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Inheritance of the fertility restoration and genotyping of rice lines at the restoring fertility (*Rf*) loci using molecular markers

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Abstract

The combination of cytoplasmic male sterility (CMS) in one parent and a restorer gene (*Rf*) to restore fertility in another are indispensable for the development of hybrid varieties. To genotype rice lines at the restoring fertility (*Rf*) loci, 38 lines were crossed with a sterile tester (*rfrf*) line. Pollen fertility test was performed to identify sterile and fertile F_1 hybrids. Seven lines were identified as restorer lines. Then these F_1 hybrids were self-pollinated to obtain F_2 seeds, and at flowering stage pollen fertility test was performed to identify sterile and fertile individuals. Bulked segregant analysis (BSA) in F_2 showed that fertility in rice WA system is controlled by more than two loci, one on the short arm of chromosome 1, one on the short arm of chromosome 10, one on the long arm of chromosome 10 and an unknown *Rf* gene in the rice genome. Results also show that lines *IR28*, *Amol1* and *Amol2* carry *Rf4* gene linked with SSR marker RM171 on the long arm of chromosome 10, lines *IR36* and *IR60966* carry *Rf3* gene linked with SSR marker RM1 on the short arm of chromosome 1, line IR62030 carries *Rf5* gene on the short arm of chromosome 10, and finally line *IR24* carries *Rf4* gene on the long arm of chromosome 10 and an unknown *Rf* gene, respectively. The results suggested the use of the method deployed here to identify restorer lines and also allelic location of fertility restoration genes in rice lines.

***Keywords*:** Molecular markers; *Rf* Genes; Rice.

Introduction

Rice has been one of the most important plants locating at the forefront of plant genomics because of its small genome size and relatively low amount of repetitive DNA, its diploid nature, and its ease of manipulation in tissue culture. In the 1990s, many advances

occurred in the application of molecular markers in rice (see reviews in Mackill and Ni 2001; Temnykh et al., 2001).

Discovery of genes controlling economically important traits can be used for applications in marker assisted selection (MAS), improving rice through transgenic approaches, and discovery of new beneficial alleles in the germplasm. A significant advance in the practical utilization of molecular markers was the development of SSR markers, also referred to as microsatellite markers (McCouch et al., 1997). These markers are highly polymorphic and easy to detect. The high polymorphism means that these markers can be used in germplasms that is closely related (Ni et al., 2002; Yang et al., 1994). Recently, a fairly dense SSR map of rice has been published (McCouch et al., 2002). Mapping agronomically important genes can provide useful information for plant breeders. This is particularly true in the case of traits that are controlled by multiple genes with similar phenotypes.

Molecular markers are particularly useful for accelerating the backcrossing of a gene or QTL into an elite cultivar or breeding line. Markers linked to the gene can be used to select plants possessing the desired trait, and markers throughout the genome can be used to select plants that are genetically similar to the recurrent parent (Hospital et al., 1992; Young and Tanksley, 1989). This approach is thought to be promising in rice because a number of rice cultivars are widely grown for their adaptation, stable performance, and desirable grain quality.

Use of MAS will be more beneficial for specific applications including selection for traits that are difficult or expensive to measure (e.g., salt tolerance, restorer genes); pyramiding multiple genes that confer a similar or identical phenotype (e.g., multiple genes for resistance to blast or bacterial blight); or selecting against the donor chromosomal segments in a backcrossing scheme (Mackill and Ni 2001; Ribaut and Hoisington 1998; Young and Tanksley, 1989).

Cytoplasmic male sterility (CMS) caused by lesions or rearrangements of mitochondrial genome is unable to produce functional pollens. But CMS can be restored by nuclear genes. The combination of cytoplasmic male sterility (CMS) in one parent and a restorer gene (Rf) to restore fertility in another are indispensable for the development of hybrid varieties. Therefore, the CMS systems are widely used for hybrid seed production (Yuan, 1992). However, searching for restorer genes is a good example where phenotyping is very time-consuming and requires determination of spikelet sterility in testcross progeny (Komori et al., 2003; Yao et al., 1997; Ahmadikhah and Karlov, 2006).

Most investigators tended to agree that restoration of wild abortive (WA) type CMS in rice is controlled by two nuclear genes (Zhang et al., 1997; Yao et al., 1997; Zhang et al., 2002). Bao Tao (BT) type CMS is restored by nuclear fertility restorer gene *Rf1*, which was mapped on chromosome 10 (Fukuta et al., 1992; Akagi et al., 1996; Yokozeki et al., 1996) and finally was cloned by several groups (Kazama and Toriyama, 2003; Komori et al., 2004; Akagi et al., 2004). HL type fertility restoration genes *Rf5* and *Rf6(t)* were also mapped on chromosome 10 (Liu et al., 2004).

The cytoplasm derived from wild rice, causes WA-type CMS in a sporophytic manner and is widely used for the production of rice hybrid seeds. Two fertility restorer genes, *Rf3* and *Rf4*, are required for the production of viable pollen in WA-type CMS. These genes

have been mapped to chromosomes 1 and 10, respectively (Yao et al., 1997; Zhang et al., 1997, 2002; Jing et al., 2001).

In this study we investigate the inheritance of the fertility restoration of WA CMS system, and attempt to genotype rice lines at the restoration fertility loci and identify molecular markers linked to the *Rf* genes.

Materials and Methods

Plant materials

Plant materials included 38 different rice lines (obtained from Rice Research Institute, Amol, Northern Iran) as paternal lines and one CMS line (*Neda-A*) as maternal line in crossing. These paternal lines were crossed to CMS line (*Neda-A*), and the F_2 generations were obtained from self-pollinating of F_1 hybrids. In the F_2 generations ~ 40-50 plants were analyzed in molecular experiments.

Screening for fertility restoration

The fertility of F_1 hybrids was analyzed using staining method of pollen grains with 1% I2-KI solution as described by Li et al. (1993). Parental lines of hybrids which had above 80 percents fertile pollens were classified as restorer lines. In the basis of the results from this test the putative restorer lines were selected and grown along with the corresponding F_2 generations. Pollen fertility test also was conducted in the F_2 generation to identify sterile and fertile individuals.

Bulked segregant analysis (BSA) and PCR conditions

Total genomic DNA was extracted using the CTAB method (Saghai-Maroof et al., 1984) with some modifications as described elsewhere (Ahmadikhah, 2006).

Two sterile and fertile bulks were formed with mixing the DNA from 10 corresponding F_2 plants. PCR amplification was performed using the DNAs from putative restorer lines, two corresponding sterile and fertile bulks and F_2 individuals. For detection of polymorphism between parental lines, their DNAs were supposed to the PCR-amplification by SSR markers RM1, RM171, RM244 and RM311. PCR was performed in 10 µl volumes containing 0.2 µM/l of each primer, 200 µM/l dNTPs, 50 mM/l KCl, 10 mM/l Tris-HCl, 1.5 mM/l $MgCl_2$, and 1 unit of Taq DNA polymerase (Qiagen). The PCR profile was 94 ^oC for 5 min (denaturation), followed by 35 cycles of 94 ^oC for 1 min, 55 ^oC for 1 min, 72 ^oC for 2 min, and finally 72 ^oC for 7 min in the final extension. The products from PCR reaction were resolved by electrophoresis in 1.5% agarose gel containing 0.5 µg/ml ethidum bromide.

Results and Discussion

The study of the inheritance of fertility restoration

Pollen fertility test of F_1 hybrids showed that seven hybrids had >80 percents fertile pollens. Therefore, their paternal lines were classified as putative restorer lines. Results from this test showed that lines *IR24*, *IR28, IR36, IR62030, IR60966, Amol1* and *Amol2* are putative male-fertility restorer lines (Table1).

Results from fertility test in F_2 populations showed that for all crosses, but for *Neda-A/IR24* in which F_2 individuals segregated into 15 fertile: 1 sterile plants, remaining F_2 populations were segregated into 3 fertile: 1 sterile plants (Table2). These results indicate that pollen fertility is controlled by one major gene in lines *IR28, IR36, IR62030, IR60966, Amol1* and *Amol2*, and by two major genes in line *IR24*.

Plants of F_2 populations have been analyzed on pollen fertility. The F_2 population from *Neda-A/IR24* cross was segregated in 109 fertile plants and 6 completely sterile plants, that corresponds to the twogenic ratio of 15:1 ($\chi^2_{fact} = 0.22$; $\chi^2_{(05,df=1)} = 3.84$) and significantly deviates from 3:1 ratio ($\chi^2_{fact} = 24.0$; $\chi^2_{(05,df=1)} = 3.84$). Further analysis in fertile class for *NedaA/IR24* cross (that is; 10-100% pollen fertility) with dividing it into 3 classes including fertile (>85%), partial fertile (70-85%) and partial sterile (>10-70%), was resulted to a good fit to digenic ratio 9:3:3:1 ($\chi^2_{fact} = 6.34$; $\chi^2_{(05, df=3)} = 7.82$, Figure 1).

Table1. The putative restorer lines identified in this study, pollen fertility of the F_1 hybrids and F_2 populations

Cross	Pollen fertility (%) (Mean±SE)	
	F_1	F_2
Neda-A / IR24	87.6 ± 2.19	77.7 ± 4.08
Neda-A / IR28	85.5 ± 1.64	64.4 ± 5.09
Neda-A / IR36	86.3 ± 2.25	61.8 ± 5.07
Neda-A / IR62030	84.7 ± 1.96	52.7 ± 5.46
Neda-A / IR60966	81.8 ± 1.74	59.4 ± 5.42
Neda-A /Amol1	83.9 ± 1.39	69.8 ± 4.92
Neda-A /Amol2	85.5 ± 2.15	65.6 ± 4.63

It seems that the restoration ability of *IR24* is governed by two independent major genes. The inheritance of fertility restoration in WA type CMS has been extensively investigated. The result obtained for *IR24* in our study is consistent with the fact that most investigators agree that restoration of WA type CMS is controlled by two nuclear genes (Zhang et al., 1997; Yao et al., 1997; Zhang et al., 2002). As shown in figure1-A, there are two picks for pollen fertility, indicating existence of two genes controlling the trait in F_2 generation, which one of these genes with higher pick has a stronger fertility restoration ability than the other, so that if both genes (*Rf3Rf3/Rf4Rf4*) are present as double dominant, fertility is like the restorer line, *IR24*; if the gene with stronger fertility restoration ability

(*rf3rf3/Rf4Rf4*) is present alone, fertility is somewhat reduced, but if the gene with weaker restoration ability (*Rf3Rf3/rf4rf4*) is present alone, plants show partial fertility ranging between 10-70 %. The plants possessing the double recessive genotype (*rf3rf3/rf4rf4*) are completely sterile like *Neda-A*.

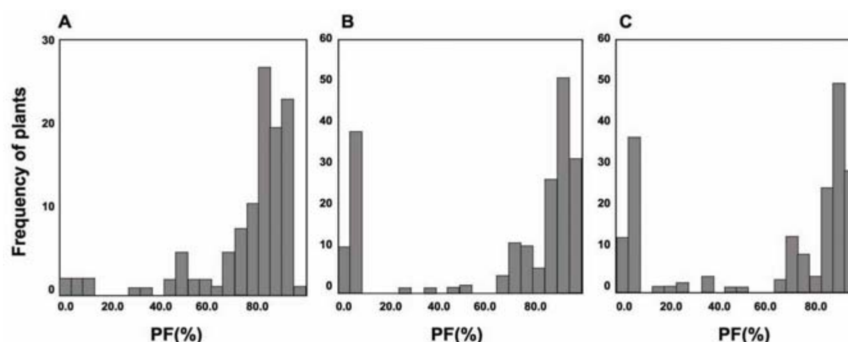

Figure 1. Distribution pattern for pollen fertility (PF) in F_2 generation of crosses: A) *NedaA*/*IR24*, B) *NedaA/IR28* and C) *NedaA/IR36*.

The F_2 population of crossing *Neda-A/IR28* was segregated into 144 fertile plants and 53 completely sterile plants, that corresponds to the monogenic ratio of 3:1 ($\chi^2_{fact} = 0.002$; $\chi^2_{05} = 3.84$) with an average value of pollen fertility of 64.4%. In F_2 population of crossing *Neda-A/IR36* it has been segregated into 139 fertile plants and 54 completely sterile plants, that also corresponds to the monogenic ratio of 3:1 ($\chi^2_{fact} = 0.08$; $\chi^2_{05}= 3.84$) with an average value of pollen fertility of 61.8 %. Results of tests on pollen fertility for other populations are given in Tables 1 and 2.

BSA analysis

For genotyping the identified putative restorer lines, PCR amplification of eight genotypes (*Neda-A*, *IR24*, *IR28*, *IR36*, *IR62030*, *IR60966*, *Amol1* and *Amol2*) were carried out, using 2 SSR primers, having a tight linkage with *Rf3* and *Rf4* genes. It has been reported that SSR marker RM1 is linked with *Rf3* gene on the short arm of 1st chromosome (He et al., 2002), and RM171 with *Rf*4 on the long arm of 10th chromosome (Jing et. al, 2001).

Table 2. Distribution of frequency of pollen fertility and segregation type in F_2 generations.

F_2 population	Fertility (%)				Segregation
	<10	10-70	70-85	>85	
Neda-A/IR24	6	18	32	59	$\chi^2_{15:1}=0.21^{n.s}$ $\chi^2_{9:3:3:1}=6.34^{n.s}$
Neda-A/IR28	49	19	24	105	$\chi^2_{3:1}=0.002^{n.s}$
Neda-A/IR36	50	24	19	100	$\chi^2_{3:1}=0.08^{n.s}$
Neda-A/IR62030	52	28	25	67	$\chi^2_{3:1}=2.51^{n.s}$
Neda-A/IR60966	12	9	11	10	$\chi^2_{3:1}=0.29^{n.s}$
Neda-A/Amol1	72	32	47	79	$\chi^2_{3:1}=0.98^{n.s}$
Neda-A/Amol2	9	9	12	11	$\chi^2_{3:1}=0.29^{n.s}$

Both primer pairs detected polymorphism between CMS line (*Neda*-A) and 7 fertile lines (Figure 2). Although for the marker loci a polymorphism between sterile line and all restorer lines were observed, the existence of a polymorphism between sterile and fertile lines is not adequate to associate this polymorphism with the existence of a linkage between marker and the given gene. In such cases that used markers are not specific for a gene, but are located in a tightly distance from, it is better to follow the co-segregation of the marker and the given trait in a segregating generation using bulked segregant analysis (BSA).

For carrying out the BSA analysis, individual F_2 plants were divided in two groups, each consisting of ten plants. Thus, groups of plants differed among themselves on alleles of the studied gene; distribution of other traits within the limits of group was random. The result of this initial testing has shown that SSR marker RM1 in populations *Neda-A/IR36* and *Neda-A/IR60966*, is linked with *Rf3* gene on the short arm of 1st chromosome, and RM171-with *Rf4* in populations *Neda-A/IR24*, *Neda-A/IR28*, *Neda-A/Amol1* and *Neda-A/Amol2* on the long arm of 10-th chromosome. In the case of population Neda-A/IR62030, both sterile and fertile groups amplified identical bands (Figure 3).

Bulked segregant analysis (BSA) in F_2 generations revealed the probable genotype of putative restorer lines. No co-segregation was observed in the F_2 generation of crosses *NedaA/IR62030*, *NedaA/IR60966* and *NedaA/IR36* between the Rf gene and the marker locus of RM171 on the long arm of chromosome 10, indicating that *Rf* gene carried by these lines is located on a chromosome other than chromosome 10 or adequately far from this locus, while in F_2 generation of crosses *NedaA/IR28*, *NedaA/IR24*, *NedaA/Amol2* and *NedaA/Amol1* at the marker locus was observed co-segregation between marker and the trait, indicating that Rf gene carried by these lines is located on chromosome 10 (Table 3).

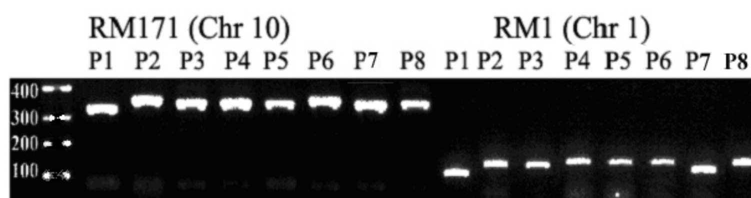

Figure 2. Polymorphism produced by SSR marker RM171 (lanes 2-9) and RM1 (lanes 10-17) between sterile line (*Neda-A*) and different restorer lines. Lane 2 and 10 are sterile line *Neda-A*. Lanes 3&11, 4&12, 5&13, 6&14, 7&15, 8&16 and 9&17, respectively, are seven restorer lines *IR24, IR28, Amol2, IR62030, IR36, IR60966* and *Amol1*.

To determine the precise genotype of each paternal line in each marker locus, a small number of plants from each F_2 population have been chosen to test. PCR amplification carried out with RM1 and RM171 markers. Results of this test are shown in table 3. As seen in the table, lines *IR36* and *IR60966* carry *Rf3* gene on the short arm of chromosome 1, lines *IR24*, *IR28*, *Amol1* and *Amol2* carry the *Rf4* gene on the long arm of chromosome 10, and line *IR62030*, by far, carries an unknown *Rf* gene (*Rf?*). As the results of the pollen fertility test specify the existence of 2 genes in line *IR24* (segregation as 15: 1 in F_2 population of *Neda-A/IR24*), this line in addition to the *Rf4* gene, carries one more unknown gene (*Rf?*) (Table 3).

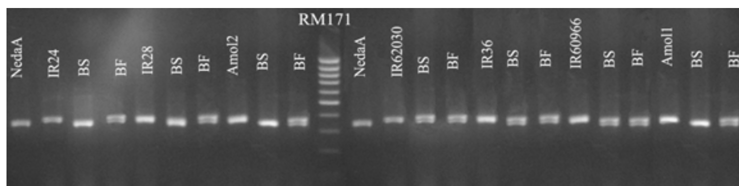

Figure 3. The BSA analysis for revealing polymorphism between SSR marker RM171 and the fertility restoration. BS and BF, corresponding bulks of sterile and fertile F_2 plants, respectively.

Table 3. Segregation at marker loci of RM1 and RM171 in various F_2 populations.

Crosses	Segregation (F:S)	RM1 (chr.1) Recombinants/ homozygote	RM171 (chr.10) Recombinants/ homozygote	Deduced genotype
Neda-A/IR24	109:6 (15:1)	4:64[a]	-[b]	*Rf4Rf4/Rf?Rf?*
Neda-A/IR28	31:10 (3:1)	1:22[a]	-[b]	*Rf4Rf4*
Neda-A/IR36	31:11 (3:1)	-[b]	1:23[a]	*Rf3Rf3*
Neda-A/IR62030	30:12 (3:1)	-[b]	-[b]	*Rf?Rf?*
Neda-A/IR60966	32:10 (3:1)	-[b]	1:22[a]	*Rf3Rf3*
Neda-A/Amol1	32:10 (3:1)	2:24[a]	-[b]	*Rf4Rf4*
Neda-A/Amol2	31:10 (3:1)	1:21[a]	-[b]	*Rf4Rf4*

[a]Presence of polymorphism between parents and two bulks of sterile and fertile F_2 plants at the given marker locus. [b]Absence of polymorphism between two bulks of sterile and fertile plants at the given marker locus.

Identification of the Rf5 gene in rice line IR62030

As seen in BSA analysis, in the population of *Neda-A/IR62030*, markers of *Rf3* and *Rf4* gene loci have not revealed polymorphism between two sterile and fertile bulks of the F_2 plants. Therefore other SSR marker, RM244, has been selected from the work of Jing et al (2001). Jing et al mapped this marker on the short arm of 10th chromosome with distance of 16.7 cM to the *Rf5* gene in rice line *IR64*. However in our work, RM244 has not revealed polymorphism between two parents even after restriction with several restriction enzymes. Therefore, another SSR marker (RM311) near to RM244 has been chosen for revealing polymorphism between the parents.

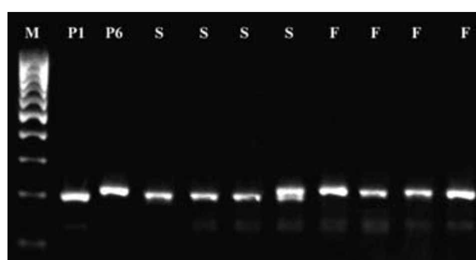

Figure 4. Sample of PCR amplification of SSR marker RM311 for two parents and 4 sterile and 4 fertile F_2 plants. M: 100bp ladder; P1: *Neda-A*; P6: *IR62030*; S: sterile plants; F: fertile plants.

SSR Marker RM311 has revealed polymorphism between *Neda-A* and *IR62030*. For the evaluation of genomic region containing the fertility restoration gene, two bulks of F_2 plants, each consisting of 10 sterile and fertile plants, were analyzed using polymorphic primer pair of RM311. This marker not only has revealed polymorphism between sterile and fertile bulks, but also has shown high correlation with the fertility restoration in F_2 population consisting of 52 homozygous sterile plants (Figure 4). The linkage analysis with package MAPMAKER 3.0 has shown that this SSR marker has been linked to the nuclear fertility restoration gene, *Rf5*. Distance of SSR marker RM311 to the *Rf5* has been estimated 2.9 cM (Figure 5).

Although SSR marker RM171 in the BSA analysis has not detected a polymorphism between two bulks irrespective of existence of polymorphism between parental lines *Neda-A* and *IR62030*, however, as it is on the same chromosome and it is visible from described SSR map of rice (McCouch et al., 2002) that it locates in the distance of ~40 cM from RM311, it was possible to map this marker in the given population. The obtained mapping results by the use of this marker have shown that RM171 locates 40.5 cM from RM311, and the *Rf5* gene locates between them. Distance of RM171 to this gene has been predicted 37.6 cM (Figure 5). A question here emerges that if we see no polymorphism between two bulks we expect to have more than 50 cM distance between *Rf5* and RM171. However, this is related to the capability of the BSA method to detect an association between a polymorph marker and the studied phenotype. As the BSA method is of high sensitivity and each bulk population contains 10-20 F_2 plants selected randomly based on phenotype, with increasing the distance between marker and the gene to be mapped the probability of existence of two marker alleles from two parental lines in each bulk population increases (note figure 3). Hence the method detects association in only a window of <20 cM length with a high confidence, and therefore, it is probable to have no polymorphism between two bulks with a marker at distances of 20-50 cM. In fact our results reflect the high sensitivity of BSA method in a restricted region of genome.

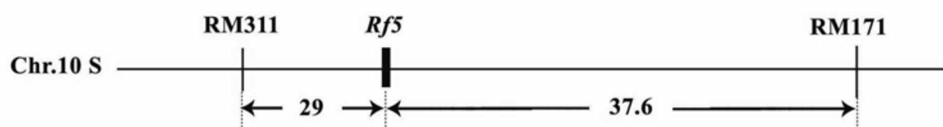

Figure 5. Genetic map of *Rf5* region on the short arm of 10 chromosome of rice in the F_2 population of cross.

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