fertility restorer gene (*Rf4*)**

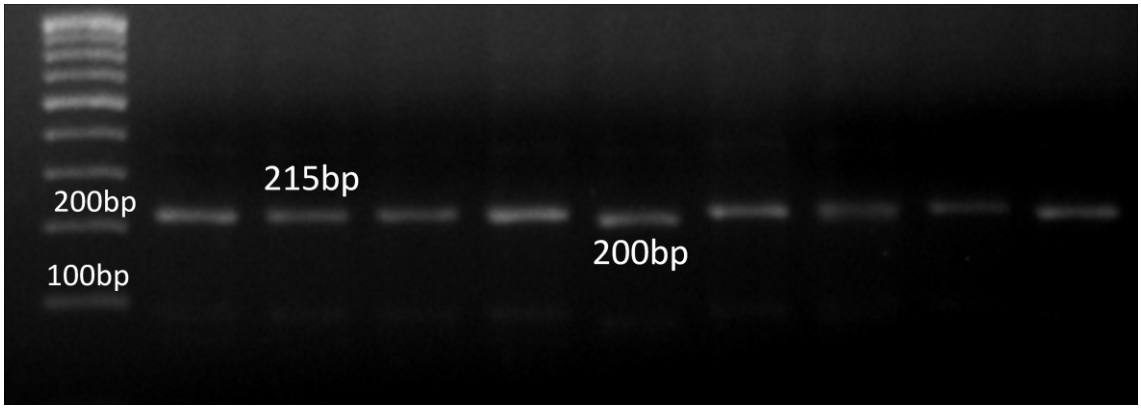


**Figure 2: RM 10313 Screening for *Rf3* gene**

**Table 3: Selected parents for crossing**

| PARENT | RM6100 | RM 10313 |
|---|---|---|
| RPHR-1124 | - | P |
| SG-27-105 | P | P |
| VG-269 | - | - |
| EPLT-109 | P | P |
| RPHR-118 | P | - |
| GQ-25 | P | P |
| GQ-37-1 | - | P |
| IR-55178 | P | P |
| RPHR 1005 | P | P |
| GQ -70 | P | P |
| BR 827-35 | - | - |
| RPHR 1096 | P | - |
| KMR 3 | P | P |
| GQ 86 | P | P |
| IR 66 | P | - |
| C 20 R | P | - |
| IBL 57 | P | P |
| IR 24 | P | P |