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Full Length Research Paper

Genetic analysis of fertility restoration genes for WA-type cytoplasmic male sterility in Iranian restorer rice line DN-33-18

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Cytoplasmic genetic male sterility system can be used to exploit heterosis in grain crops only when the effective restorer lines are available. Crosses between an Iranian CMS line Neda A with three newly developed lines DN-33-1, DN-33-18 and DN-32-6 showed that only the cross of Neda-A/DN-33-18 had more than 80% pollen and grain fertility in F_1 generation. The inheritance of fertility restoration in F_2 population of this cross was evaluated at flowering and grain filling stages. Pollen staining test with 1% I_2KI solution showed segregation ratio of 15:1 (fertile: sterile), representing two nuclear independent dominant genes controlling the trait carried by fertile parent DN-33-18. Segregation for spikelet fertility in F_2 confirmed the results of pollen fertility test. Molecular tagging of fertility restorer genes with SSR markers showed that four markers RM258, RM171, RM591 and RM3148 produced polymorphic bands between two parents. Linkage analysis on F_2 recessive class showed that RM258 and RM171 (on chromosome 10) flanked to restorer gene *Rf4* at the distances of 3.1 and 6.3 cm, respectively. In this study polymorphism was not detected for *Rf3* gene using SSR markers RM1, RM443, RM315 and RM294 on chromosome 1 of rice but we found new SSR marker RM3148 linked with *Rf* locus at a genetic distance of 19.7 cm.

Key words: Rice, cytoplasmic male sterility (CMS), inheritance, molecular tagging, simple sequence repeat (SSR) markers.

INTRODUCTION

Plant cytoplasmic male sterility (CMS) caused by lesion or rearrangement of mitochondrial genome is unable to produce functional pollens. But CMS can be restored by nuclear genes. Therefore, the CMS systems are widely used for hybrid seed production. In rice, more than 90% of the rice hybrids developed over these years belongs to wild abortive cytoplasmic source (Yao et al., 1997).

Hybrid breeding based on CMS/*Rf* system has achieved great success all over the world. Three primary types of CMS in rice are known, and their heritance habits and physiological characteristics have been extensively investigated. They are Wild-rice abortive (WA), BaoTai (BT) and HongLian (HL). WA type CMS belongs to sporophytic abortion, which fails to produce normal pollen and finally forms typical abortive pollen. In contrast, BT (*japonica*) and HL type CMS (*indica*) belong to gametic abortion, but they are also greatly different in terms of abortive phenotype, relationship of restoration, and maintenance (Sattari et al., 2008).

BT type CMS is restored by nuclear fertility restorer gene *Rf1*, which was mapped on chromosome 10 (Shinjio, 1975; Fukuta et al., 1992; Akagi et al., 1996; Yokozeki et al., 1996). HL type fertility restoration gene *Rf5* was also mapped on chromosome 10 (Huang et al., 2003). The inheritance of fertility restoration in WA-CMS system has been extensively investigated. Using RFLP markers, Zhang et al. (1997) mapped one of the two *Rf* loci (*Rf3*) on chromosome 1 between RG140 and RG532

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Abbreviations: CMS, Cytoplasmic male sterility; **WA,** wild-rice abortive; **BT,** BaoTai; **HL,** HongLian; **RAPD,** randomly amplified polymorphic DNA; **RFLP,** restriction fragment length polymorphism; **MAS,** marker assisted selection; **SSR,** simple sequence repeat; **NILs,** near isogenic lines.

Table 1. List of the used SSR primer pairs with their chromosomal locations and annealing temperature.

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
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 tcg c	55
RM244	10	ccg act gtt cgt cct tat ca	ctg ctc tcg 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 tcg ccg gtc aag aac	55
RM7003	12	ggc aga cat aca gct tat agc	tgc aaa tga acc cct cta gc	55

at a distance of 1.9 cm from each. Using RAPD and RFLP markers, Yao et al. (1997) confirmed the location of *Rf3* on chromosome 1 and mapped the second *Rf* locus (Rf_4) on chromosome 10 at 3.3 cm from G4003. Jing et al. (2001) mapped an *Rf* locus (Rf_4) 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.

The most straightforward method of marker identification would be finding different banding patterns in two sets of lines representing contrasting phenotypic classes. The main precondition for starting molecular analysis aimed at finding useful markers for a given trait is a basic knowledge of its inheritance. This is usually gained through classical population genetic analysis. Recognition of the gene or genes underlying the trait under study opens the possibility of looking for linked molecular markers. Detection of segregation mode of fertility restoring genes in newly developed Iranian *indica* rice DN-33-18 and identifying molecular markers linked to that trait for use in marker assisted selection (MAS) was the aims of this study.

MATERIALS AND METHODS

Plant materials and population development

In this study an Iranian CMS-WA line Neda A, with newly developed cultivars DN-33-1, DN-33-18 and DN-32-6 were crossed in 2007 and F_1 seeds were obtained. Neda A has been improved through backcross method using IR58025A from IRRI (Nematzadeh et al., 2006). DN-33-1, DN-33-18 and DN-32-6 are advanced lines under release in north of Iran, Mazandaran province which improved through pedigree-backcross method from the cross between Sepidroud and Sang Jo. Only the cross of Neda-A / DN-33-18 had more than 80% pollen and grain fertility in F_1 generation. So, the inheritance of pollen fertility restoration in F_2 population of this cross including 328 individual plants was evaluated at flowering and grain filling stages.

Evaluation of pollen and spikelet fertility

In pollen fertility test, 3 panicles per plant were selected to prepare pollen samples and 1% I2-KI solution was used to identify fertile and sterile pollens. Pollen fertility was evaluated by shape, size and stained color: Fertile-grain with morphologically spherical and darkly stained, and sterile-grain with spherical but small and stained in light brown. In spikelet fertility test, 3 panicles per plant were bagged with paper pocket to prevent outcrosses and percentage of fertile spikelets was calculated. X^2 test was used to test fitness of observed genetic ratio with expected ratio in F_2 population.

DNA extraction and PCR analysis

Total genomic DNA was extracted according to Dellaporta et al. (1983). PCR amplification was performed using the DNAs from parental lines Neda A and DN-33-18 along with completely sterile plants from F_2 population of Neda A/DN-33-18. The DNAs were subjected to the amplification by 11 SSR markers (Table 1). A 25 µl mixture was prepared for the PCR assay containing 50 ng template DNA, 2.5 µl of 10X buffer, 0.3 µl of 10 mM dNTPs, 1 µl of 50 mM $MgCl_2$, 1 µl of each primer, and 1 unit of *Taq* polymerase. The PCR reaction was performed at 94°C for 5 min (initial denaturation); then for 35 cycles of 94°C for 1 min; 50 - 67°C for 1 min; 72°C for 2 min followed by 72°C for 5 min. PCR products were resolved by electrophoresis in 3.5% agarose gel containing 0.5 µg/ml ethidium bromide.

Linkage analysis

Map distances were based on the Kosambi function (Kosambi, 1944). Linkage groups were assigned to 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.

Table 2. Testcross of advanced lines with Neda A for determination of their ability to restoration of fertility in CMS-WA system.

Cross	Pollen fertility	Spikelet fertility	Ability to restoration of fertility
NedaA/ DN-33-1	<80	<80	Partial restorer
NedaA/ DN-33-18	>80	>80	Restorer
NedaA/ DN-32-6	<80	<80	Partial restorer

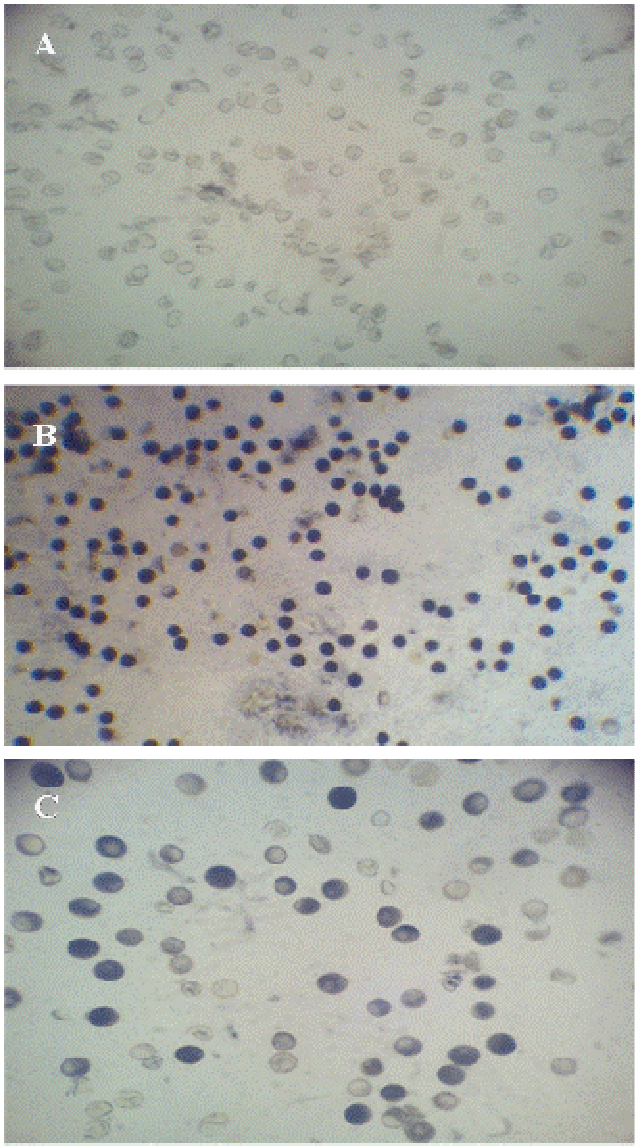

Figure 1. Segregation of pollen fertility in F_2 population of NedaA/DN-33-18 using 1% I_2KI solution. Completely sterile plants (A) vs. completely and partial fertile plants (B, C).

RESULTS

Segregation of fertility restoration

Taking seed setting rate as fertility criterion, the F_1 Neda A/DN-33-18 showed more than 80% fertility (Table 2) so this cross was chosen to study of genetic bases of fertility restoration for Neda A with WA type of sterile cytoplasm. Lines DN-33-1 and DN-32-6 showing partial fertility in test cross with CMS line Neda A.

The pollen fertility test in F_2 population of Neda A/DN-33-18 revealed segregation into 312 fertile and 16 completely sterile plants (Figure 1), indicating that the expected 15 : 1 (fertile: sterile) ratio is good (Table 3).

Detection of fertility restoration genes in DN-33-18

Eleven microsatellite primers that were reported to link with fertility restoring genes in different chromosomal locations (chromosomes 1, 7, 10 and 12) were screened for polymorphism between the parents. Microsatellite primers RM171, RM258 and RM591 all on chromosome 10 of rice produced polymorphic products. Then these primers were used in linkage analysis with 16 completely sterile plants from F_2 population of NedaA/DN-33-18. Linkage analysis on recessive class showed that RM258 and RM171 closely linked to restorer gene *Rf4* at the intervals of 3.1 and 6.3 cm from it (Table 4). In this study, analysis of SSR markers for *Rf3* on chromosome 1 shows no polymorphism using RM1, RM443, RM315 and RM294 markers. Extra analysis with other SSR markers achieved for detection of chromosomal location of second *Rf* gene in Iranian restorer line DN-33-18. We detected new SSR marker RM3148 on chromosome 1 (Figure 2) linked with second *Rf* gene at a genetic distance of 19.7 cm in line DN-33-18 (Figure 3).

DISCUSSION

Classical analysis of fertility restoring genes showed that fertility restoration for the Neda A with WA type of cytoplasm is controlled by two dominant genes carried by Iranian *indica* line DN-33-18. The study result is in accordance with several studies on *indica* rice (Zhou, 1983; Young and Virmani, 1984; Govinda Raj and Virmani, 1988; Teng and Shen, 1994).

This study, successfully tagged two *Rf* genes in restorer line DN-33-18 for WA type of CMS system. One *Rf* gene on rice chromosome 10 flanked by RM258 and RM171 markers. Sattari et al. (2008) clarified that *Rf* genes for WA-, HL- and BT-type CMS restoration systems were located on chromosome 10, and this *Rf*

Table 3. Distribution for pollen and spikelet fertility (numbers in parentheses) in parents, F_1 and F_2 population of the cross Neda A/DN-33-18.

Progeny	Fertile	Completely sterile	Genetic ratio	$\chi^{2\,a}$
Neda A	-	10		
DN-33-18	10	-		
F_1	10	-		
F_2	312 (316)	16 (12)	15: 1	X^2=1.054 ^{ns} (3.760 ^{ns})

[a] Critical χ^2 for 1 degree of freedom is 3.84 (p = 0.05).

ns: Not significant at 5% statistical level.

Table 4. Recombination frequencies and genetic distances between the positive markers and the *Rf* locus calculated using Maximum likelihood method, 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	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

LOD, Limit of detection.

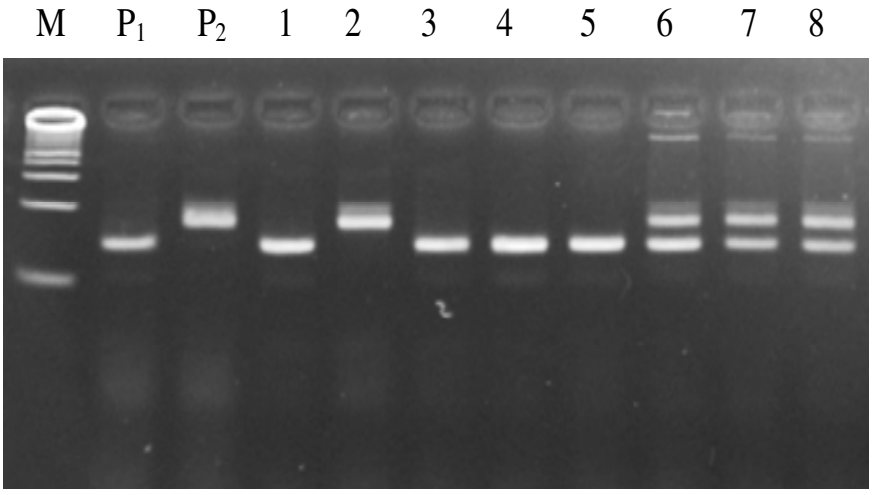

Figure 2. Linkage analysis on sterile plants from F_2 population of Neda A/DN-33-18 using SSR marker RM3148 located on chromosome 1 of rice. M: 100bp ladder, P_1: Neda A, P_2: DN-33-18 and numbers 1 to 8 are some of individual sterile plants.

gene that was detected is located at the same region. The second *Rf* gene located on chromosome 1 of rice was not linked with SSR markers that was reported to be link with *Rf3* gene but this study found a new marker RM3148 on short arm of chromosome 1 at a distance of 19.7 cm. Therefore, the inheritance mechanism and fine mapping of this gene and its relation with *Rf3* is yet to be elucidated.

Marker assisted selection (MAS) is being explored as an important supplement to phenotypic selection in rice breeding. PCR based markers offers great potential to enhance the efficiency of MAS. SSR markers have the advantages of rapidity, straight, and simplicity of RAPD, and the stability, reliability, and repeatability of RFLP. The results presented here clearly indicate that the micro-satellite markers RM258, RM171 and RM3148 will be facilitating MAS of restorer lines in CMS-WA system from large source nurseries to avoid routine testcrosses in hybrid breeding programs. It is also expected that the use of these microsatellite markers in MAS integrated with

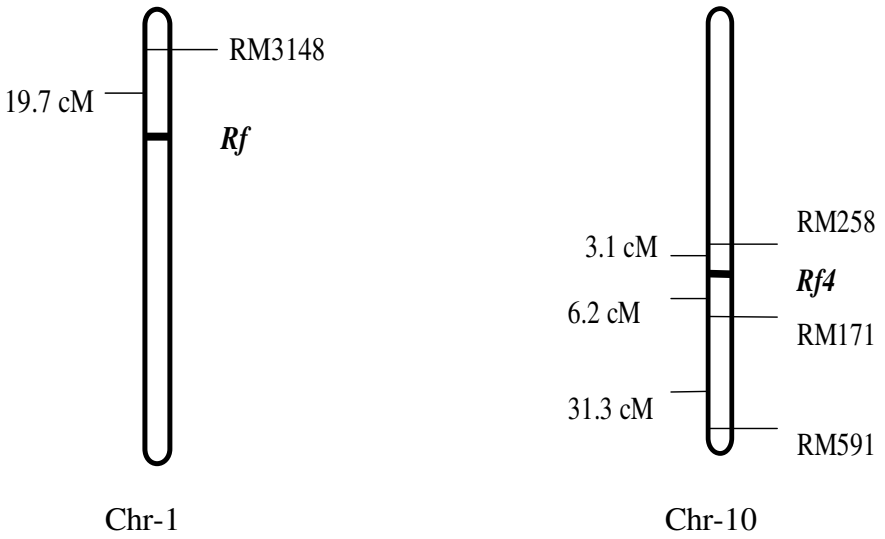

Figure 3. Linked SSR markers with fertility restoring genes on chromosomes 1 (left) and 10 (right) of rice.

backcross breeding will produce near isogenic lines (NILs) of fertility restorer lines for genetic research.

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