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Mapping fertility-restoring genes of rice WA cytoplasmic male sterility using SSLP markers

Runchun Jing, Xiaoming Li, Ping Yi, and Yingguo Zhu*

The Key Laboratory of MOE for Plant Developmental Biology and Institute of Genetics, College of Life Sciences, Wuhan University, Wuhan, 430072, P.R. China

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Abstract. Rice wild abortive (WA) type cytoplasmic male sterility (CMS) is commercially used for production of hybrid seeds in China. Two fertility restorer genes in the CMS-WA system were detected with SSLP markers in this study. An F_2 population consisting of 210 excessive sterile individuals from a cross between Zhenshan 97A and a strong restorer line IR24 was used for mapping of *Rf4*. The genetic distance from *Rf4* locus to RM171 (OSR33) and RM228 on long arm of chromosome 10 was 3.7 cM and 3.4 cM, respectively, which were the two closest SSLP markers flanking the *Rf4* locus. The two SSLP markers gave promise of application in molecular marker-assisted selection (MAS) for fertility restorer lines of the CMS-WA system. RM244, another SSLP marker on the short arm of chromosome 10, was found to be linked with a fertility restorer locus in an F_2 population consisting of 30 excessive sterile individuals from a cross between Zhenshan 97A and a weak restorer line IR64. The genetic relationship among fertility restorer genes *Rf1*, *Rf4* and *Rf5(t)* for three rice CMS system is also discussed.

Keywords: Fertility restorer gene; Genetic linkage map; Molecular marker-assisted selection; *Oryza sativa* L.; SSLP 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, hybrid rice varieties developed based on wild abortive (WA) type CMS accounted for approximately 90% of hybrid rice in China (Yuan, 1992) and inheritance of fertility restoration for WA type CMS has been extensively studied. Most researchers conclude that the fertility restoration of WA type CMS is controlled by two independent nuclear loci. Besides CMS-WA, other CMS systems have also been developed and their fertility restoring genes were termed. *Rf1*, *Rf2*, *Rf3* and *Rf4* were the terms for CMS-BT, CMS-L and CMS-WA, respectively (Shinjyo, 1975; Zhang et al., 1997; Shinjyo and Sato, 1994). These genes were mapped on different chromosomes based on trisomic and RFLP analyses. *Rf1* and *Rf2* genes were mapped on chromosomes 10 and 2 by trisomic analyses, respectively (Shinjyo, 1975; Shinjyo and Sato, 1994). *Rf3* and *Rf4* were located on chromosomes 1 and 10 by trisomic and RAPD/RFLP analyses (Bharaj et al., 1995; Zhang et al., 1997; Yao et al., 1997). *Rf3* was flanked by RFLP markers RG458/RG140 and RG532, which had a genetic distance of 3.8 cM (Zhang et al., 1997). *Rf4* was located in a 22.4 interval between G4003 and C234 (Yao et al., 1997). Tan et al. (1998) reported that one QTL, tightly linked to RFLP marker C1361 on chromosome 10, explained 71.5% of phenotypic variance (Tan et al., 1998). Huang et al. (2000) mapped a nuclear fertility restorer gene *Rf5(t)* on chromosome 10 for Honglian (HL) type CMS, another CMS type applied in commercial hybrid rice seed production in China (Huang et al., 2000).

Simple Sequence Length Polymorphism (SSLP) provides a new tool for gene mapping and marker-assisted selection (MAS). SSLPs are the results of variable numbers of repeated units within the microsatellite structure and are widely distributed in the rice genome (McCouch et al., 1997). SSLP can be easily and economically analyzed by Polymerase Chain Reaction (PCR) and generally behaves as a co-dominant marker, which often detects higher levels of allelic variation than RFLP or RAPD markers. SSLP markers are being successfully applied in fingerprinting and variety identification, gene and QTL mapping, and marker-assisted selection (MAS) in plants.

The studies reported in this paper were to determine locations of *Rf* loci in the rice genome based on linkage maps of SSLP markers (Akagi et al., 1996a; Panaud et al., 1996; Chen et al., 1997; Temnykh et al., 2000).

*Corresponding author. Tel: +86-27-87684560; Fax: +86-27-87882661; E-mail: ygzhu@hotmail.com

Materials and Methods

Mapping Population Development and Fertility Scoring

Two mapping populations, constructed in 1998, were used in this study. The first one consisted of 210 F_2 extremely sterile individuals from about 10,000 F_2 plants derived from a cross between Zhenshan 97A and IR24. The second one consisted of 30 F_2 extremely sterile individuals from about 1,000 F_2 plants derived from a cross between Zhenshan 97A and IR64. Zhenshan 97A is an elite male sterile line of WA type. IR24 is a strong restorer line, and IR64 is a weak restorer line. Pollen fertility was investigated by staining with 1% I_2-KI, and seed setting rates of bagged panicles were evaluated at maturity. Sterile individuals contained less than 5% dark-stained pollens and didn't produce fertile seed.

Genomic DNA Extraction

Total genomic DNA was isolated using the SDS method as described previously by Dellaporta et al. (1983). About 4 g of leaves were ground in liquid nitrogen to a very fine powder and incubated with 25 ml of extraction buffer (100 mmol/ Tris-HCl (pH 8.0), 50 mmol/l EDTA, 500 mmol/l NaCl, 1.25% SDS (W/V), 0.38 g/l $NaHSO_4$) at 65°C for 20 min. Then 10 ml of 5 mol/l KAc was added and incubated on ice for 20 min. After centrifugation, the supernatant was collected, and two-thirds volume of pre-chilled isopropanol was added to precipitate DNA. The DNA pellets were washed with 70% ethanol and dissolved in TE buffer, to which was added 15 µl of RNase (10 mg/ml) before incubation at 37°C for 30 min. The DNA was re-extracted with 2 volumes of chloroform/isopentanol (24/1) and precipitated by absolute ethanol. After washing twice with 70% ethanol, the DNA was re-dissolved in TE buffer for use.

SSLP Analysis

One hundred and sixty-three microsatellite primer pairs were used in this study. They were synthesized by Sangon (Shanghai) Co. according to sequences distributed from Temnykh et al. (2000). Polymerase chain reaction (PCR) was performed in 25 µl volume containing 0.2 µmol/ l of each primer, 200 µmol/l deoxyribonucleotides, 50 mmol/ l KCl, 10 mmol/l Tris-HCl (pH 8.3), 1.5 mmol/l $MgCl_2$ and 1 unit of Taq DNA polymerase. The PCR profile was 94°C for 5 min (denaturation), followed by 40 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, and finally 72°C for 5 min in the final extension. The PCR reaction was performed on a Perkin Elmer DNA Thermal Cycler 480. Products from PCR reaction were resolved by electrophoresis in 4% agrose gel containing 0.5 µg/ml ethidum bromide or in 5% polyacrylamide gel with detection by silver staining according to the Progema technical manual.

Linkage Map Construction

The linkage map was constructed with MAPMAKER EXP3.0 on a personal computer (Lincoln et al., 1992). Map distances were based on the Kosambi function (Kosambi, 1944). Linkage groups were assigned to corresponding chromosomes based on SSLP markers mapped by Akagi et al. and Temnykh et al. (Akagi et al., 1996a; Temnykh et al., 2000). 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

Detection of Rf Genes in IR24

For detection of fertility restoring genes in IR24 with SSLP markers, 163 pairs of microsatellite primers were used to screen polymorphism between Zhenshan 97A and IR24. Among the 163 primers, 48 primers showed polymorphism between Zhenshan 97A and IR24, polymorphic frequency was 29.4%. To assess the possibility that a genomic region contains a gene for fertility restoration, 42 extremely sterile plants of the 210 F_2 extremely sterile population were assayed individually with each pair of the polymorphic SSLP markers. SSLP markers on chromosome 1, including RM1, RM9, RM14, RM24, RM238A, RM243, OSR3, OSR23, and OSR27 detected polymorphism between Zhenshan 97A and IR24; however, no SSLP marker, including RM1, which was next to RG532 on the genetic map (Chen et al., 1997; Temnykh et al., 2000), was found to link with the fertility restorer gene.

However, microsatellite primers RM258, RM171 (OSR33) and RM228 on the long arm of chromosome 10 not only showed polymorphic bands between Zhenshan 97A and IR24 but also showed high correlations with a fertility restorer gene (Table 1). For instance, with RM171 (OSR33), of all the 210 sterile progenies, 15 plants were heterozygous, containing two alleles of the two parents (Figure 1). The linkage analysis with MAPMAKER EXP

Table 1. Recombination frequencies and genetic distances between a positive marker locus and *Rf* locus were calculated using Maximum likelihood method, based on the assumption that all the extremely sterile plants are homozygous for the recessive allele at each of the targeted loci.

Locus	Chromosome	Recombination freq. (%)	Genetic distance (cM)	LOD score
RM258	10	10.00	11.2	67.14
RM171	10	3.57	3.7	98.33
RM228	10	3.33	3.4	99.78

3.0 revealed that SSLP markers RM258, RM171 (OSR33), and RM228 were linked with a nuclear fertility restorer gene, *Rf4*. The distances from *Rf4* to the two closest SSLP markers, RM171 (OSR33) and RM228 were 3.7 cM and 3.4 cM, respectively (Figure 2). The two SSLP markers gave promise of application in molecular marker-assisted selection (MAS) for restorer lines in CMS-WA system. In addition, the positive SSLP markers on other chromosomes did not detect any *Rf* locus in the experimental population.

Identification of Fertility Restoring Gene in IR64

Mean pollen fertility of F_1 generation from a cross between Zhenshan 97A and IR64 was 87.27±7.77%, but natural seed setting rate was only 47.39±11.14% in Wuhan. For mapping the weak restorer genes in IR64, 163 pairs of microsatellite primers were used to screen polymorphism between Zhenshan 97A and IR64. Among the 163 primers, 46 primers showed polymorphism between Zhenshan 97A and IR64. Polymorphic frequency was 28.2%. On chromosome 1, RM1, RM5, RM14, RM81A, RM237, RM243, OSR3, OSR23, and OSR27 showed polymorphism between Zhenshan 97A and IR64, but no SSLP marker was found linked with a fertility restorer gene. Although RM258 and RM228, linked with a restorer gene of IR24, showed polymorphic bands between Zhenshan 97A and IR64, they did not show linkage with a restorer gene in the 30 extremely sterile plants from the cross between Zhanshan 97A and IR64. In the same chromosomal region, RM171 (OSR33) and RM294A, which revealed polymorphism between IR24 and Zhenshan 97A, did not show polymorphism between IR64 and Zhenshan 97A (Figure 3). This suggested that origination of the chromosome segment carrying the *Rf4* of IR24 was different from IR64.

However, RM244 on the short arm of chromosome 10 did not only reveal polymorphism but also showed a correlation with fertility restoration. Of the 30 sterile progenies, 1 plant was homozygous as IR64, 8 plants were heterozygous containing two alleles of the two parents. Single-marker analysis revealed that a nuclear restorer gene of IR64 was linked to RM244 and recombination frequency was 16.7%; genetic distance was 17.3 cM; LOD score was 6.32. This restorer gene was designated *Rf6(t)* temporarily. In the same way, the positive SSLP markers on other chromosomes did not detect any *Rf* locus in this experimental population.

Discussion

This study located nuclear fertility restorer genes of rice CMS-WA system with SSLP markers on the short arm and the long arm of chromosome 10. Interestingly, Akagi et al. (1996b) also mapped a nuclear fertility restorer gene of BT type CMS on chromosome 10, 3.7 cM from OSR33 (Akagi et al., 1996b), and Huang et al. (2000) also mapped a nuclear fertility restorer gene *Rf5(t)* of HL type CMS on chromosome 10, 3.6 cM and 7.8 cM from OSR33 and RM258, respectively (Huang et al., 2000). Comparison of various molecular marker linkage maps suggested that the three

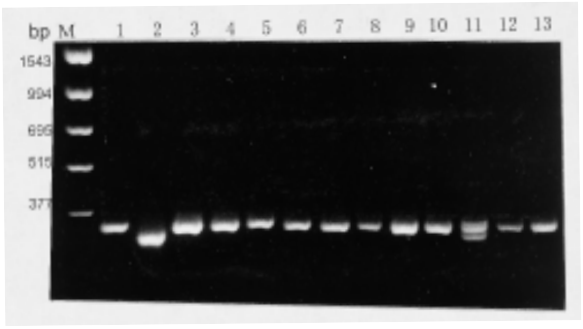

Figure 1. SSLP pattern of microsatellite primer pair RM171 (OSR33). The PCR products were resolved on 4% agarose gel. Lanes 1 and 2 are Zhenshan 97A and IR24, respectively. Lanes 3-13 are extremely sterile individuals. Lane M contains PCR Markers (SABC). Molecular sizes are shown on the left.

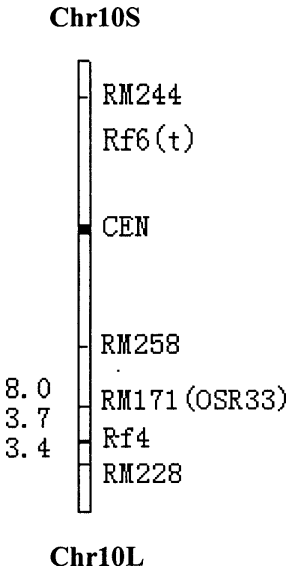

Figure 2. Mapping of *Rf4* on chromosome 10. Markers are indicated on the right, and map distances (in cM) based on the kosambi function are on the left.

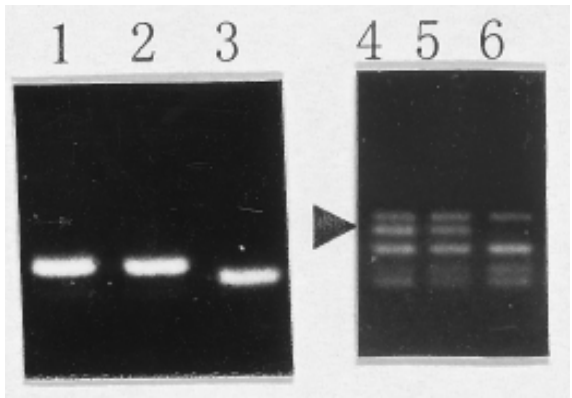

Figure 3. SSLP analysis of RM171 and RM294 on three parents. Lanes 1, 2, 3 were amplified using RM171, 4, 5, 6 were amplified using RM294. Lanes 1 and 4 are zhenshan 97A, 2 and 5 are IR64, 3 and 6 are IR24.

fertility restorer genes for the three types of CMS were located closely on the long arm of chromosome 10. Recently, Borner et al. (1998) found that *Rfg1*, determining the restoration of cytoplasmic genic male sterility caused by the G-type cytoplasm, may be allelic to a gene determining the restoration of rye CMS caused by the P-type cytoplasm, and allelic to *Rfc4* on rye addition lines of chromosome 4RL restoring fertility of hexaploid wheat with *T. timopheevi* cytoplasm (Borner et al., 1998). Jia et al. (1997) mapped four nuclear fertility restorer genes of bean (*Fr*, *Fr2*, $Fr_{PI207228}$ and Fr_{XR235}) on the same linkage group and proposed that they may be allelic (Jia et al., 1997). Li et al. (1998) reported that nuclear fertility restorer genes (*Rfp* and *Rfn*), for two different types of CMS (nap and pol), were allelic and represented different alleles or haplotypes of a single nuclear locus. A single restorer locus can influence transcripts of three different mitochondrial genes, and restore fertility of different CMS forms (Li et al., 1998). These results suggested that the three rice nuclear fertility restorer genes (*Rf1*, *Rf4*, and *Rf5(t)*) for the three types of rice CMS (BT, WA, and HL) may be allelic, and different alleles or haplotypes of a single nuclear locus can restore the fertility of different types of CMS. Searching for markers more tightly linked to the *Rf* locus and construction of a high-resolution regional map will be helpful to resolve the relationship of the three rice nuclear fertility restorer genes.

Molecular markers close to a target gene may be useful for selection during breeding (Paran and Michelmore, 1993). SSLP markers have the advantages of both the rapidity, straight, and simplicity of RAPD, and the stability, reliability, and repeatability of RFLP. Although molecular weight differences of SSLP markers are small between different genotypes, our results suggest that the difference could be resolved by agarose gel or polyacrylamide gel. The results presented here clearly indicate that the SSLP markers RM171 and RM228 will facilitate molecular marker-assisted selection (MAS) of restorer lines in CMS-WA system, which will promote the development of hybrid rice. It is also expected that the use of RM171 (OSR33) and RM228 in MAS integrated with backcross breeding will produce near isogenic lines (NILs) of fertility restorer lines for genetic research.

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水稻野敗型細胞質雄性不育恢復基因 Rf4(t) 的 SSLP 標記遺傳連鎖圖

景潤春　李小明　易　平　朱英國

武漢大學植物發育生物學教育部重點實驗室和生命科學院遺傳研究所

水稻野敗型細胞質雄性不育系被廣泛用於中國雜交水稻生產。本研究用 SSLP 標記定位了兩個水稻野敗型細胞質雄性不育恢復基因。珍汕 97A 與其強恢復系 IR24 配組的 210 株 F_2 代極端不育群體作為恢復基因作圖群體用於定位 *Rf4*。結果發現，位於水稻基因組第 10 染色體長臂上的 RM171 (OSR33) 和 RM228 位於 *Rf4* 基因兩側，距 *Rf4* 的遺傳距離分別為 3.7 cM 和 3.4 cM，這兩個距 *Rf4* 最近的 SSLP 標記有助於對水稻野敗型細胞質雄性不育恢復系的分子標記輔助選擇。另一個位於第10染色體短臂上的 SSLP 標記，RM244，在珍汕 97A 與其弱恢復系 IR64 配組的 30 株 F_2 代極端不育群體中與恢復基因座位 *Rf6(t)* 連鎖。本文還對珍汕 97A、IR64 和 IR24 在恢復基因 *Rf4* 所在的染色體區段的 SSLP 標記多態性與恢復基因間的關係進行了分析。

關鍵詞：水稻；恢復基因； SSLP標記；遺傳連鎖圖；分子標記輔助選擇。