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Mapping of the *Rf-3* nuclear fertility-restoring gene for WA cytoplasmic male sterility in rice using RAPD and RFLP markers

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Abstract The cytoplasmic male sterility (CMS) of wild-abortive (WA) cytoplasm has been widely used for breeding hybrid rice. Two restorer genes for the CMS have been found by traditional genetic analysis. To tag the restorer genes we used a set of near-isogenic lines (NILs) of Zhenshan 97 carrying different genotypes for fertility restoration from IR24, to perform RAPD analysis. From the survey of 720 random primers, six RAPD markers were identified to be associated with *Rf-3*. Three of these OPK05-800, OPU10-1100 and OPW01-350, were mapped on chromosome 1. Two F_2 populations from the crosses between Zhenshan 97 A and a near-isogenic restorer line ZSR21 and between Zhenshan 97 A and IR24 were used for mapping *Rf-3*. The three RAPD markers and three RFLP markers, RG532, RG140 and RG458, were found to be closely linked to *Rf-3* in the two F_2 populations. The same location of *Rf-3* was also found in a BC_1 population from the cross of IR58025 A//IR36/IR58025 B. At the RG532 locus, different alleles were found between two CMS lines, Zhenshan 97 A and IR58025 A, and between two restorer lines, IR24 and IR36. The use of these molecular markers closely linked to *Rf-3* in facilitating the development of hybrid rice is discussed.

Key words Restorer gene · Near-isogenic lines · Random amplified polymorphic DNA · Molecular mapping · *Oryza sativa*

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Introduction

Cytoplasmic male sterility (CMS), which is characterized by the inability to produce functional pollen, is caused by an interaction between a male-sterile cytoplasm and nuclear genes. In rice, a large number of male-sterile cytoplasms have been found in *Oryza sativa*, *O. glaberrima* and some wild species (Li and Zhu 1988; Virmani and Wan 1988). The male sterility of 'wild abortive' cytoplasm (cms-WA) is restored by two sporophytic restorer genes, giving rise to a 15:1 F_2 ratio. Their effects could be additive (Gao 1981; Li and Yuan 1986; Zhang and Lu 1987). The two restorer genes in line IR36 for the male sterilitty of (cms-WA) IR58025 A have been located on chromosome 7 and chromosome 10, respectively based on trisomic analysis (Bharaj et al. 1995). For more elaborate genetic analysis, a set of near-isogenic lines (NILs) has been developed by backcrossing five times and nine times to a CMS line WA-Zhenshan 97 A using IR24 as the donor for the two restorer genes (Lu and Zhang 1986; Zhang et al. 1994b).

The nomenclature of fertility-restoring genes needs clarification. *Rf-1* restores male sterility in cms-B and is located on chromosome 10 (Shinjyo 1975). *Rf-2* restores male sterility in cms-L and is located on chromosome 2 (Shinjyo and Sato 1994). To distinguish these from the two genes that restore male fertility of WA cytoplasm, we have renamed the genes designated as *Rf-2* and *Rf-1* in the NILs of Zhenshan 97 (Zhang et al. 1994b) as *Rf-3* and *Rf-4*, respectively, in this paper.

CMS is widely used for hybrid rice breeding (Yuan and Virmani 1988). Hybrid rice is developed by using three lines: cytoplasmic male-sterile, maintainer and restorer lines. However, the genotypic identity of maintainer and restorer lines cannot be determined without a generation of testcrossing with CMS lines. Therefore, breeding of the three lines is laborious and time-consuming.

Molecular markers are becoming useful in facilitating plant breeding. The identification of molecular

markers that are closely linked to genes of agronomic importance represent an important step toward increasing the efficiency of selection in breeding. Restriction fragment length polymorphism (RFLP) markers have been widely used to construct genome maps. Two saturated RFLP maps of the rice genome have been constructed (Causse et al. 1994; Kurata et al. 1994). Random amplified polymorphic DNA (RAPD) has also been used as genetic markers (Williams et al. 1990), and used successfully to construct a genetic map (Reiter et al. 1992). One of the most useful applications of RAPD markers is the rapid generation of markers within a genomic region of interest using NILs (Klein-Lankhorst et al. 1991; Martin et al. 1991; Paran et al. 1991; Penner et al. 1993; Zhang et al. 1994a).

The identification of molecular markers closely linked to restorer genes will facilitate the breeding of hybrid varieties. Recently, two restorer genes in maize were mapped using RFLP markers (Wise and Schnable 1994). RAPD markers linked to a restorer gene (*Rfo*) in rapeseed were identified (Delourme et al. 1994). Here, we report the identification of RAPD markers linked to one of two restorer genes for cms-WA using a set of NILs and the mapping of the gene using three segregating populations.

Materials and methods

Plant materials

Near isogenic lines (NILs)

A sporophytic CMS line with WA cytoplasm, Zhenshan 97 A (ZSA), a maintainer line Zhenshan 97 B (ZSB) and a set of NILs for fertility restoration were used. ZSA and ZSB carry the genotypes *rf-3/rf-3 rf-4/rf-4*. The set of NILs included 2 lines, ZSR1 and ZSR2, carrying genotypes *Rf-3/Rf-3 rf-4/rf-4*, 3 lines ZSR3, ZSR5 and ZSR7 carrying *rf-3/rf-3 Rf-4/Rf-4* and 3 lines ZSR11, ZSR19 and ZSR21 carrying *Rf-3/Rf-3 Rf-4/Rf-4*.. All NILs were developed by backcrossing nine times to ZSA using IR24 as the donor for the two pairs of restorer genes (Zhang et al. 1994b). Another NIL, ZSR, was developed by backcrossing five times from the same backcross (Lu and Zhang 1986).

F_2 populations

Two F_2 populations were developed from the crosses ZSA/ZSR21 and ZSA/IR24. Previous studies showed that fertility restoration is controlled by two pairs of restorer genes. In F_2 populations derived from the two crosses, the ratio of fertile (and partially fertile) plants to sterile plants was 15:1 (Zhang and Lu 1987; Zhang et al. 1994b). Because of the continuous variation in fertility, only the sterile plants could be clearly identified in the two F_2 populations. Thirty-six sterile plants were selected from 640 F_2 plants derived from ZSA/ZSR21 and 27 sterile plants were collected from 510 F_2 plants derived from ZSA/IR24.

BC_1 population

A BC_1 population was developed from the cross IR58025 A//IR36/IR58025 B. IR58025 A is a CMS line with WA cytoplasm; IR 58025 B is a maintainer line; and IR36 is a restorer line. The BC_1 population was originally prepared for trisomic analysis of fertility restoration. IR36 in the cross was a trisomic plant. The BC_1 population used in this study was developed from three primary trisomics, Triplo 6, Triplo 8 and Triplo 9 of IR36 (Bharaj et al. 1995). In the F_1 of IR36/IR58025 B, only disomic plants were selected to backcross to IR58025 A. From 313 BC_1 plants, 92 plants were identified as being sterile. The segregation of fertile (and partially fertile) plants and sterile plants was in the ratio of 3:1 (Bharaj et al. 1995). In the BC_1 population, only sterile plants were used in the study. From 92 sterile BC_1 plants, only 39 sterile plants were successfully ratooned to produce enough green leaves to prepare DNA samples.

RAPD analysis

DNA from ZSA, ZSB, 9 NILs and IR24 were used as templates. For each amplification, 20 ng DNA was used as template in 25 µl of reaction volume. The reaction conditions were as described in Williams et al. (1990). A total of 720 arbitrary 10-base oligonucleotide primers (Operon Technologies) were surveyed for their ability to amplify polymorphic bands among the DNA samples. The positive polymorphic bands were isolated from gels and then used as templates for reamplification using the corresponding RAPD primers. After three rounds of amplification, a single DNA band corresponding to the original fragment size was obtained in each case. For use as probes, the specific DNA fragments were radioactively labeled using the random primer method (Feinberg and Vogelstein 1983).

To determine the chromosomal location of the *Rf-3* gene, we used three RAPD markers, OPK05-800, OPU10-1100 -and OPW01-350, as hybridizing probes to filters containing DNA from 135 doubled haploid lines derived from a cross IR64/Azucena, from which an RFLP map consisting of 135 RFLP markers had been constructed (Huang et al. 1994). The scores were joined to the data pool on the RFLP map. The three RAPD markers were then mapped onto the RFLP map using the MAPMAKER program (Lander et al. 1987).

RFLP analysis

Procedures for DNA extraction, restriction enzyme digestion and Southern blotting were as described in McCouch et al. (1988). All RFLP markers used in this study were kindly provided by S. D. Tanksley, Cornell University, New York, USA.

Results

Identification of RAPD markers linked to *Rf-3*

DNA from 12 lines (a CMS line ZSA, a maintainer line ZSB, 9 NILs and a donor restorer line IR24) was used as a template in polymerase chain reactions (PCRs) with each of 720 arbitrary 10-mer oligonucleotide as primers. Six primers generated polymorphic bands between the genotypes *rf-3/rf-3* and *Rf-3/Rf-3*. Four primers, OPK05, OPN13, OPU10 and OPAA12, each generated one specific DNA fragment which was associated with the fertility restorer allele *Rf-3*. The sizes of the specific DNA fragments were about 800, 600, 1100 and 700 bp, respectively. Two primers, OPW01 and OPY16, each generated one specific DNA fragment (OPW01-350 and OPY16-550, respectively), which was associated with the sterility maintainer allele *rf-3* (Table 1). From the survey of the 720 random primers, however, we failed to identify any RAPD markers associated with the restorer gene *Rf-4*.

Three of the six RAPD markers, OPK05-800, OPU10-1100 and OPW01-350 (Fig. 1), were isolated from gels and used as probes for hydridization to filters containing DNA from the 12 lines. Polymorphisms

Table 1 RAPD markers associated with the *Rf-3* locus that were identified from ZSA, ZSB, NILs and IR24

Marker	*rf3 rf3 rf4 rf4*		*Rf3 Rf3 rf4 rf4*		*rf3 rf3 Rf4 Rf4*			*Rf3 Rf3 Rf4 Rf4*				
	ZSA	ZSB	ZSR1	ZSR2	ZSR3	ZSR5	ZSR7	ZSR11	ZSR19	ZSR21	ZSR	IR24
OPK05-800	–[a]	–	+	+	–	–	–	+	+	+	+	+
OPN13-600	–	–	+	+	–	–	–	+	+	+	+	+
OPU10-1100	–	–	+	+	–	–	–	+	+	+	+	+
OPW01-350	+	+	–	–	+	+	+	–	–	–	–	–
OPY16-550	+	+	–	–	+	+	+	–	–	–	–	–
OPAA12-700	–	–	+	+	–	–	–	+	+	+	+	+

[a] +/–, Presence/absence of the DNA fragment, respectively

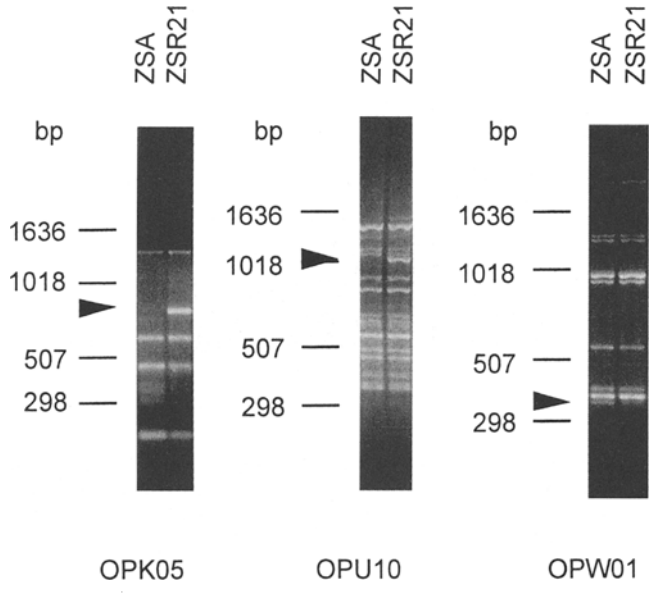


Fig. 1 DNA amplification patterns with RAPD primers OPK05, OPU10 and OPW01 on DNA of CMS line Zhenshan 97 A (*ZSA*) and near-isogenic restorer line ZSR21. The polymorphic DNA fragments are indicated by *arrows*

between the genotypes were detected when DNA was hybridized with OPK05-800 on 4 restriction digests (*Bgl*II, *Eco*RV, *Pst*I and *Xba*I), hybridized with OPU10-1100 on 5 restriction digests (*Dra*I, *Hae*III, *Hin*dIII, *Sca*I and *Xba*I) and hybridized with OPW01-350 on 4 restriction digests (*Bgl*II, *Eco*RV, *Sca*I and *Xho*I) (Fig. 2). All three RAPD markers generated the same segregating pattern and segregation at the marker loci was completely associated with that at the *Rf-3* locus in the 12 lines. Using a mapping population as reference (Huang et al., 1994), we mapped all three RAPD markers at the same positions on chromosome 1 between RG173 and RG246 and close to RG532 (Fig. 3).

Analysis of introgressed segments on chromosome 1 in NILs

On the basis of the RAPD analysis, 15 RFLP markers between RZ276 and RG246 on chromosome 1 were selected for hydridization to the filters containing DNA from the 12 lines (Table 1). Among the RFLP markers, 9 markers generated polymorphisms between ZSA and IR24, and 7 markers generated polymorphisms among the NILs. With RG532, RG140 and RG458, all NILs carrying *Rf-3* showed the same band pattern as IR24, while all NILs carrying *rf-3* showed the same pattern as ZSA (data not shown). Hybridization with RZ382, RZ489, RZ449 and RG173 also revealed polymorphisms among NILs, but only ZSR2, ZSR19 and ZSR21 carried the marker allele from IR24 while other NILs carried the marker allele from ZSA in each case. RZ276 and RG246 generated polymorphism between ZSA and IR24, but no polymorphism was found among the NILs. From the RAPD and RFLP analyses, it is evident that the chromosomal segments introgressed from IR24 were found from RZ382 to RG458 in ZSR2, ZSR19 and ZSR21, and from OPW01-350 to RG458 in ZSR1, ZSR11 and ZSR. No introgressed segments from IR24 on the same chromosomal region were found in ZSR3, ZSR5 and ZSR7 (Fig. 4).

Mapping of *Rf-3*

Thirty-six sterile F_2 plants from the cross ZSA/ZSR21 and 27 sterile F_2 plants from ZSA/IR24 were used as mapping populations. Three RAPD markers and six RFLP markers which generated polymorphisms between the sterile parent ZSA and restorer parents ZSR21 and IR24 were used to hybridize to filters containing DNA from the sterile F_2 plants of the two crosses. Two similar maps were constructed from the two F_2 populations (Fig. 5). Three RAPD markers, OPW01-350, OPU10-1100 and OPK05-800, and three RFLP markers, RG532, RG140 and RG458 were found to be closely linked to the *Rf-3* locus on each of the maps. In the F_2 population of ZSA/ZSR21, *Rf-3* was inferred to be located in the same position as OPK05-800. They are flanked by RG532, RG140 and RG458 (Fig. 5A). In the F_2 population of ZSA/IR24, no recombinants were found between RG532 and OPK05-800 and between RG140 and RG458. The *Rf-3* locus therefore resides between the two marker pairs (Fig. 5B).

Thirty-nine sterile BC_1 plants derived from IR58025 A//IR36/IR58025 B were also used for mapping *Rf-3*.

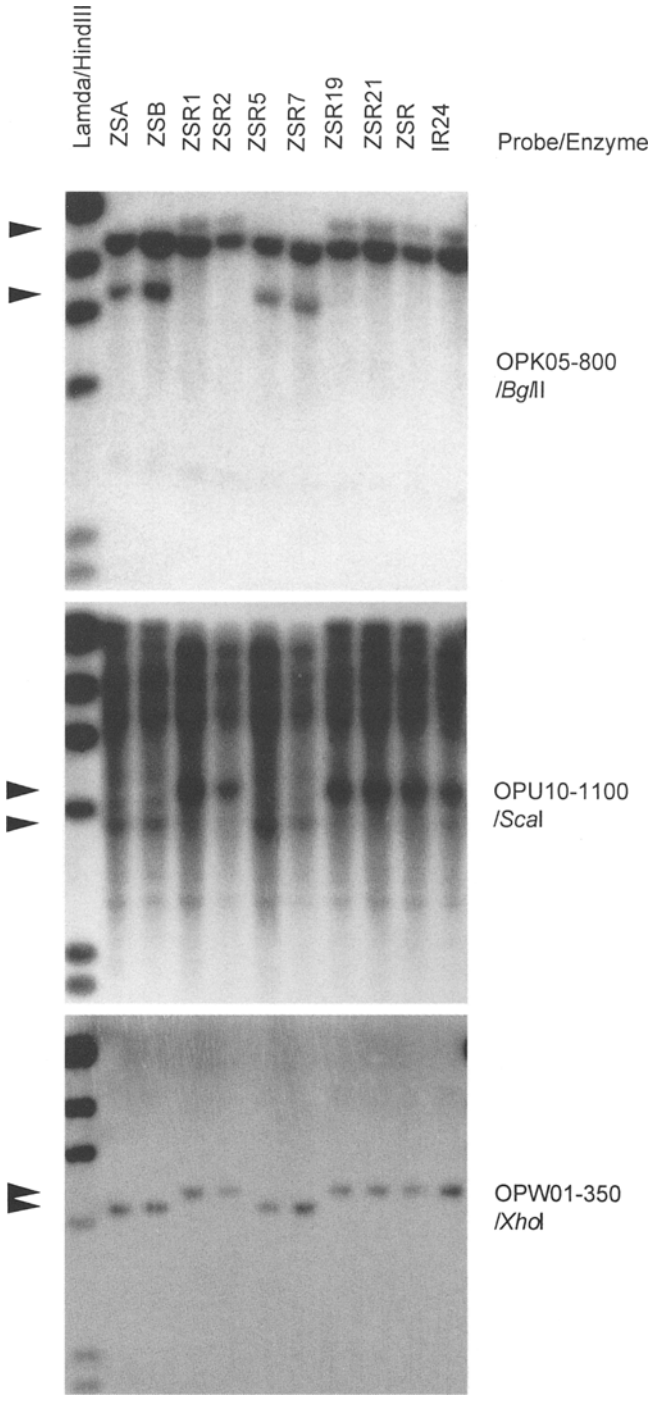


Fig. 2 Hybridization patterns with the three RAPD markers of the near-isogenic lines with different genotypes (see Table 1). The polymorphic bands are indicated by *arrows*

Three RAPD markers, OPK05-800, OPU-10-1100 and OPW01-350, and eight RFLP markers on the region from RZ382 to RG246 on chromosome 1 were used to survey the parents on six restriction enzymes (*Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Sca*I and *Xba*I). OPK05-800 and RG532 generated polymorphism between IR58025 A and IR36. The two markers were then used to survey the mapping population consisting of 39 sterile BC_1 plants. *Rf-3* was located between RG532 and OPK05-800, and the distances between *Rf-3* and RG532 and

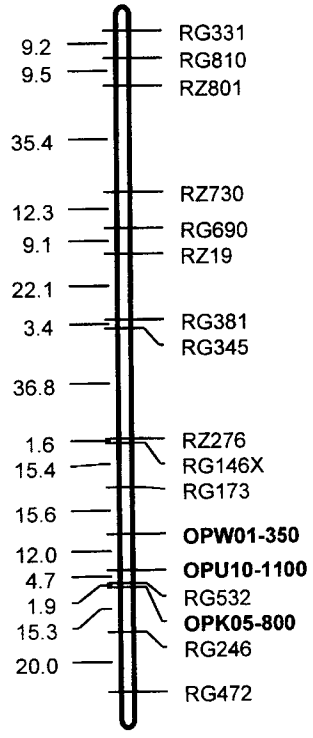


Fig. 3 Locations of the three RAPD markers on chromosome 1 (*boldface*) in a population of doubled haploid lines derived from the cross IR64/Azucena

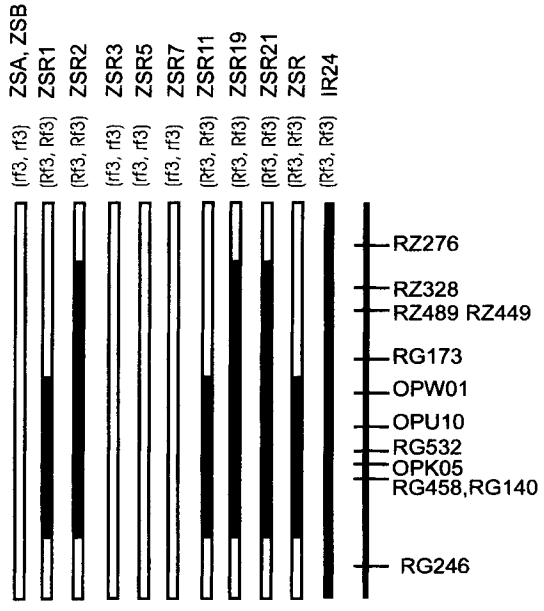


Fig. 4 Chromosomal segments introgressed from IR24 (*black bars*) on chromosome 1 in the near-isogenic lines with different genotypes. The positions of markers are indicated on the *right*. RFLP markers are positioned on the basis of the Cornell genetic map of rice (Causse et al. 1994) and RAPD markers are positioned on the basis of their locations shown in Fig. 3

between *Rf-3* and OPK05-800 were found to be 2.6 and 5.5 cM, respectively (Fig. 5C).

A comparison of the three maps showed that *Rf-3* is located within 3 cM of RG532. RG140 and RG458 were located on the other side of *Rf-3* in the F_2 populations of ZSA/ZS21 and ZSA/IR24. The position of OPK05-800 is closely linked to *Rf-3* on each of the three maps.

Multiple alleles at the RG532 locus

Multiple alleles were found at the RG532 locus in the four surveyed parents. Polymorphism at RG532 not only occurred between CMS lines and restorer lines but also between the two CMS lines ZSA and IR58025 A and between the two restorer lines IR24 and IR36. ZSA and IR58025 A with sterility allele *rf-3* carried different alleles at the RG532 locus, and IR24 and IR36 with fertility restorer allele *Rf-3* also carried different marker alleles at the locus for each of the six restriction digests (*Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Sca*I and *Xba*I). For example, digested with *Dra*I, DNA fragments hybridized with RG532 from ZSA, IR58025 A, IR24 and IR36 were 5.5, 6.6, 3.5 and 4.0 kb, respectively (Fig. 6).

Discussion

Several studies indicate that the fertility restoration of WA-CMS is controlled by two pairs of restorer genes (Gao 1981; Zhou 1983; Li and Yuan 1986; Zhang and Lu 1987; Govinda Raj and Virmani 1988; Bharaj et al. 1991). Recently, two restorer genes from IR36 were located on chromosome 7 and chromosome 10 through trisomic analysis (Bharaj et al. 1995). We initially took on this assumption and made an attempt to survey the introgressed segments carrying the restorer genes in Zhenshan 97 NILs using available RFLP markers on chromosomes 7 and 10. However, we failed to map the two restorer genes. RAPD analysis was therefore conducted using the NILs. Three RAPD markers closely linked to the *Rf-3* locus were identified and located on chromosome 1. Furthermore, three RFLP markers on the same chromosomal region were also found to be closely linked to the *Rf-3* locus. However, we failed to map the *Rf-4* gene.

The RAPD method has been used to quickly generate markers within a genomic region of interest using NILs (Klein-Lankhorst et al. 1991; Martin et al. 1991; Paran et al. 1991; Penner et al. 1993; Zhang et al. 1994a). In this study, six RAPD markers were found to be linked to the *Rf-3* locus in the NILs from the 720 surveyed random primers, and three of them were mapped to chromosome 1 using a doubled haploid population. The results indicate that the RAPD-NILs system is an efficient way of gene tagging. However, no RAPD markers were found to be linked to another restorer gene, *Rf-4*. The failure to identify RAPD markers linked to the gene of interest may be due to a short introgressed segment carrying the restorer gene, low polymorphism on the chromosomal region between the parents and/or few annealing sites for the primers in the chromosomal region of interest.

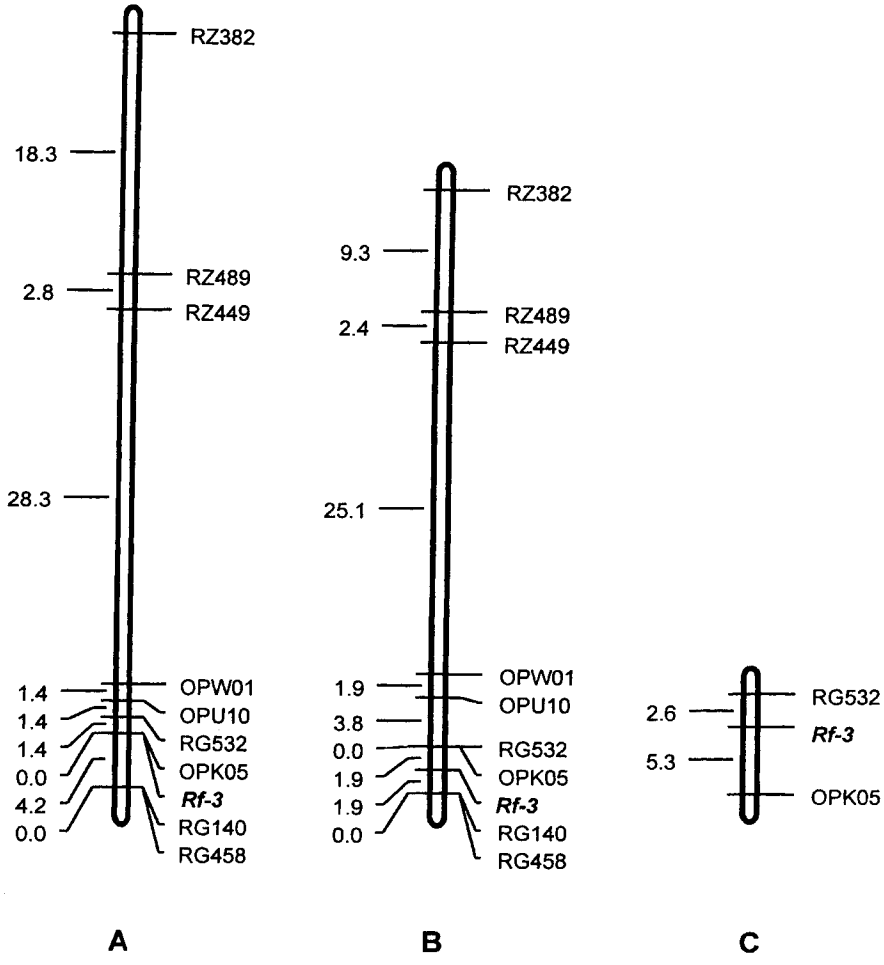
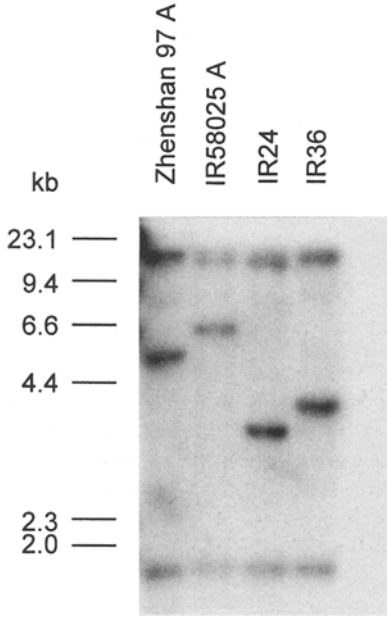


Fig. 5A–C Genetic maps of the *Rf-3* region on chromosome 1. Markers are indicated on the *right* and map distances (in cM) based on the kosambi function are on the left. **A** A map constructed from a population consisting of 36 sterile F_2 plants derived from the cross ZSA/ZSR21. **B** A map constructed from a population consisting of 27 sterile F_2 plants derived from the cross ZSA/IR24. **C** A map constructed from a population consisting of 39 sterile BC_1 plants derived from IR58025 A//IR36/IR58025 B

Fig. 6 Band patterns of two CMS lines, Zhenshan 97 A and IR 58025 A, and Two restorer lines, IR24 and IR36, whose DNA was digested with *Dra*I and hybridized with the *Rf-3* gene-linked marker RG532



WA cytoplasm is the most important source of male-sterile cytoplasm used for developing hybrid rice. A large number of CMS lines with WA cytoplasm and their restorer lines have been developed. Differences in sterility maintenance and fertility restoration were detected in the CMS system. CMS lines differ in their degree of sterility, and some restorer lines are more effective than others (Li and Zhu 1988; Virmani and Wan 1988). For example, Er-jiu-ai 4 A and Zhenshan 97 A have the same male-sterile cytoplasm (WA), but the percentage of typical abortive pollen grains (irregular in shape and unstainable with I-KI solution) in Er-jiu-ai 4 A is higher than that in Zhenshan 97 A. When IR24 was used as the restorer, the mean pollen fertility in F_1, F_2 and BC_1 plants derived from Er-jiu-ai 4 A was lower than those derived from Zhenshan 97 A in each generation (Zhang and Lu 1987). The difference in sterility maintenance and fertility restoration among the CMS lines is believed to be due to the presence of minor fertility genes (Virmani et al. 1986) or to a different genetic background in maintainers and restorers (Li and Zhu 1988). In our study, we found multiple alleles at the RG532 locus that are closely linked to the *Rf-3* locus. It is possible that the *Rf-3* locus also has multiple alleles. In this case, different sterility maintainer alleles could possess different capaci-

ties for maintaining male sterility, and different fertility restorer alleles could differ in the ability to restore fertility. Based on this hypothesis, it is important to select more effective alleles for sterility maintenance and fertility restoration in the breeding of hybrid rice.

Molecular marker-assisted selection (MAS) is a promising approach for integrating biotechnology with traditional plant breeding. MAS may be especially useful for characters that are difficult or expensive to measure, such as fertility restoration for CMS, where the presence of restorer genes in breeding lines cannot be detected through traditional approaches without a generation of progeny testing. Identification of DNA markers linked to *Rf-3* provides useful tools for MAS for the sterility maintainer genes and the fertility restorer genes. The multiple marker alleles at the RG532 locus closely linked to the *Rf-3* locus provide a possibility for marker-assisted screening for more effective sterility maintainer alleles and fertility restoration alleles from rice germplasm if multiple alleles at the locus are identified. The RG532 marker, which generates multiple alleles, could allow identification of multiple alleles at the *Rf-3* locus. It is expected that marker-assisted identification of effective alleles and the use of MAS in breeding programs will facilitate the development of hybrid rice.

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