

RESEARCH NOTES

Validation of SSR Markers Linked to Restoring Fertility (*Rf*) Genes and Genotyping of Rice Lines at *Rf* Loci

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ABSTRACT

The present study was carried out with the objective of validating linked SSR markers to *Rf* genes and adopting Marker Assisted Selection (MAS) for restorer/non-restorer line detection in Wild Abortive (WA) type of Cytoplasmic Male Sterility (CMS). Twelve SSR markers reported to be linked to *Rf* genes were analyzed in the mapping population of NedaA/Pajouhesh. Among these, three markers, namely, RM258, RM171, and RM3148 proved to be associated with *Rf* genes. In this study, on a set of rice lines including 2 restorers, 4 maintainers, and 9 conventional varieties (totally 15 genotypes of rice), MAS with RM258 and RM171, a major *Rf* locus on chromosome 10, and RM3148, another *Rf* locus on chromosome 1, both of the *Rf* alleles in Hashemi and Deylamani varieties were amplified the same as restorer lines. However, Pouya, Khazar, and Shastak had one *Rf* locus (partial restorer). Cultivars Shiroudi, Tabesh, Fajr, and Shafaq were identified as non-restorer (maintainer) lines. Results demonstrated that these markers could be used for screening of genotypes to identify restorers and non-restorer lines in hybrid rice breeding programs.

Keywords: Maintainer, Marker assisted selection, Restorer, Rice, WA-CMS.

INTRODUCTION

Rice is staple food in Iran cultivated on 570,000 hectares of irrigated land and 2.4 million tons of milled rice is produced (Nematzadeh *et al.*, 2006). Iran still largely depends on rice imports amounting to 0.8 million tons each year to meet the domestic consumers' demand. Therefore, hybrid rice with an average 20-25% higher grain yield over conventional varieties seems to be a viable option to enhance the production and productivity levels, since the area under rice cultivation cannot be further increased due to water shortages (Nematzadeh *et al.*, 2006).

Cytoplasmic-genetic Male Sterility (CMS) combined with a fertility restoration

system has been found to be the most efficient genetic tool to exploit hybrid vigor on a commercial scale in rice (Lin and Yuan, 1980; Virmani and Wan, 1988). Wild Abortive (WA) is a widely used CMS source that accounted for approximately 90% of the rice hybrids produced in China and 100% of the hybrids developed outside China (Sattari *et al.*, 2008).

The inheritance of fertility restoration in the WA-CMS system has been extensively investigated and genetic analyses have made it clear that two major genes are generally involved (Govinda Raj and Virmani, 1988; Bharaj *et al.*, 1991, 1995; Teng and Shen, 1994). Using RFLP markers, Zhang *et al.* (1997) mapped one of the two *Rf* loci (*Rf3*) on chromosome 1 between RG140 and

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RG532 at a distance of 1.9 cM from each other. Using RAPD and RFLP markers, Yao *et al.* (1997) confirmed the location of *Rf3* on chromosome 1 and mapped the second *Rf* locus (*Rf4*) on chromosome 10 at 3.3 cM from G4003. Jing *et al.* (2001) mapped *Rf4* governing fertility restoration on the long arm of chromosome 10 using SSLP markers. Zhang *et al.* (2002) also mapped the *Rf4* gene on chromosome 10 at 0.9 cM from the marker Y3-8 and anchored to the RFLP marker S10019. Bazrkar *et al.* (2008) tagged four *Rf* genes for WA –CMS system using SSR markers on chromosomes 1 (*Rf3*), 7 (*Rf4*), 10 (*Rf6*) and 12 (*Rf7*) by recessive class analysis.

Attempts were made to use these two major *Rf* genes (*Rf3* and *Rf4*) for Marker Assisted Selection (MAS) to identify restorer lines possessing *Rf* genes for WA-CMS to expedite phenotype-based screening. Ichikawa *et al.* (1997) proposed a simple PCR-mediated system for the selection of rice lines containing the *Rf1* gene. In the study of Prakash (2003), microsatellite marker RM6100 located on chromosome 10 was identified as closely linked to *Rf* gene at a distance of 7 cM. Assessment of this marker for utilization in identification of restorers with a set of 175 lines showed high accuracy of 97.4% in identifying restorer lines.

Microsatellite (RM6100/RM25654) and GC based marker systems (TMPPR3) were also evaluated for their selection efficiency by Sheeba *et al.* (2009) and Ngangkham *et al.* (2010). Suresh *et al.* (2012) validated selection efficiency of SSR markers in a set of restorer lines. They reported DRCG-RF4-14/DRCG-RF4-8 for the *Rf4* locus and DRRM-RF3-5/DRRM-RF3-10 for the *Rf3* locus, with the maximum efficiency of 92 percent for identification of restorers.

The markers which have been reported to be linked to the *Rf* genes have not been validated in alternate populations and have not been characterized for their allelic status with respect to these markers. Therefore, the present study was undertaken to validate linkage of these markers in mapping

populations involving fertility restorer lines Pajouhesh for WA-cytoplasm and to analyze the allelic status of the closely linked markers in a set of rice lines used for hybrid rice breeding in Iran.

MATERIALS AND METHODS

Plant Materials and Population Development

Plant materials used in this study are presented in Table 1. In this study, CMS-WA line ‘Neda A’ was crossed with restorer line ‘Pajouhesh’ and F₁ seeds were obtained. The F₂ population of this cross, including 328 plants, was used for genetic mapping of the restorer gene and validation of the candidate restorer gene-based markers. ‘Pajouhesh’ (Nematzadeh *et al.*, 2010) improved through pedigree-backcross method from the cross between ‘Sepidroud’ and ‘Sang Jo’. ‘Neda A’ is a CMS line with WA type of cytoplasm improved through backcross using ‘IR58025A’. Also, two known restorers, namely, Pajouhesh and Sepidroud (Alahgholipour *et al.*, 2007) along with 4 maintainer lines, namely, Neda, Nemat, Dasht, and Champa and 9 conventional rice lines including Pouya,

Table 1. Plant materials used in this study with their pedigree and origin.

Variety name	Pedigree	Origin
Neda A	Improved cultivar	Mazandaran, Iran
Pajouhesh	Improved cultivar	Mazandaran, Iran
Sepidroud	Improved cultivar	Guilan, Iran
Neda	Improved cultivar	Mazandaran, Iran
Nemat	Improved cultivar	Mazandaran, Iran
Dasht	Improved cultivar	Mazandaran, Iran
Champa	Local	Guilan, Iran
Pouya	Improved cultivar	Mazandaran, Iran
Hashemi	Local	Guilan, Iran
Shiroudi	Improved	Mazandaran, Iran
Tabesh	Improved cultivar	Mazandaran, Iran
Shastak	Local	Guilan, Iran
Fajr	Improved cultivar	Mazandaran, Iran
Khazar	Improved cultivar	Guilan, Iran
Shafaq	Improved cultivar	Mazandaran, Iran
Deylamani	Local	Mazandaran, Iran

Table 2. List of the used SSR primer pairs with their chromosomal locations (Bazrkar *et al.*, 2008).

SSR Marker	Chromosome	Forward primer	Reverse primer	Annealing temperature (°C)
RM1	1	gcg aaa aca caa tgc aaa aa	gcg ttg gtt gga cct gac	55
RM443	1	ggg agt tag ggt ttt gga gc	tcc agt ttc aca ctg ctt cg	55
RM315	1	cgg tca aat cat cac ctg ac	caa ggc ttg caa ggg aag	55
RM294	1	ttg gcc tag tgc ctc caa tc	gag ggt aca act tag gac gca	55
RM3148	1	gac tat tgc tgc aac act ttg	ttg tct tgc ttt ggt att tgc	55
RM6344	7	aca cgc cat gga tga tga c	tgg cat cat cac ttc ctc ac	55
RM171	10	aac gcg agg aca cgt act tac	acg aga tac gta cgc ctt tg	67
RM258	10	tgc tgt atg tag ctc gca cc	tgg cct tta aag ctg tgc c	55
RM244	10	ccg act gtt cgt cct tat ca	ctg ctc tgc ggt gaa cgt	55
RM591	10	cgg tta atg tca tct gat tgg	ttc gag atc caa gac tga cc	55
RM3123	10	att tcc cac aca tct cgc tg	gtg tgc ccg gtc aag aac	55
RM7003	12	ggc aga cat aca gct tat agc	tgc aaa tga acc cct cta gc	55

Hashemi, Shastak, Deylamani, Shiroudi, Tabesh, Fajr, Khazar, Shafaq were totally 16 genotypes used for the study of MAS efficiency using linked SSR markers.

DNA Extraction and PCR analysis

Total genomic DNA was extracted according to Dellaporta *et al.* (1983). PCR amplification was performed using 12 linked SSR markers (Table 2) in F₂ population of 'Neda A'/'Pajouhesh' for validation of their association with *Rf* loci. A 25 µL mixture was prepared for the PCR assay which contained 50 ng template DNA, 2.5 µL of 10X buffer, 0.3 µL of 10 mM dNTPs, 1 µL of 50 mM MgCl₂, 1 µL of each primers (2 µM), and 1 unit of *Taq* polymerase. The PCR reaction was performed at 94°C for 5 minutes; then, for 35 cycles of 94°C for 1 minute; 50-67°C for 1 minute; 72°C for 2 minutes followed by 72°C for 5 minutes. PCR products were resolved by electrophoresis in 3.5% agarose gel containing 0.5 µg mL⁻¹ ethidium bromide. Restorers were identified by 350 and 150 bp bands using SSR marker RM171 and RM258, respectively.

Linkage Analysis

Polymorphic primers between parents Neda A and Pajouhesh were used for linkage

analysis on completely sterile plants from the F₂ population of Neda A / Pajouhesh. Linkage groups were assigned to the corresponding chromosomes based on SSR markers mapped by McCouch *et al.* (2002). For single-marker analysis, the recombination frequency between a positive marker and an *Rf* locus was calculated using maximum likelihood estimator (Allard, 1956), assuming that all the extremely sterile individuals were homozygous at the targeted *Rf* locus.

RESULTS

Polymorphism Detection

Twelve microsatellite primers that were reported to be linked with fertility restoring genes in different chromosomal locations (chromosomes 1, 7, 10, and 12) (Bazrkar *et al.*, 2008) were employed for polymorphism survey between the parents of 'Neda A' and 'Pajouhesh'. Microsatellite primers RM171 and RM258, both located on chromosome 10 of rice, and RM3148 on chromosome 1 showed polymorphism for *Rf4* and *Rf3* loci, respectively (Figure 1). In this study, SSR markers RM1, RM443, RM315 and RM294 did not show any polymorphism for *Rf3* located on chromosome 1.

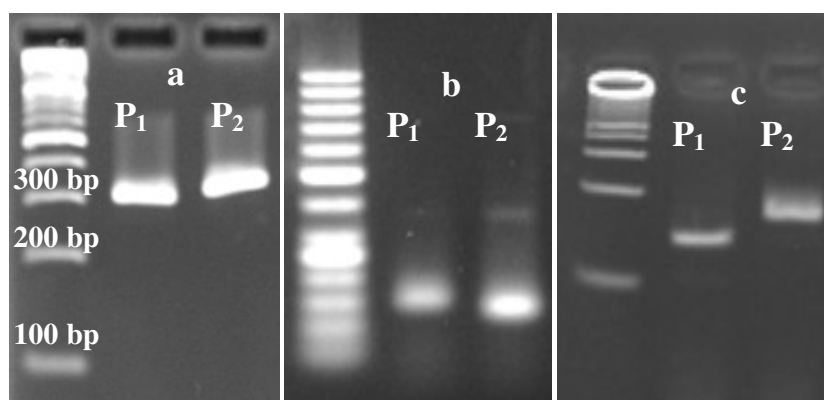


Figure 1. Polymorphism detection on parental lines Neda A (P₁) and Pajouhesh (P₂) using SSR markers RM171 (a), RM258 (b) and RM3148 (c). Lane 1, 100bp ladder.

Linkage Analysis

We used polymorphic primers for linkage analysis on completely sterile plants from F₂ population of 'Neda A' / 'Pajouhesh' and found that RM258 and RM171 were closely linked to restorer gene *Rf4* at the intervals of 3.1 and 6.3 cM from it (Table 3). In addition, SSR marker RM3148, which was previously mapped on chromosome 1, was also proved to be linked with *Rf3* gene at a genetic distance of 19.7 cM.

Genotyping of Rice Lines for *Rf* Loci

DNA Marker Assisted Selection (MAS) was used for WA fertility restoration in a set of 15 rice genotypes to determine the precise genotype of some rice cultivars in each marker locus. The major gene, *Rf4*,

underwent MAS using SSR marker RM171 and RM258. Using these two markers, restorers were identified by 350 and 150 bp bands when resolved in an agarose gel, respectively. Results of this test in comparison with two known restorers (Sepidroud and Pajouhesh) showed that some of these genotypes, such as Pouya, Hashemi, Khazar, and Deylamani, carried *Rf4* gene on the long arm of chromosome 10 based on presence of restorer bands with either or both of RM258 and RM171 (Table 4). MAS was used for another fertility restoring gene i.e. *Rf4*, using SSR marker RM3148 located on short arm of chromosome 1. Cultivars Hashemi, Shastak and Deylamani possess *Rf4* allele in their genome (Figure 2). This study revealed that cultivars Hashemi and Deylamani carry both of *Rf* genes considered as suspected restorers and suggested to be test crossed with CMS lines to assess the restoration of fertility in their F₁ offsprings.

Table 3. Recombination frequencies and genetic distances between the positive markers and the *Rf* locus based on the assumption that all the extremely sterile plants are homozygous for the recessive allele at targeted loci.

Locus	Chromosome	Recombination frequency (%)	Genetic distance (cM)	LOD score
RM258	10	3.12	3.13	3.85
RM171	10	6.25	6.28	3.19
RM591	10	37.5	37.95	0.22
RM3148	1	18.75	19.71	1.46

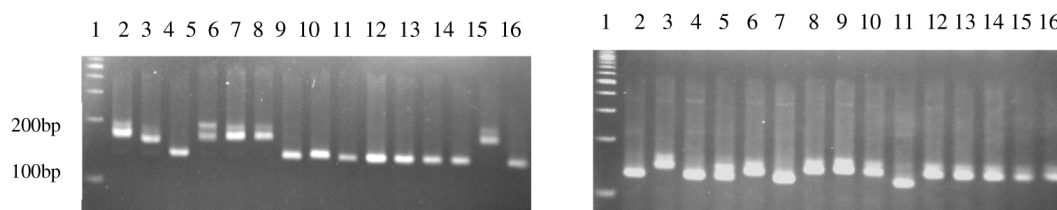


Figure 2. Molecular assay using markers RM258 (right) and RM3148 (left) for *Rf* genes in rice lines. Lane 1 is 100 bp ladder, lanes 2 to 16 are Pajouhesh (Restorer), Sepidroud (Restorer), Pouya, Hashemi, Shastak, Deylamani, Shiroudi, Tabesh, Fajr, Khazar, Shafaq, Neda (B-Line), Nemat (B-Line), Dasht (B-Line) and Champa (B-Line), respectively.

Table 4. Molecular screening of rice lines for validation of SSR markers for identification of *Rf3* and *Rf4* genes.

Line	Type	Chromosome 10		Chromosome 1
		RM258	RM171	RM3148
Sepidroud	Restorer	NR ^a	R ^b	R
Pajouhesh	Restorer	R	R	R
Neda	Maintainer	NR	NR	NR
Nemat	Maintainer	NR	NR	NR
Dasht	Maintainer	NR	NR	R
Champa	Maintainer	NR	NR	NR
Pouya	Conventional	R	NR	NR
Hashemi	Conventional	R	NR	R
Shiroudi	Conventional	NR	NR	NR
Tabesh	Conventional	NR	NR	NR
Shastak	Conventional	NR	NR	R
Fajr	Conventional	NR	NR	NR
Khazar	Conventional	R	NR	NR
Shafaq	Conventional	NR	NR	NR
Deylamani	Conventional	R	NR	R

^a non-restorer band, ^b restorer band,

DISCUSSION

Genetic analysis of fertility restoring genes revealed existence of two dominant genes. One *Rf* gene was on rice chromosome 10 and the second on chromosome 1, in accordance with several other researches (Govinda Raj and Virmani, 1988; Teng and Shen, 1994). Conventionally, restorers are identified by test-crossing a large number of genotypes with CMS lines and then evaluating their progeny for pollen and spikelet fertility. This method is laborious, time-consuming, and less accurate. There is,

therefore, a need to identify molecular markers that are tightly linked to *Rf* genes so that MAS can be routinely done to identify restorers more quickly and more efficiently. MAS has been successfully used for restorer gene detection by several researchers (Ichikawa *et al.*, 1997; Wang *et al.*, 2012; Suresh *et al.*, 2012).

The result of molecular assay on Pouya cultivar revealed monogenic nature for its fertility restoration ability, which is in consistent with the findings of Bagheri and Babaeian-Jelodar (2011) who have reported a single dominant *Rf* gene for Pouya in F₂ and BC₁ segregating populations. In



addition, the result of molecular assay on Fajr cultivar revealed maintainer nature for its fertility restoration ability, which is in consistent with the findings of Nematzadeh and Sattari (2003) who have reported maintainer genomic nucleolus for that variety. MAS also showed that cultivars Shiroudi, Tabesh, Fajr, and Shafaq were non-restorer (maintainer) lines. Thus, backcross breeding method can be applied on these lines for transferring sterile cytoplasm to them for development of new CMS lines.

The results this study indicate that the microsatellite markers RM258, RM171 and RM3148 are suited for marker assisted selection of restorer lines from large source nurseries. Using these markers, primary selection can be carried out at seedling stage, hence, works required for test crosses by breeders would be reduced. Further studies are needed to confirm the efficiency of MAS through crossing between suspected restorers with CMS lines and fertility evaluation of F₁ plants.

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اعتبار سنجی نشانگرهای SSR همبسته با ژن‌های اعاده کننده باروری (*Rf*) و تعیین

ژنوتیپ لاین‌های برنج در مکان‌های ژنی *Rf*

غ. کیانی

چکیده

مطالعه حاضر با هدف اعتبار سنجی نشانگرهای SSR همبسته با ژن‌های *Rf* و استفاده از انتخاب به کمک نشانگر برای تشخیص لاین‌های اعاده کننده و غیر اعاده کننده باروری در سیستم نرعقیمی



سیتوپلاسمی نوع WA انجام گرفته است. دوازده نشانگر SSR که همبستگی آنها با ژنهای Rf گزارش شده است در جامعه نقشه کشی نداA/پژوهش مورد ارزیابی قرار گرفتند. از بین آنها سه نشانگر RM258، RM171 و RM3148 با ژنهای Rf همبسته بودند. MAS با استفاده از RM258 و RM171 (مهمترین مکان ژنی Rf روی کروموزم ۱۰) و RM3148 (دیگر مکان ژنی Rf روی کروموزم ۱) روی مجموعه‌ای از لاین‌ها شامل ۲ اعاده کننده، ۴ نگهدارنده و ۹ رقم معمولی (جمعاً ۱۵ ژنوتیپ)، هر دو آلل Rf در ارقام هاشمی و دیلمانی مشابه لاین‌های اعاده کننده باروری تکثیر شدند. درحالیکه ارقام پویا، خزر و شصتک دارای یک مکان ژنی Rf (اعاده کننده ناقص) بودند. ارقام شیرودی، تابش، فجر و شفق به عنوان لاین‌های غیر اعاده کننده (نگهدارنده) شناسائی شدند. نتایج نشان داد که از این نشانگرها برای غربالگری ژنوتیپ‌ها به منظور شناسائی لاین‌های اعاده کننده از غیر اعاده کننده در برنامه‌های اصلاح برنج هیبرید می‌توان استفاده نمود.