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

Analisis Keragaman dan Kapabilitas Gen Pemulih Kesuburan pada Mandul Jantan Padi Berdasarkan Marka SSR

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ABSTRACT

Development of hybrid rice depends on the effectivity 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 abbortive/WA Kalinga and Gambiaca) as female parents and to identify diversity of genes controlling fertility restoration in rice. The study used nine F_1 hybrids and F_2 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 F_1 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]

ABSTRAK

*Keberhasilan pengembangan padi hibrida bergantung pada ketersediaan galur/mandul jantan (*cytoplasmic male sterility*, CMS) dan galur pemulih kesuburan (*restorer*) yang efektif. Teknik molekuler dapat digunakan untuk membantu pemulia dalam menentukan galur-galur tetua yang tepat untuk perakitan padi hibrida. Penelitian ini bertujuan untuk mengetahui hubungan genetik tiga tipe sitoplasma padi (*wild abortive */WA, Kalinga, dan Gambiaca) dan mengidentifikasi keragaman gen yang mengendalikan pemulihan kesuburan. Penelitian menggunakan sembilan hibrida F_1 dan populasi F_2 yang diperoleh dari tiga tipe sitoplasma (IR58025A-WA, IR80156A-Kalinga, dan IR80154A-Gambiaca) yang disilangkan dengan tiga galur pemulih kesuburan (PK90, PK12, dan BP11). Sebanyak 15 marka SSR digunakan untuk menyeleksi daerah genom pada kromosom 1 dan 10 di mana gen* Rf3 *and* Rf4 *berada. Hasil penelitian menunjukkan bahwa pemulihan kesuburan pada galur CMS-WA dan CMS-Gambiaca didominasi oleh dua gen independen* Rf3 *and* Rf4*, sedangkan pada galur CMS-Kalinga oleh gen tunggal. Proses biologi yang mengendalikan pemulihan kesuburan tiga tipe CMS pada semua hibrida F_1 adalah sama berdasarkan tingkat fertilitas polen dan spikelet, yaitu masing-masing 76,1–78,3% dan 69,1–76,6%. Galur mandul jantan PK12 memiliki kemampuan memulihkan kesuburan lebih kuat dibandingkan dengan PK90 dan BP11. Marka SSR RM490 dan RM258 berpotensi menjadi penanda untuk gen* Rf3 *dan* Rf4 *untuk memulihkan kesuburan CMS tipe WA. Penggunaan galur mandul jantan tipe WA dianjurkan untuk mengidentifikasi galur R yang mampu memulihkan kesuburan.*

[*Kata kunci:* mandul jantan sitoplasmik, galur pemulih kesuburan, padi hibrida, marka SSR**]**

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-*Oryza 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 F_1 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 F_1 hybrids. This hand-crossing was conducted during November –February 2015 at Sukamandi Field Station, Indonesian Center for Rice Research (ICRR). It is located at $107^0$39′ longitude and $06^0$20′ S latitude, at an elevation of 16 m above sea level. Prior to anthesis, 2–3 panicles of each F_1 population were wrapped with paper bags to avoid cross-pollination from other plants until harvested as F_2 populations.

Twenty-one-day old seedlings of 100 F_2 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 F_2 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 F_1 and F_2 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_2 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_2 populations using 100 µL 0.25 N NaOH. The young leaves were treated by 33 Tissue Lyser System (Qiagen, Tokyo, Japan) for 2 min at speed of 25 vibrations per second, twice. The isolated leaves were then added with 400 µL 0.1 M Tris-HCl 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 $\mu g.ml^{-1}$ 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_2 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 IR801564A/PK90, IR80156A/PK12) 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 cytoplasms as reported by Sahu et al. (2014) who found that Baghdiand 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

Table 1. Pollen and spikelet fertilities of F_1 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	

lines IR58025A (WA-CMS) and IR75601A (Gambiaca-CMS). However, there were differences in the degree of pollen and spikelet fertilities among the hybrids resulted from the crossing combinations. Das et al. (2013) reported that fertility restoration varied according to the background of cytoplasm and restoring ability of the lines used. Genetically, variations of fertility restoration obtained may be due to the maternal effects and/or the ability of pollen fertility restoring genes in different CMS systems (Jayasudha and Sharma 2010) or sterility levels of intervarietal hybrid developed (Govinda Raj and Virmani 1988).

Pollen Fertility Analysis in F_2 Populations

Spikelet fertility is influenced by physiological and environmental factors, and spikelet fertility data cannot be used to study the inheritance of fertility restoration trait. Therefore, to obtain information regarding the number of genes and their actions in controlling fertility restoration, a value of pollen fertility at the F_2 population is usually used. Table 2 shows the results of a Chi-square analysis of fertility restoration trait resulted from each of the crossing combinations.

Pollen fertility in F_2 populations derived from a cross of IR58025A/PK90 (WA-CMS) ranged from 0 to 93.3%. Out of 100 F_2 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 F_2 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 ($\chi^2 = 1.29$, $P \geq 0.05$). 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 F_2 population derived from IR80154A/PK12 (Gambiaca-CMS) showed 96 fertile : 10 sterile and it indicated a good fit to 15 fertile : 1 sterile ($\chi 2 = 2.40$, $P \geq 0.05$). 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 F_2 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

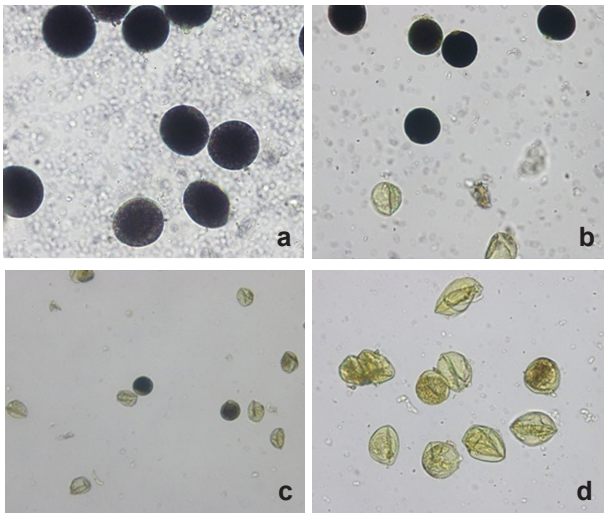

Figure 1. Segregation of pollen fertility in F_2 population of rice stained with 1% iodine potassium iodide solution: a = fully fertile, b = partially fertile, c = partially sterile, d = fully steril

Table 2. Chi-square analysis for pollen fertility restoration trait in F_2 populations of rice.

Hybrid combination	Pollen type	Number of plants		χ2 test	Genetic ratio
		Observed	Expected		
IR58025A/PK90	Fertile	91	94	1.29^{ns}	15 : 1
	Sterile	9	6		
IR80154A/PK90	Fertile	97	94	1.80^{ns}	15 : 1
	Sterile	3	6		
IR80156A/PK90	Fertile	85	94	13.07*	15 : 1
	Sterile	15	6		
IR58025A/PK12	Fertile	90	94	2.40^{ns}	15 : 1
	Sterile	10	6		
IR80154A/PK12	Fertile	90	94	2.40^{ns}	15 : 1
	Sterile	10	6		
IR80156A/PK12	Fertile	88	94	5.64*	15 : 1
	Sterile	12	6		
IR58025A/BP11	Fertile	86	75	3.41*	3 : 1
	Sterile	17	25		
IR80154A/BP11	Fertile	82	75	2.61^{ns}	3 : 1
	Sterile	18	25		
IR80156A/BP11	Fertile	80	75	1.33^{ns}	3 : 1
	Sterile	20	25		

^{ns}not significant at 5% statistical level; * significant at 5% statistical level.

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 F_2 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 ($\chi2 = 1.33$, $P \geq 0.05$). 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 Ahmadikhah 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 F_2 progenies derived from crossing involving restorer line PK90, i.e. RM490 (*Rf3*), RM1059 (*Rf3*), RM258 (*Rf4*) and RM228(*Rf4*). A total of 100 F_2 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 F_2 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 F_2 segregation ratio of 9 : 6 : 1 ($\chi^2 = 3.72$, and χ^2 0.05 at $p \geq 0.05$) 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 ($\chi^2 = 3.72$, and χ^2 0.05 at $P \geq 0.05$). 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.

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 *Rf3* and *Rf4* 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 RM 258 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 *Rf3* and *Rf4* 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 Sawant 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 (*Rf3*) was not significantly different at an expected ratio of 1 : 2 : 1 (χ2 = 5.62, P ≥ 0.05), while that of RM228 *(Rf4*) 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

Table 3. Segregation pattern of SSR markers for pollen fertility in F_2 rice population derived from three types of cytoplasmic male sterility (CMS).

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
	RM490 (R*f3*)	RM258 (R*f4*)		RM1059 (R*f3*)	RM1108 (R*f3*)		RM1059 (R*f3*)	RM228 (R*f3*)	
RfRf	45	51	56	39	41	56	35	33	25
Rfrf	45	37	37	50	50	37	41	41	50
rfrf	9	12	6	11	9	6	24	26	25
P value	0.01	0.01		0.85	0.85		0.49	0.36	
R^2 value	0.26	0.71		0.001	0.03		0.02	0.09	
x^2 value	3.72^{ns}	0.05^{ns}		9.46^{*}	8.30^{*}		5.62^{*}	4.18^{ns}	

* Significantly different and ^{ns} not significantly different at 5% statistical level χ2 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.

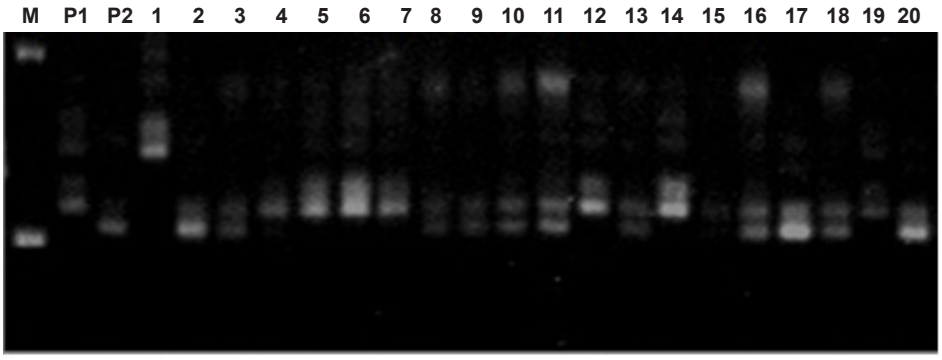

Figure 2. Linkage analysis on sterility of F_2 population of IR58025A/PK90 using SSR marker RM258 located on chromosome 10 of rice. M = 100bp ladder, P1 = IR58025A, P2 = PK90, 1 to 20 = number of individual plants.

fertility showed that markers RM1059 and RM228 were not associated significantly (P<0.01) to pollen fertility trait with R^2 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 F_1 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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