DIVERSITY AND CAPABILITY ANALYSES OF FERTILITY RESTORER GENES OF CYTOPLASMIC MALE STERILE RICE LINES USING SSR

Analysis of Diversity and Capabilities of Fertility Restorer Genes in Cytoplasmic Male Sterile Rice Lines Based on Markers SSR

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ABSTRACT

Development of hybrid rice depends on the effectiveness of cytoplasmic male sterility (CMS) and restorer (R) lines. The molecular genetic approach is expected to help the breeder in identification of suitable parental lines to hybrid rice improvement. The study aimed to assess genetic relationship among three types of CMS systems (wild abortive/WA Kalinga and Gambiaca) as female parents and to identify diversity of genes controlling fertility restoration in rice. The study used nine F1 hybrids and F2 populations obtained from the hybridization of three different CMS lines (IR58025A-WA, IR80156A-Kalinga and IR80154A-Gambiaca) with three restorer lines (PK90, PK12 and BP11). Fifteen SSR markers were used to select genomic regions of chromosome 1 and 10 on which Rf3 and Rf4 genes located in the hybrids. The results showed that fertility restoration in CMS-WA and CMS-Gambiaca was governed by two independent and dominant genes (Rf3 and Rf4), while in CMS-Kalinga the fertility restoration was controlled by one single dominant gene. Biological processes occurred in the fertility restoration of the hybrids were the same based on the pollen and spikelet fertilities of F1 hybrids derived from three CMS and R lines, i.e. 76.1–78.3% and 69.1–76.6%, respectively. A restorer line PK12 had a higher capability in fertility restoration than PK90 and BP11. The SSR primers RM490 and RM258 were capable of identifying the Rf3 and Rf4 genes controlled fertility restoration in CMS-WA. The study supports the use of male sterile WA in rice hybridization.

Keywords: cytoplasmic male sterile, fertility-restorer gene, hybrid rice, SSR/markers

INTRODUCTION

Hybrid rice technology is considered as one of the promising options to increase rice yield. In Indonesia the hybrid rice breeding program so far has used three lines, i.e. cytoplasmic male sterility or CMS (A), maintainer (B) and restorer (R) (Satoto and Suprihatno 2008). The CMS line is unable to produce functional pollens, but it can be restored by nuclear genes controlling fertility restoration in restorer line. The maintainer line is genetically the same as that of CMS line, but the line itself can produce fertile pollens. The CMS and restoring fertility systems are important mechanisms in hybrid rice breeding programs (Virmani and Wan 1988). CMS inherited maternally through the disability of the plant to produce normal pollen and it is...
related with open reading frames (ORFs) in mitochondria genome. CMS can be restored by fertility-restorer (Rf) genes (Chase and Babay-Laughnan 2004).

More than 20 cytoplasm sources of CMS have been identified in rice. These include a wild abortive (WA), Dissi, Gambiaca, Boro Type II (BT), Kalinga (Ka) and Honglian (HL) (Pradhan et al. 1992; Fujii et al. 2010). The CMS systems of WA (Indica-Orzya rufipogon Griff.), Dissi (Indica, variety DS 97A from Senegal), and Gambiaca (Indica from West Africa) were categorized as sporophytic CMS systems typical with aborted pollens. Among the types of cytoplasm sources, WA-CMS has been extensively used in seed production of hybrid rice (Xie 2010; Huang et al. 2014).

Study on restoring fertility in WA-CMS has been reported by many researchers. A major study revealed that inheritance of restoring fertility in WA-CMS is controlled by two genes, i.e. Rf3 and Rf4 (Shah et al. 2012). The effect of Rf4 (located on chromosome 10) was greater than that of Rf3 (chromosome 1) on its ability for restoring pollen fertility of WA-CMS (Yao et al. 1997). These two gene mechanisms are similar to those reported by Nematzadeh and Kiani (2010). However, other studies demonstrated the different mechanisms of pollen fertility restoration, such as monogenic (Tan et al. 2008) and trigenic (Hossian et al. 2010).

The WA-CMS was controlled by Rf3 and Rf4 genes. Sattari et al. (2008) reported that the effect of Rf3 on pollen fertility appeared to be stronger than the effect of Rf4 gene Kalinga-CMS has not been identified yet of its fertility restoring genes, but some studies showed the similarity level of fertility restoration between WA-CMS and Kalinga-CMS (Khera et al. 2012; Das et al. 2013). Sahu et al. (2014) reported that several restorer lines could restore the fertility of pollen grains and spikelet on both CMS systems.

In Indonesia, approximately 99% of F1 hybrid rice varieties were developed using a wild abortive type of CMS and its Rf genes. The use of only one cytoplasm source for a long time and applied in wide areas worries breeders over the potential genetic vulnerability of the WA cytoplasmic lines to biotic and abiotic stresses similar to those occurred in maize (Ullstrup 1972) and millet (Kumar et al. 1983). It is important, therefore, to use CMS lines originated from different cytoplasms such as Kalinga and Gambiaca. However, information on the genetic mechanism for Kalinga and Gambiaca CMS systems is still lacking.

The use of molecular markers for detecting restoration genes in different cytoplasmic systems has been reported previously (Seesang et al. 2014; El. Namaky et al. 2016). In their study, some of SSR markers could be used to identify genetic variability of CMS and restorer lines to support hybrid rice breeding program.

The study aimed to assess genetic relationship among three types of CMS systems (WA, Kalinga and Gambiaca) as female parents and to identify diversity of genes controlling fertility restoration in rice.

**MATERIALS AND METHODS**

**Plant Materials and Population Development**

Rice genotypes used in this study were three CMS lines with different cytoplasm sources, namely IR58025A (WA-CMS), IR80154A (Gambiaca-CMS) and IR80156A (Kalinga-CMS) as female parents and three restorer lines namely PK90, PK12 and BP11 as male parents. Crosses were carried out in all combinations between CMS lines and restorer lines, resulted in nine F1 hybrids. This hand-crossing was conducted during November -February 2015 at Sukamandi Field Station, Indonesian Center for Rice Research (ICRR). It is located at 10°31’39’’ longitude and 06°20’ 5’ latitude, at an elevation of 16 m above sea level. Prior to anthesis, 2-3 panicles of each F1 population were wrapped with paper bags to avoid cross-pollination from other plants until harvested as F2 populations.

Twenty-one-day old seedlings of 100 F1 plants for each population and parental lines were planted in the field by single seedling per hill at a spacing of 20 cm x 20 cm during March-July 2015. At 30 days after planting, few leaves were picked and used for DNA isolation. The other F2 plants and their parents were raised following the recommended agronomic practices. At the early flowering stage, ten of matured but not opened spikelets were taken from the middle to the top of panicles. The pollens were then collected and put in vials containing 70% alcohol to determine pollen fertility. At the time of maturity, the panicles were sampled and analyzed their fertility.

**Evaluation of Pollen and Spikelet Fertility in F1 and F2 Populations**

At flowering before anthesis, about 15 spikelets per plant were collected in vial bottles containing 70% alcohol. Five spikelets were randomly taken from the bottom, middle and above of the main panicles. Pollen grains were stained with 1% iodine potassium iodide solution and pollen fertilities were determined on a microscopic slide-glass and counted under an optical microscope at 40x magnifications. Pollen fertility percentage was determined by counting the sterile and fertile pollen grains divided by the total number of pollen grains.
observed. Pollen fertility was grouped into four classes based on Chaudary et al. (1981), i.e. fully fertile (FF = >60% pollen fertile), partially fertile (PF = 30–60% pollen fertile), partially-sterile (PS = 1–30% pollen fertile) and fully sterile (FS = 0% pollen fertile). A Chi-square ($\chi^2$) analysis was done to test the goodness of fit of the tested hypothesis. Pollen fertility data in F$_1$ populations were classified into two groups, i.e. fertile (FF + PF + PS) and sterile (FS) (Seesang et al. 2014).

To estimate spikelet fertility, the main panicles of all segregating family derived from nine crosses were wrapped with butter paper bags prior to anthesis. At the time of maturity (25–30 days after flowering), the panicles were harvested. The filled and unfilled grains in the panicles were counted and the fertility percentage was calculated from 15 spikelets. They were then classified into four classes as described by Chaudary et al. (1981). A Chi-square analysis was performed to test the goodness of fit of the genetic hypothesis by dividing the data of spikelet fertility into two groups, i.e. fertile (spikelet fertility $>2\%$) and sterile (spikelet fertility $<2\%$).

**Fertility Restoring Gene Analyses Using SSR Markers**

The genomic DNA isolation and PCR amplification were conducted at the molecular biology laboratory of the Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development during August – December 2015. The total genomic DNA was isolated from the ± 4 cm fresh leaves of the parents and individual plants of nine F$_1$ populations using 100 µL 0.25 N NaOH. The young leaves were treated by 33°C for 2 min and centrifuged at 14,000 rpm for 15 min.

The isolated DNAs were subjected to the PCR amplification using 15 SSR markers. The PCR was carried out under the following conditions: a total of 35 cycles comprising one minute at 94°C, one minute at 55°C and two minutes at 72°C. PCR reaction was performed in 10 µL volume containing 5 µL of mix PCR KAPA 2G Fast Ready Mix with Dye, 2 µL DNA, 1 µL nano-pure water, and 1 µL each of forward and reverse primers (10 µM). The PCR products were then electrophoresed using 2% (w/v) non-denaturing 8% polyacrylamide gels, stained with 0.5 µg.ml$^{-1}$ ethidium bromide solution (Model MGV, CBG Scientific Co.), and visualized under UV light using a chemidoc gel system.

A Chi-square ($\chi^2$) analysis was performed to test the goodness of fit of the F$_1$ populations for the phenotypic and marker data by comparing an observed frequency distribution with an expected one. The marker-trait association was analyzed by using single marker analysis method to obtain association strength between the markers and the fertility restoration trait.

**RESULTS AND DISCUSSION**

**Pollen and Spikelet Analysis in F$_1$ Populations**

Pollen fertility of F$_1$ population ranged from 71.7% to 89.9%, while spikelet fertility was between 63.7% and 79.9%. All cross combinations showed more than 60% pollen fertility (fully fertile) and fully fertile spikelet (71–100%) except IR80154A/PK90 and IR80156A/PK90 (Table 1). It suggests that pollen fertility had a little bit difference from that of spikelet fertility at the time of maturity. It might be due to the pollen abortion at different cell division stages. The study showed that differences in pollen fertility among hybrids and response of fertility restoration varied according to the background of cytoplasm and restoring ability.

F$_1$ plants derived from crosses involving A and R lines of the respective cytoplasm and their cross-combination (IR58025A/PK12, IR58025A/BP11; IR80154A/PK90, IR80154A/BP11; and IR80156A/PK90, IR80156A/BP12) showed similar pollen fertilities (Table 1), indicating the similar biological process which affects fertility restoration in CMS types of WA, Gambiaca and Kalinga in combination with restorer lines PK12 and BP11. It indicated that Rf genes in PK90 (WA-CMS), PK12 (Gambiaca-CMS) and BP11 (Kalinga-CMS) might be allelic and could functionally supplement each other to restore the same level of pollen and spikelet fertilities. If the Rf genes were not allelic, this would mean that each restorer line carries different restorer genes for WA, Gambiaca and Kalinga CMS systems. Li and Zhu (1988) reported that Gambiaca-CMS has the same restoration system with those of WA-CMS and Kalinga-CMS (Khera et al. 2012).

Among the three restorer lines, PK12 showed higher fertility restoration than those of PK90 and BP11. The averages of pollen and spikelet fertilities of PK12 were 78.3% and 76.6%, respectively, which were higher than those shown by PK90 (76.1% and 69.1%) and BP11 (77.7% and 73.7%) (Table 1).

The same restorer lines could restore CMS lines with different cytoplasmics as reported by Sahu et al. (2014) who found that Baghdian restorer line could restore IR58025A (WA-CMS) and CRMS32A (Kalinga-CMS). A similar result was reported by Sattari et al. (2008) who showed that restorer line IR34686R could restore CMS
Pollen and spikelet fertilities of F₁ plants derived from A/R cross-combinations for wild abortive (WA), Gambiaca and Kalinga cytoplasmic male sterility (CMS) systems using three different restorer lines.

| CMS (female parent)/ restorer (male parent) | Fertility (%) of F₁ Hybrids | Average (%) |
|--------------------------------------------|-----------------------------|-------------|
|                                            | PK90 (WA)                   | PK12 (Gambiaca) | BP11 (Kalinga) |       |
| IR58025A (WA)                              | Pollen 80.9 ± 0.7           | 78.7 ± 1.6   | 78.6 ± 1.8   | 79.4  |
|                                            | Spikelet 76.9 ± 1.2         | 77.6 ± 2.4   | 71.3 ± 1.7   | 75.3  |
| IR80154A (Gambiaca)                        | Pollen 75.7 ± 0.8           | 75.9 ± 1.8   | 75.5 ± 1.9   | 75.7  |
|                                            | Spikelet 63.7 ± 2.9         | 72.2 ± 1.7   | 71.9 ± 1.4   | 69.3  |
| IR80156A (Kalinga)                         | Pollen 71.7 ± 2.3           | 80.2 ± 1.5   | 79.0 ± 1.3   | 77.0  |
|                                            | Spikelet 66.8 ± 1.8         | 79.9 ± 1.9   | 77.8 ± 2.5   | 74.8  |
| Average                                    | Pollen 76.1                 | 78.3         | 77.7         |       |
|                                            | Spikelet 69.1               | 76.6         | 73.7         |       |

Spikelet fertility in F₂ populations derived from a cross of IR58025A/PK90 (WA-CMS) ranged from 0 to 93.3%. Out of 100 F₂ plants, 9 progenies showed complete pollen sterility and 91 progenies were classified as FF, PF and PS. The test showed that segregation of pollen fertility in the F₂ population of IR58025A/PK90 revealed 91 fertile and 9 completely sterile plants (Figure 1), which were not significantly different from the expected ratio of 15 fertile : 1 sterile ($\chi^2 = 1.29$, P ≥ 0.05). This indicates the presence of digenic inheritance and epistatic dominant duplicate gene action controlling fertility restoration in the WA-CMS system.

Segregation pattern observed in F₂ population derived from IR80154A/PK12 (Gambiaca-CMS) showed 96 fertile : 10 sterile and it indicated a good fit to 15 fertile : 1 sterile ($\chi^2 = 2.40$, P ≥ 0.05). This suggests that the restorer line used carried two independent dominant fertility restoring genes, and one of the two genes had a duplicate effect that showed higher expression than the other gene and it alone could restore fertility. The findings are in line with those reported by Sattari et al. (2008), Seesang et al. (2014) and Hasan et al. (2015) who found the duplicate dominant epistatic gene action at the fertility restoration inheritance of WA and Gambiaca CMS.

The F₂ populations from IR80154A/PK90 (WA-CMS) and IR80156A/PK90 (Gambiaca-CMS) segregated and fitted well to 15 fertile : 1 sterile. Such a segregation pattern in different genetic backgrounds indicated the presence of two duplicate dominant genes in controlling fertility restoration.

Figure 1. Segregation of pollen fertility in F₂ population of rice stained with 1% iodine potassium iodide solution: a = fully fertile, b = partially fertile, c = partially sterile, d = fully sterile.
fertility restoration trait. Variation and fertility restoration of the restorer lines to the CMC lines could be due to the different penetrances and expressivities of these restorer genes depending on the nuclear genotypes of the female parents (Table 2).

The F₂ population involving IR80156A/BP11 cross segregated into 80 fertile : 20 sterile and this ratio did not significantly different from the expected ratio of 3 : 1 (χ² = 1.33, P ≥ 0.05). This indicates that restorer line for Kalinga-CMS was controlled by a single gene inheritance. Similar results were observed on two other crosses derived from restorer line BP11, i.e. IR58025A/BP11 and IR80154A/BP11 which were also controlled by a single dominant gene action. This ratio denoted that there was a dominant locus in the BP11 line. Bagheri and Jelodar (2011) reported that restorer lines with dominant allele at homozygote or heterozygote conditions would be fertile, while the one that was in homozygote recessive would be sterile. Similar results were demonstrated by Ahmadikkah et al. (2007), Alavi et al. (2009) and Hossain et al. (2010) who reported a complete dominant gene action at a ratio of 3 fertile : 1 sterile in the studied CMS-Rf system.

### Genotyping Analysis of Different CMS Systems for Rf Genes

Among the 15 SSR markers used, 4 markers were polymorphic between the parents and F₂ progenies derived from crossing involving restorer line PK90, i.e. RM490 (Rf3), RM1059 (Rf3), RM258 (Rf4) and RM228(Rf4). A total of 100 F₂ progenies derived from restorer line PK12 were genotyped using the above three SSR markers, i.e. RM490 (Rf3), RM1059 (Rf3) and RM1108 (Rf4) of which all of the three SSR markers showed polymorphism between the two tested parent genotypes. Furthermore, RM490 (Rf3) and RM228 (Rf4) revealed polymorphism between the parents in the F₂ progenies derived from crosses involving the restorer line BP11. These SSR primers were then used for genotyping analysis involving individual F₂ plants showing sterile and fertile phenotypes. The genotyping analyses were done at three populations according to cytoplasmic systems, i.e. IR58025A/PK90 (WA-CMS), IR80154A/PK12 (Gambiaca-CMS), and IR80156A/ BP11 (Kalinga-CMS).

The SSR analysis showed an F₂ segregation ratio of 9 : 6 : 1 (χ² = 3.72, and χ² = 0.05 at p ≥ 0.05) for IR58025A/PK90 (WA-CMS) cross (Table 3). The markers RM490 (Rf3) and RM258 (Rf4) showed good fit with the expected ratio and were not significantly different from the tested ratio of 9 : 6 : 1 (χ² = 3.72, and χ² = 0.05 at P ≥ 0.05). The results indicated the involvement of digenic supplementary or an epistatic with recessive gene action for pollen fertility trait. Assuming that two dominant fertility restoring genes involved in this action, one of the two genes appeared more effective than the other gene.

| Hybrid combination     | Pollen type | Number of plants | χ² test | Genetic ratio |
|------------------------|-------------|------------------|---------|---------------|
| IR58025A/PK90          | Fertile     | 91               | 1.29*** | 15 : 1        |
|                        | Sterile     | 9                |         |               |
| IR80154A/PK90          | Fertile     | 97               | 1.80*** | 15 : 1        |
|                        | Sterile     | 3                |         |               |
| IR80156A/PK90          | Fertile     | 85               | 13.07***| 15 : 1        |
|                        | Sterile     | 15               |         |               |
| IR58025A/PK12          | Fertile     | 90               | 2.40*** | 15 : 1        |
|                        | Sterile     | 10               |         |               |
| IR80154A/PK12          | Fertile     | 90               | 2.40*** | 15 : 1        |
|                        | Sterile     | 10               |         |               |
| IR80156A/PK12          | Fertile     | 88               | 5.64*** | 15 : 1        |
|                        | Sterile     | 12               |         |               |
| IR58025A/BP11          | Fertile     | 86               | 3.41**  | 3 : 1         |
|                        | Sterile     | 17               |         |               |
| IR80154A/BP11          | Fertile     | 82               | 2.61**  | 3 : 1         |
|                        | Sterile     | 18               |         |               |
| IR80156A/BP11          | Fertile     | 80               | 1.33**  | 3 : 1         |
|                        | Sterile     | 20               |         |               |

*a* not significant at 5% statistical level; *b* significant at 5% statistical level.

Table 2. Chi-square analysis for pollen fertility restoration trait in F₂ populations of rice.
The F$_2$ segregation ratio involving epistatic gene has been reported earlier by Jing et al. (2001) and Hossain et al. (2010) in WA type sources of the CMS lines. Association of pollen fertility and SSR markers demonstrated low and moderate values. The R$^2$ of IR58025A/PK90 were 0.26 and 0.71 for SSR markers RM490 and RM258, respectively (Table 3). The markers showing polymorphisms between the male sterile lines and the restorer line, also demonstrating an association with the pollen fertility trait, should be applicable for tagging the genes controlling fertility restoration trait in rice (Singh et al. 2013). Based on these results, SSR markers RM490 and RM258 should be applicable for R$_f$3 and R$_f$4 genes, respectively, for fertility restoration in the WA-CMS system. Among the 15 SSR markers used, two markers were polymorphic between two parents IR58025A and PK90. One of the markers namely RM258 was polymorphic between the sterility and fertility spikelet in F2 population of IR58025A/PK90 (Figure 2).

In the F$_2$ population of IR80154A/PK12 (Gambiaca-CMS), both of SSR markers RM1059 and RM1108 for R$_f$3 and R$_f$4 genes, respectively, were significantly different at a ratio of 9 : 6 : 1 (χ$^2$ = 9.46 and χ$^2$ 8.30, P ≥ 0.05). Although there was a false deviation to the ratio, genotypic segregation in Gambiaca-CMS indicated two independent dominant genes that control restoration fertility like in WA-CMS. Similarities in fertility restoration of WA and Gambiaca were previously reported by Savant et al. (2006) and Sattari et al. (2008).

The association analysis of SSR markers with pollen fertility trait showed that SSR markers RM1059 and RM1108 were not significantly associated (P<0.01) to pollen fertility trait with R$^2$ of 0.001 and 0.03, respectively. Therefore, it is essential to screen more SSR markers that have close association with fertility restoration for Gambiaca-CMS.

In the F$_2$ population of IR80156A/BP11 (Kalinga-CMS), the ratio of marker RM1059 (R$_f$3) was not significantly different at an expected ratio of 1 : 2 : 1 (χ$^2$ = 5.62, P ≥ 0.05), while that of RM228 (R$_f$4) was not significantly different at an expected ratio of 1 : 2 : 1 (χ$^2$ = 4.18. P ≥ 0.05). Segregation pattern of 1 : 2 : 1 indicated the incomplete dominant gene action in the population. The association of SSR markers with pollen

| Genotype of F2 individuals | IR58025A/PK90 (WA-CMS) | IR80154A/PK12 (Gambiaca-CMS) | IR80156A/BP11 (Kalinga-CMS) |
|----------------------------|-------------------------|-------------------------------|-------------------------------|
|                            | Observed frequency      | Expected frequency            | Observed frequency            | Expected frequency            | Observed frequency            | Expected frequency            |
| RfRf                       | 45 51                   | 56 39                        | 41 56                        | 35 33                        | 25 33                        |
| RfRF                       | 45 37                   | 37 50                        | 37 50                        | 41 41                        | 50 45                        |
| RfRf                       | 9 12                    | 6 11                         | 9 6                         | 24 26                        | 25 26                        |
| P value                    | 0.01 0.01               | 0.85 0.85                    | 0.001 0.03                   | 0.02 0.09                    | 0.49 0.36                    |
| R$^2$ value                | 0.26 0.71               | 0.001 0.03                   | 9.46* 8.30*                 | 5.62* 4.18*                 |
| x$^2$ value                | 3.72* 0.05**            | 9.46* 8.30*                 | 5.62* 4.18*                 |

*Significantly different and ** not significantly different at 5% statistical level χ$^2$ value = 5.99.

RfRf defines the presence of fertile parent alleles in homozygous conditions; RfRf defines the presence of both sterile and fertile alleles in heterozygous conditions and rfrf defines the presence of sterile parent alleles in homozygous conditions.

![Figure 2](image_url) Linkage analysis on sterility of F$_2$ population of IR58025A/PK90 using SSR marker RM258 located on chromosome 10 of rice.

M = 100bp ladder, P1 = IR58025A, P2 = PK90, 1 to 20 = number of individual plants.
fertility showed that markers RM1059 and RM228 were not associated significantly (P<0.01) to pollen fertility trait with R² of 0.02 and 0.09, respectively.

Khera et al. (2012) and Das et al. (2013) identified restorer and maintainer lines for development of hybrid rice using Kalinga source of CMS lines, but inheritance of fertility restoration remains unknown. The restorer fertility in Kalinga-CMS system displayed epistasis with incomplete dominant gene. The results indicated that the SSR markers used, i.e. RM490 and RM258, were suited for marker assisted selection (MAS) of restorer lines in WA-CMS system. By using these markers, primary selection can be carried out at seedling stage, hence, works required for testing crosses by breeders would be reduced. MAS is being explored as an important supplement to phenotypic selection in rice breeding. PCR-based markers offer great potential to enhance MAS efficiency. Further studies are needed to confirm the efficiency of MAS through crossing between suspected restorers and CMS lines and fertility evaluation of F₂ plants. In addition to their use in MAS procedures, these markers can also be used to transfer Rf genes into adapted cultivars through a backcrossing in an active hybrid rice breeding program.

CONCLUSION

Cytoplasmic male sterility (CMS) in wild abortive (WA) and Gambiaca rice lines were controlled by a pair of dominant genes, i.e. Rf3 and Rf4, whereas in Kalinga was controlled by one gene. Biological processes occurred in fertility restoration of the hybrids were the same. A restorer line PK12 has a higher capability in fertility restoration than PK90 and BP11. The SSR primers RM490 and RM258 are potential markers of Rf3 and Rf4 genes for restoring fertility of WA system.

The study supports the use of male sterile WA in rice hybridization. Further study is required to improve marker-assisted selection of cytoplasmic male sterile lines for hybrid rice breeding program.

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