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Delineating an important *Rf* locus in rice using molecular markers

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Abstract

Cytoplasmic male sterility (CMS) is a common phenomenon that has been extensively used for production of hybrid seeds in various crops. *Rf* genes are needed for restoring fertility to CMS lines. Searching for and molecular tagging of restorer genes is of high importance where phenotyping is very time consuming and requires the determination of spikelet sterility in testcross progeny. In this study we attempted to map a fertility restorer gene using SSR and CAPS markers in rice line IR36 in a F_2 population developed from the cross Neda- A×IR36. The genetic linkage analysis indicated that thee SSR markers (RM1, RM3233, RM3873) and one CAPS marker (RG140/*Eco*RI) on the short arm of chromosome 1 were linked to *Rf3*. *Rf3* flanked by tow SSR markers RM1 and RM3873 at distances of 5.6 and 14 cM, respectively. The use of identified markers give promise for their application in molecular marker assisted selection (MAS).

Keywords: CMS, fertility restoration, SSR marker, Rf3 gene.

INTRODUCTION

Rice is one of the most important agricultural products in the world earning substantial foreign exchange and is a staple food crop in densely populated Asia. 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). Cytoplasmic Male Sterility (CMS) in plants caused by lesion or rearrangement of the mitochondrial genome is unable to produce functional pollens (Jing et al., 2001). Nuclear genes are required to restore pollen fertility to CMS lines. Therefore, the CMS systems are widely used for hybrid seed production. In hybrid seed production using three-line system, the combination of a CMS line, a maintainer line and a restorer line carrying the restorer gene (*Rf*) to restore fertility is indispensable for the development of hybrid varieties (Virmani et al., 2003).

Cytoplasmic male sterility (CMS) and nucleus controlling fertility restoration are widespread plant reproductive features that provide useful tools to exploit heterosis in crops. Rice wild abortive (WA) type cytoplasmic male sterility (CMS) is commercially used for production of hybrid seeds in Asia. Within rice there are several types of CMS/Rf system, among them important is wild abortive (WA), BaoTai (BT) and Honglian (HL) which popularly are applied in commercial hybird rice seed production (Virmani and Shinjyo, 1998) . In rice, hybrid rice varieties developed based on wild abortive (WA) type CMS accounted for approximately 90% of hybrid rice in china. So, inheritance of fertility restoration in the WA type CMS has been extensively investigated. Most of the investigators tended to agree that the restoration of WA type CMS is controlled by two nuclear genes and their chromosomal locations have resolved (Yao et al., 1997; Zhang et al., 1997; Komori et al., 2003; Ahmadikhah et al., 2006).

WA-type CMS is due to the cytoplasm derived from wild rice, called WA acting in a sporophytic manner and is widely used for hybrid seed production in the indica subspecies. Tow major fertility-restorer genes, *Rf3* and *Rf4*, are required for the production of viable pollen in WA-type CMS and the genes have been mapped to chromosomes 1 and 10 respectively (Yao et al., 1997; Zhang et al., 1997; Ahmadikhah et al., 2006; Ahmadikhah and Alavi, 2009). Searching for restorer genes is a good

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approach where phenotyping is very time-consuming and requires the determination of spikelet sterility in testcross progeny.

Molecular markers are particularly useful for accelerating the process of introducing a gene or Quantitative Trail Loci (QTL) into an elite cultivar or breeding line via backcrossing. When introducing a gene or a trait, 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 (Young and Tanksley, 1989; Hospital et al., 1992; Semgan et al., 2006). A significant advance in the practical utilization of molecular marker was the development of SSR markers, also referred to microsatellite markers (McCouch et al., 2002). 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. In this line, our objective was to map an important *Rf* locus in rice using molecular markers.

MATERIALS AND METHODS

Mapping population and fertility scoring

Mapping population was derived from a cross between Neda-A (CMS) and IR36 and consisted from 6 extremely sterile and 85 extremely fertile individuals Neda-A is an elite male sterile line of WA-type. IR36 is a strong restorer line for WA-CMS (Ahmadikhah et al., 2007). For the analysis of pollen fertility, panicle from the main tiller of each plant was selected and several spikelets were randomly selected from different positions in the panicle. The anthers from each spikelet were squashed in a drop of 1% Iodine Potassium Iodide (I-KI) solution on a glass slide separately and observed under a light microscope. F2 plants were classified into different groups based on proportion of stained-round pollen grains as completely sterile (< 5% fertility), partially sterile (5.1 - 50% fertility), partially fertile (50.1 - 80%) and fertile (> 80%), as proposed by Ahmadikhah et al. (2006). Pollen fertility was investigated at flowering time. The seed setting rates of bagged panicles were evaluated at maturity. Sterile plants contained less than 5% stainable pollen and produced no fertile seed. All the others were treated as the fertile individuals.

Genomic DNA extraction

Young leaves were collected from the parental lines and respective 209 individual F_2 plants from cross Neda-AxIR36 and subsequently screened for pollen fertility. Total genomic DNA was isolated from the leaves according to CTAB method (Saghai-Maroof et al., 1984) with some modifications (Ahmadikhah, 2009) . Leaves were ground in liquid nitrogen using mortar and pestle to a very fine powder. It was then transferred to pre-warmed extraction buffer and incubated at 65°C for 45 min and vortexed for 60 s, then added an equal amount of chloroform: isoamyl alcohol (24:1), mixed well by gentle inversion and centrifuged. The supernatant was transferred to a fresh tube and DNA was precipitated by adding 0.7 volume of cold isopropanol to precipitate DNA. After centrifugation, the pellet was dried and dissolved in water. DNA was quantified on the agarose gel, diluted and used in PCR.

Bulked segregant analysis (BSA)

Tow bulk one sterile and one fertile were formed by mixing the DNA from 10 fully fertile (pollen fertility > 90%) and 10 fully sterile (pollen fertility 0%) from corresponding F2 plants was used to constitute fertile and sterile bulks, respectively, for BSA. Polymerase chain reaction (PCR) was performed using DNAs from sterile line Neda-A and restorer line IR36 corresponding sterile and fertile bulk and F2 individuals The parental lines IR36 and Neda-A along with the bulks were simultaneously screened with rice SSR and CAPS markers to find informative. In addition 3 SSR markers RM1, RM3233, RM2873 and one CAPS marker derived from RFLP clones RG140 reported to be linked with the fertility restorer genes in other source were also used in BSA

PCR condition

Polymerase chain reaction (PCR) was performed in 15 l volumes containing 0.75 M/l of each primer, 7.5 l master mix (200 M/l dNTPs, 50 mM/l KCl, 10 mM/l Tris HCl, 1.5 mM/l MgCl2, and 1 unit of Taq DNA polymerase (Cinnagen)) 5 l H2O and 1 l DNA. The PCR profile was 94°C for 5 min (denaturation), followed by 35 cycles of 94°C for 1 min, 50, 55 or 60°C (depend on the melting temperature of the primer pairs) for 1 min, 72^{o}C for 2 min and finally 72°C for 7 min in the final extension. The products from PCR reaction were resolved by electrophoresis in 2/5% agarose gel containing 0.5 g/ml ethidum bromide.

Linkage map construction

JoinMap software (Stam, 1995) was used to calculate the marker distances and to assign the linked markers to linkage groups on a personal computer. 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 and fertile individuals were homozygous at the targeted *Rf* locus.

RESULTS AND DISCUSSION

Segregation of the F_2 population for fertility restoration

Mean pollen fertility for F_1 hybrids of Neda-A x IR36 was calculated as high as 86.3%, indicating that paternal parent IR36 is a strong restorer line. However, mean pollen fertility of F_2 was 73.6% and ranged between 0% and 99%. Phenotypic distribution in F_2 is shown in Figure 1 that shows pollen fertility skewed toward parent IR36. Since one major peak is seen in Figure 1, in this step of analysis we can conclude that one powerful locus exists in IR36 and confers fertility restoration to CMS.

The F_2 population was segregated into 202 fertile and 7 sterile plants that well fitted with 15:1 ratio (X^2_{fact} = 3.0;

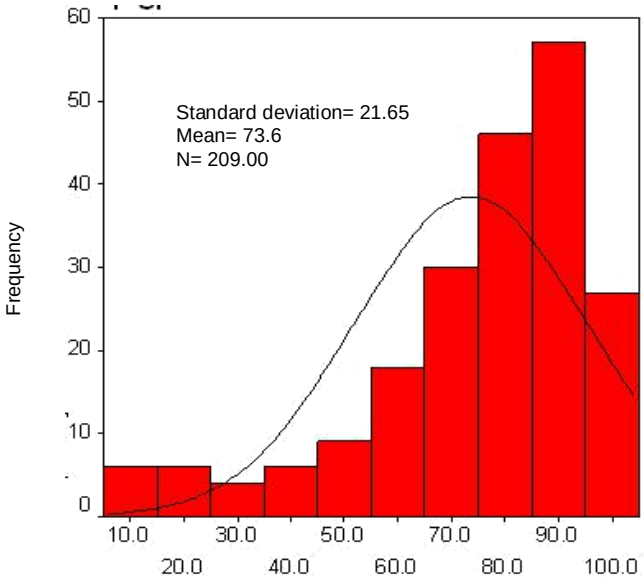

Figure 1. Frequency distribution of pollen fertility (Fr on vertical axis) in F_2 population of Neda-AxIR36.

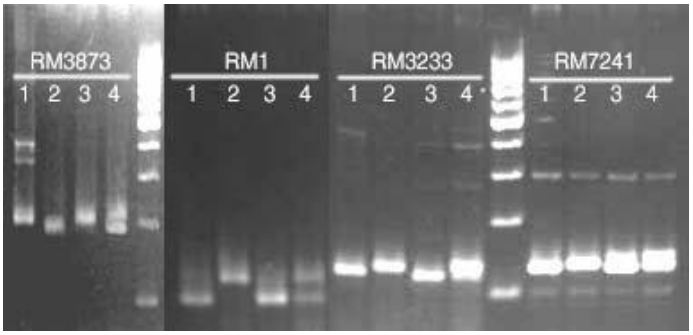

Figure 2. BSA analysis for confirmation of linkage of markers to *Rf3* locus.

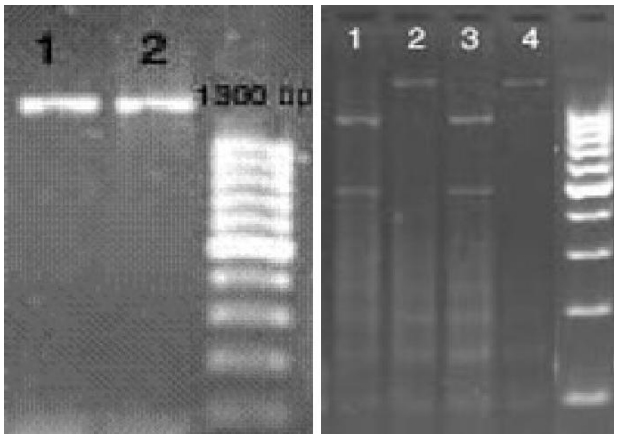

Figure 3. The banding pattern of RG140-based primer pair (left), and BSA analysis of RG140/*Eco*RI after digestion with restriction enzyme *Eco*RI (right); 1 = Neda-A, 2 = IR36, 3 = sterile bulk and 4 = fertile bulk

X^2_{05} = 3.84) for two dominant genes interacting in duplicate fashion. Therefore, in this step, we can conclude that IR36 carries two *Rf* genes. However, whether they act independently or dependently is yet unknown until test it with theoretical ratio 9:3:3:1 for two independent genes. By testing dependence/independence of these two genes using chi square statistic (X^2_{fact} = 72.74; $X^2_{05/df = 3}$ = 7.82), indicated that these genes were not independent and acted dependently; that is, these two genes locate on a linked segment of chromosome. Thus, according to first conclusion and considering second one, we can conclude that one complex locus consisted of two linked genes in IR36 restores fertility to CMS line.

Bulked segregant analysis (bsa)

SSR markers

For detection of the fertility-restoring genes in IR36, 26 pairs of microsatellite primers (Table 1) were selected to screen polymorphism between Neda-A and IR36. Among SSR primers, seven primer pairs (RM1, RM3783, RM 3233, RM7241 on chromosome 1 and RM171, RM6100, RM228 on the chromosome 10) showed polymorphism between Neda-A and IR36 (Table 2). To assess the possibility that a genomic region contains a locus for fertility restoration, tow bulks (Bf and Bs, for fertile bulk and sterile bulk, respectively), each consisting of 12 extremely fertile and 6 sterile plants was assayed with each pair of the polymorphic markers between population mapping parents.

To confirm the linkage of the mentioned primers to fertility restoration, they were tested in PCR with DNA from two parents and respective bulks (Bf and Bs). Results of BSA analysis revealed that microsatellite primers RM1, RM3233, RM3783 on the short arm of chromosome 1 not only showed polymorphic bands between Neda-A and IR36, but also were linked to a fertility-restorer locus *Rf3*.

Development of a caps marker

In addition to SSR markers mentioned above, we used one RFLP-based primer pair (named RG140/*Eco*RI), for detection of polymorphism among parental lines. After amplification of the DNA from Neda-A and IR36 in PCR with RG140-based primers, we did not detect any polymorphism between parental lines and in PCR was produced a monomorphic band (Figure 2). Therefore, to detect probable polymorphism in the PCR product we digested it with restriction enzyme *Eco*RI. This digestion produced polymorphism between two parents; so that the PCR product from CMS line Neda-A was cleaved into two fragments and that of restorer line IR36 stayed intact (Figure 3). Then, BSA analysis was conducted with RG140/*Eco*RI that revealed this CAPPS marker was linked to fertility restoration locus *Rf3*.

Table 1. The sequence information of primer pairs used in this study.

Name	Sequence (5' to 3')	Chr.
RM7241	CAGTCGCACTAACTGAACAACACC CACGGACAGATCAGTTTCTTTCG	1
RM1003	GATTCTTCCTCCCCTTCGTG TTCCTGTCAGAACAGGGAGC	1
RM272	AATTGGTAGAGAGGGGAGAG ACATGCCATTAGAGTCAGGC ACAAGGCCGCGAGAGGATTCCG	1
RM134	GCTCTCCGGTGGCTCCGATTGG	7
RM1335	GCATGCATGAATATGATGG AGATCGAACAAGAAGAGTGG	7
RM226	GAAGCTAAGGTCTGGGAGAAACC AATGGCCTTAACCAAGTAGGATGG	1
RM171	AACGCGAGGACACGTACTTAC ACGAGATACGTACGCCTTTG	10
RM3019	AAAGGTGTTGTAGGAGTCGAGGTTGG ACGCATTCGCCTTTGACATGC	1
RM7180	GTGTTTATAGGGGTGCCACG TGTTGGTGGTGCAGGTAAAG	
RM6737	GCACGTAAATGATAGGCACCATTGC CACAAGGTGGTGTGGGCTAGACG	10
RM6100	TTCCCTGCAAGATTCTAGCTACACC TGTTCGTCGACCAAGAACTCAGG	10
RM1146	TCTCCCTATTCCCGTGTAAATCG CCCGATGATCGATTGTACCTAGC	10
RM228	TCTAACTCTGGCCATTAGTCCTTGG AAGTAGACGAGGACGACGACAGG	10
RM5841	TGAGAGTTACCGTCCATCTAGC GAGTACAGTGAGTGCCCTACG	10
RM10355	GGACCCATATGCTTCATGTCACC CTCACGTTCTCCTTCACCAAAGC	1
RM10346	GCTTGATCTGCCCTTGTTTCTTGG AACTCGAGCGGCCTTCTCAGC	1

Table 1. Contd.

Name	Sequence (5' to 3')	Chr.
M302	TCATGTCATCTACCATCACAC ATGGAGAAGATGGAATAC	1
RM543	CTGCTGCAGACTCTACTGCG AAATATTACCCATCCCCCCC	1
RM319	ATCAAGGTACCTAGACCACCAC TCCTGGTGCAGCTATGTCTG	1
RM522	ACCAGAGAAGCCCTCCTAGC GTTCTGTGGTGGTCACGTTG	1
RM311	TGGTAGTATAGGTACTAAACAT TCCTATACACATACAAACATAC	
SSR061	GTCTAATTTTCCTCCCTCCT TGATGTTGGCTTTGTTATTG	1
RM151	GGCTGCTCATCAGCTGCATGCG TCGGCAGTGGTAGAGTTTGATCTGC	1
RM3233	GAAATTCGAAATGGAGGGAGAGC GGTGAGTAAACAGTGGTGGTGAGC	1
RM3873	GCTATAGACGCCTCCTCCTTATCC AAAGCTAGCTAGGACCGACATGC	1
RM1	GCGAAAACACAATGCAAAAA GCGTTGGTTGGACCTGAC	1
RG140	GTACATAGTAGCACCTGCTC TCCCTAGTTTGTGCTACTC	1

Table 2. SSR markers which showed polymorphism between parental lines.

Location (chromosome)	Size (base pair)	Name
1	100-113	RM1
1	120-130	RM3233
1	185-200	RM3873
1	120-130	RM7241
10	320-340	RM171
10	160-170	RM6100
10	110-120	RM228

Mapping SSR and CAPS markers

After confirmation of linkage of the SSR primer pairs (RM1, RM3233 and RM3873 and CAPS marker Rg140/*Eco*RI)

Table 3. Result of single marker analysis.

	RM1	RG140	RM3873	RM3233
Rf3	6.49 (29.72)	12.53 (14.49)	15.83 (12.24)	16.96 (10.8)
RM1	-	10.43 (15.54)	18.48 (9/59)	15.21 (11.39)
RG140		-	23.31 (6.55)	18.13 (9.14)
RM3873			-	32.13 (2/31)

Note: Distances are expressed in cM and LOD scores are shown in parenthesis.

to *Rf3*, we selected 91 F_2 plants (including 6 extremely homozygous sterile individuals and 85 extremely homozygous fertile ones) for calculating linkage distance in the mapping population. In single marker analysis RM1 in distance ~6.5 cM, RG140 in distance of ~12.5 cM, RM3873 in distance of ~15.8 cM and RM3233 in distance of ~17 cM were mapped with *Rf3* locus (Table 3).

However, when all the marker loci together with *Rf3* locus were selected for mapping purpose in JoinMap3.0, SSR markers RM1, RM3233 and RG140 located on one side and RM3783 alone on the other side of *Rf3* (Figure 4).

As seen in Figure 3 the *Rf3* was flanked by two SSR markers RM1 and RM3783, at distances of 5.6 and 14 cM, respectively.

In this study, molecular markers linked to fertility restoration in rice WA-CMS system were successfully identified and mapped on the short arm of chromosome 1 by the use of SSR markers. Some workers located Rf3 on the short arm of chromosome 1, too (Yao et al., 1997; Zhang et al., 1997; He et al., 2002). For example, He et al. (2002) found a SSR marker RM1 linked o Rf3 gene in the short arm of chromosome 1. In our work also we showed that RM1 was linked to Rf3 gene. We also developed a CAPS marker namely RG140/EcoRI on the basis of RFLP sequences in this region and showed that this marker was linked to Rf3. Zhang et al. (1997) mapped Rf3 between RFLP markers RG140 and RG532. Ahmadikhah and Alavi (2009) also reported that CAPS marker RG140/EcoRI was linked to Rf3 on the short arm of chromosome 1. The identification of closely linked markers would be valuable for use in MAS strategy and finally help in map- based cloning of the fertility restorer gene in near future (Ahmadikhah and Karlov, 2006). For example, expected efficiency of RM1 and RM3873 in MAS when they are used alone is 89.1 and 74.0%, respectively. However, when they are used together, their expected efficiency in MAS will be 99.2%.

ACKNOWLEDGMENT

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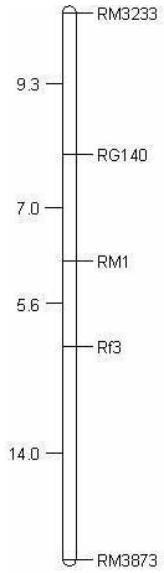

Figure 4. Molecular mapping of *Rf3* locus on the short arm of chromosome 1 of Indica rice. Marker loci and *Rf3* are shown on the right and distances (in cM) on the left.

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