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Molecular mapping of the reverse thermo-sensitive genic male-sterile gene (*rtms1*) in rice

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Abstract TGMS (thermo-sensitive genic male-sterile) rice is widely used in hybrid rice production. Because of a specific temperature requirement, it can be used only in a narrow rice-growing zone in Asia. A newly discovered reverse thermo-sensitive genic male-sterile line, J207S, has an opposite phynotype compared to the normal TGMS lines. J207S is completely sterile when the temperature is lower than 31°C. Thus, it can be widely used in a larger area. Genetic analysis indicated that the sterility of J207S was controlled by a single recessive gene which was first named as *rtms1*. An F_2 population from the cross between J207S and E921 was developed and used for molecular mapping of the *rtms1* gene. The AFLP (amplified fragment length polymorphism) technique, combined with BSA (bulked segregant analysis), was used to screen markers linked to the target gene, and eight polymorphic AFLP loci were identified. Co-segregating analysis using the F_2 population showed that two of them, Rev1 and Rev7, were closely linked to the target gene with a recombinant rate of 3.8% and 7.7%, respectively. Both Rev1 and Rev7 were found to be single-copy sequences through Southern analysis. Rev1 was subsequently mapped on chromosome 10 with a doubled-haploid mapping populations derived from the cross CT9993 × IR62266 available at Texas Tech University. RM222 and RG257 were linked to Rev1 at a distance of 11.8 cM and 4.6 cM, respectively. Additional SSR markers from the rice map of Cornell University, RFLP markers from the map of RGP in Japan and the map of Texas Tech University were selected from the region surrounding Rev1 on chromosome 10 to conduct the fine-mapping of the *rtms1* gene. Presently, *rtms1* was mapped between RM239 and RG257 with genetic distance of 3.6 cM and 4.0 cM, respectively. The most-closely linked AFLP marker, Rev1, 4.2 cM from the *rtms1* gene, was sequenced and converted into a SCAR (sequence characterized amplified region) marker which could facilitate marker-assisted selection of the *rtms1* gene.

Keywords Rice · Reverse TGMS gene · AFLP · MAS · Molecular mapping

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Introduction

The discovery and application of thermo-sensitive genic male-sterile (TGMS) lines have simplified the procedure of rice hybrid seed production because TGMS lines can be used not only as male-sterile lines but also as maintainer lines. Additionally, they are highly valued because of the wide spectrum of restorers and their great potential in hybrid seed production. Up to now, TGMS lines have made both a great contribution and economical benefits to the hybrid rice production of the two-line system. Most TGMS lines are sterile at high temperature (>25°C), but fertile at a lower temperature. The sterility is controlled by a recessive gene. Recently, some TGMS genes in different TGMS lines have been discovered and studied. So far, six TGMS genes, *tms1*, *tms2*, *tms3*, *tms4*, *tms5* and *ms-h*, have been mapped on chromosomes 8, 7, 6, 2, 2 and 9, respectively (Maruyama et al. 1991; Wang et al. 1995; Subudhi et al. 1997; Yamagushi et al. 1997; Koh et al. 1999; Dong et al. 2000; Jia et al. 2000). There were also some reports on thermo-sensitive male sterility controlled by dominant genes such as the Pingxiang male-sterile line (Yan et al. 1989) and the 8987 male-sterile line (Li et al. 1999), though this kind of male-sterile line was difficult to apply in rice breeding.

Owing to the limitation of the temperature requirement, TGMS rice lines can only be used in a very narrow zone for growing rice. If the TGMS rice is sterile at a lower temperature, and the sterility is controlled by a recessive gene, it can be used in a much larger area. During the last decade, many rice breeders had been focusing on the development of such kinds of TGMS lines. One such TGMS line, J207S, is completely sterile when the temperature is lower than 31°C, but fertile when the temperature is higher than 31°C. And thus it is named as the reverse TGMS line. It can be safely used for hybrid seed production in a much larger area by adjusting its sowing time. Genetic study indicated that the sterility of J207S is controlled by a recessive gene which was named as *rtms1*. The application of the reverse TGMS line will compensate the TGMS lines in rice production and facilitate the application of the two-line system in rice. This paper reports the tagging and mapping of a rice reverse thermo-sensitive male-sterile gene (rtmsl) by using a molecular-marker technique combined with bulked segregant analysis.

Materials and methods

Plant materials and fertility characterization

Line J207S, obtained from the spontaneous mutation of the progeny from the cross [(IR64 × Zhachanglong) × Jing101], was crossed with a high yielding cultivar, E921, in 1997 and 1999. Pollen and spikelet fertility were determined in the F_1 and F_2 generations at the anthesis stage using a 1% iodine – potassium iodide (I-KI) solution. Plants with no stained pollen were considered as completely male sterile, whereas plants having more than 95% stained pollen were classified as fertile. At the same time, self-pollination was tested to confirm the sterility and fertility. This fertility characterization was performed at the Tianjin Agricultural College and Hainan Island of China in 1997 and 1999. The F_2 population from the cross J207S × E921 consisted of 315 individuals; χ^2 tests were performed to determine the goodness of fit of the F_2 generation to a 3:1 ratio. Out of the 315 plants, 52 (27 sterile plants and 25 fertile plants) were used for AFLP analysis.

DNA preparation

DNA from each individual, along with both parents, was isolated following the method of McCouch et al. (1988). The DNA concentration was determined according to genomic DNA samples of known concentration. Bulk F was made by mixing equal amounts of DNA from 15 fertile F_2 plants and bulk S by mixing equal amounts of DNA from 15 sterile F_2 plants.

AFLP analysis

AFLP analysis was performed following the procedure of Vos et al. (1995) with minor modifications. The Gibco-BRL AFLP System II kit was used and [γ-^{33}P] ATP was purchased from Amersham. Amplification products were separated on a 6% PAGE sequencing gel, at 100 W and 2,000 V for about 2.5 h. The gel was dried and the X-ray film was exposed for 3–5 days at –70°C.

Recovery and cloning of the target AFLP fragments

After development, the autoradiogram was aligned with the gel to recover the target AFLP fragments from the gel. The gel slice containing DNA fragments were excised, eluted with 400 µl of high salt buffer (containing 20% ethanol, 1 M LiCl, 10 mM Tris-HCl, pH 7.5) and then incubated at room temperature for 24 h and at 65°C for 2 h. The DNA was precipitated and one-quarter of the re-suspended DNA was re-amplified using the same primer combination and PCR conditions as that of the selective amplification. The re-amplified DNA fragment was purified using a Gene Clean Kit and then cloned to the pGEM-T easy vector (Promega).

Mapping of the AFLP marker and the *rtms1* gene

A double-haploid (DH) population from the cross between CT9993 and IR62266 (Zheng et al. 1998) was used for mapping the AFLP markers with the MAPMAKER program (Lander et al. 1987). After the AFLP markers were mapped on the chromosome, a number of SSR and RFLP markers in this region were selected for fine mapping of the *rtms1* gene. Restriction digestion, gel electrophoresis and Southern hybridization followed standard procedures (Sambrook et al. 1989). Genomic DNA was digested with the restriction enzymes *Eco*RI, *Hin*dIII, *Eco*RV, *Bam*HI, *Xba*I and *Dra*I, respectively. SSR amplification was performed as described by Wu and Tanksley (1993) and Akagi et al. (1996). The amplified products were resolved on a 6% denaturing polyacrylamide gel. The phynotype, RFLP, AFLP, and SSR data were combined for linkage analysis using the MAPMAKER program and a partial linkage map of the region on the chromosome surrounding the *rtms1* gene was constructed.

Sequencing, primer designing and PCR analysis

The AFLP fragments, which were cloned in the pGEM-T easy vector system, were sequenced with an ABI 373 system. Primers were designed and synthesized according to the sequence. Then PCR reactions were performed among the two parents, the sterile bulk, the fertile bulk, ten fertile individuals and ten sterile individuals in order that the cloned AFLP marker could be converted into a SCAR marker. The PCR amplification was performed in 20 µl of reactions containing 25 ng of template DNA, 10 mM Tris HCl, pH 8.3, 50 mM KCl, 2.0 mM $MgCl_2$, 100 µM of each dNTP, 15 ng of primer and 1 unit of *Taq* DNA polymerase. After an initial heat denaturation at 94°C for 3 min, the reaction mixture was subjected to amplification in a MJ PTC-100 thermocycler for 30 cycles consisting of 1 min at 94°C, 1 min at 62°C, and 1.5 min at 72°C. Final extension was for 5 min at 72°C. Amplified products were separated on 1.2% agarose gels, stained with ethidium bromide and visualized by illumination with ultraviolet light, then photographed or scanned with a Bio-Rad DOC-1000 scanner.

Results

Genetic analysis of the *rtms1* gene

The results from the field characterization in 1997 and 1999 showed that the sterility of J207S had no relationship with the anthesis stages. All of the F_1 plants were fertile at 31°C, like the parent E921. The segregation of fertile to sterile plants in the F_2 population followed a 3:1 ratio. The results of self-pollination in the F_2 population also confirmed that the gene controlling the sterility in J207S, *rtms1*, was recessive.

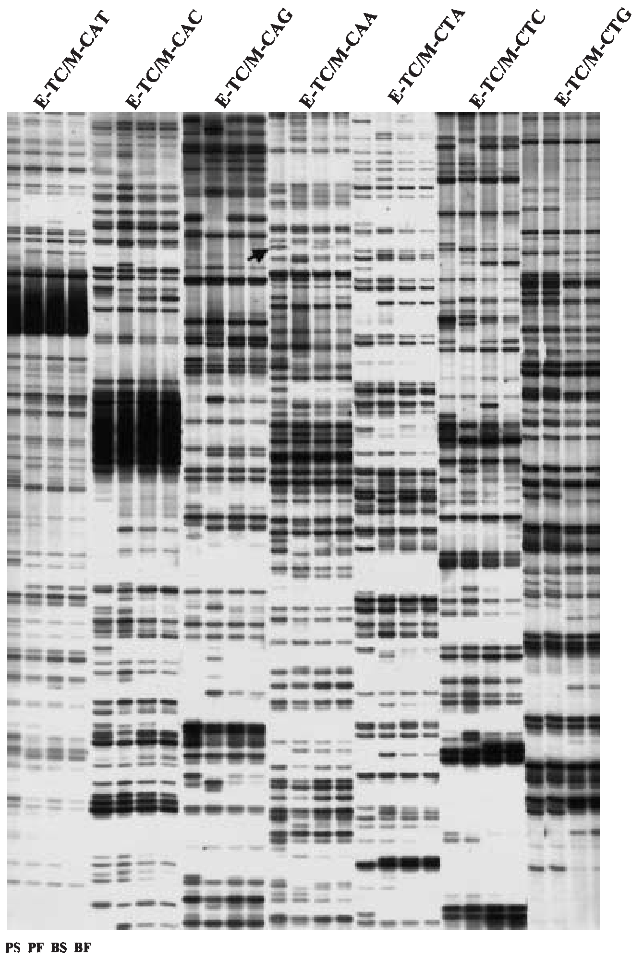

Fig. 1 BSA analysis of the *rtms1* gene using different AFLP primer combinations. The samples are the sterile parent J207S (*PS*), the fertile parent E921 (*PF*), the sterile bulk (*BS*) and the fertile bulk (*BF*). The *arrow* indicates the polymorphic band Rev1

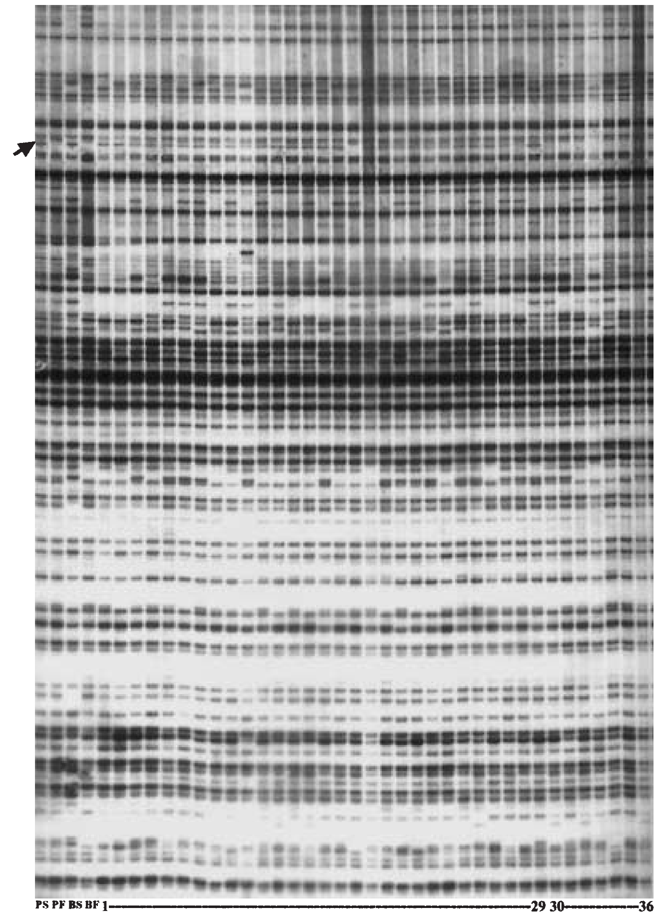

Fig. 2 Partial results from F_2 progeny analysis of Rev1. The samples in each lane are: J207S (*PS*), E921 (*PF*), the sterile bulk (*BS*), the fertile bulk (*BF*) and F_2 individuals. The *arrow* indicates the specific band Rev1

Screening AFLP markers linked to the *rtms1* gene

The AFLP analysis showed that there were about 60–100 bands in each 2+3 primer combination when labeled with [γ-^{33}p] ATP. In the bulked segregant analysis (Michelmore et al. 1991), 64 primer combinations were employed and eight polymorphic AFLP bands were obtained, which were designated as Rev1 to Rev8, respectively. Out of these, five were from the sterile plant and three from the fertile plant (Fig. 1). These results reconfirmed that AFLP combined with bulked segregant analysis was effective in finding closely linked markers to the target gene (Tanksley et al. 1995), and that the simultaneous use of both methods reduced the risk of false positives.

The F_2 progeny analysis was conducted to identify the linkage between the AFLP markers and the target gene. Out of the F_2 population from the cross of J207S and E921, 52 individuals consisting of 27 sterile plants and 25 fertile plants were used for progeny analysis. The results showed that only two or four individuals in the 52 plants were identified as recombinants for Rev1 and Rev7, respectively. The recombinant value between the two markers and the *rtms1* gene was about 3.8% and 7.7%, respectively. Rev1 and Rev7 were amplified with the E-TC/M-CAA and E-TA/M-CAT primer combinations. Figure 2 shows the partial results of the progeny analysis of Rev1. The recombinant values of the other six polymorphic bands were over 11.5%. Southern analysis showed that both the Rev1 and Rev7 markers were single-copy sequence in the genome, so they could be used for mapping the *rtms1* gene.

Mapping Rev1 and the *rtms1* gene onto a specific chromosome

To determine the chromosome location of Rev1, progenies from a DH population of a cross between CT9993 and IR62266 were used. After analysis with MAPMAKER software Rev1 was finally mapped on the

short arm of chromosome 10 near the markers RM222 and RG257 at distances of 11.8 and 4.6 cM, respectively (Fig. 3a). Two AFLP markers, Me5_16 and EMP2_9, were at 0.3 and 0.0 cM on the map, respectively, with the mapping population from the cross CT9993 × IR62266.

To further confirm the map location of the *rtms1* gene, co-segregating analysis was conducted with the F_2 population from the cross of J207S and E921. Five RFLP markers (L169, S10906, R2965, G89B and S10265B) kindly provided by the MAFF DNA Bank in Japan (Harushima et al. 1998), and six microsatellite markers (RM244, RM216, RM239, RM184, RM269, RM271) available from the web site (http://ars-genome.cornell.edu/rice/allmicrosat.txt/) (Temnykh et al. 2000), were selected from the region surrounding Rev1 on chromosome 10. RG257 used for mapping Rev1 was selected for mapping *rtms1*. The two microsatellite markers, RM216 and RM239, showed linkage with the target gene at a distance of 14.2 and 3.6 cM respectively. The linkage distance of Rev1 with *rtms1* was 3.6 cM. On the other side of *rtms1* was RG257 which was located 4.0 cM from *rtms1*(Fig. 3b). Figure 4 showed the co-segregant analysis of RM239 with the *rtms1* gene.

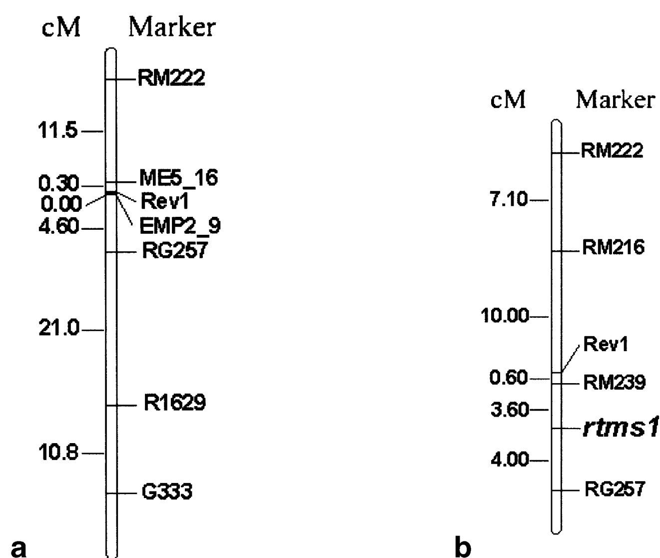

Fig. 3a A partial linkage map of the region surrounding Rev1 on chromosome 10, derived from the cross CT9993 × IR62266. **b** Mapping results of the *rtms1* gene on chromosome 10 with the F_2 mapping population from the cross J207S × E921

Converting the AFLP marker to a SCAR marker

The AFLP marker Rev1, which was closely linked to the *rtms1* gene, was sequenced. Then the following primers were designed according to Paran and Michelmore (1993):

PRev1-reverse:
5′-CACGAACAAGAAGGAATGAG-3′,

and

PRev1-forward:
5′-CAGGTCCCTAACCCTTAGCAAAG-3′.

Using this primer pair, PCR reactions were performed among the two parents, bulk F, bulk S, ten individual fertile plants and ten individual sterile plants. The results indicated that the fragment amplified by PRev1 existed only in sterile plants and the sterile bulk with the same fragment length as Rev1 (Fig. 5). Thus, the AFLP marker Rev1 was converted into a SCAR marker and this SCAR marker could be used in the marker-assisted selection of the *rtms1* gene. The DNA sequence of Rev1 has been deposited in GenBank under Accession Number AF255611. In our study, other AFLP markers failed to be converted into a SCAR marker.

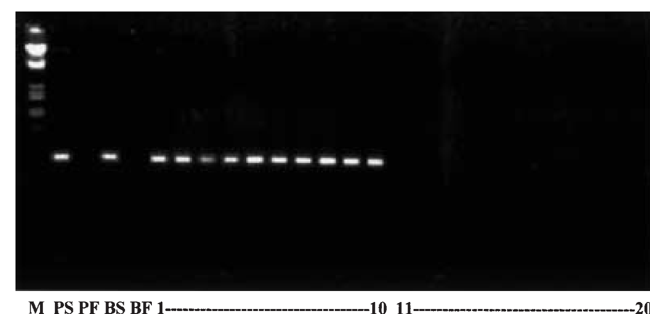

Fig. 5 The SCAR marker converted from Rev1. *Lanes: M* molecular-weight marker/lambda DNA digested with *Eco*RI and *Hin*dIII; *PS* sterile parent J207S; *PF* fertile parent E921; BS sterile bulk; *BF* fertile bulk; *1–10* F_2 sterile individuals; *11–20* F_2 fertile individuals

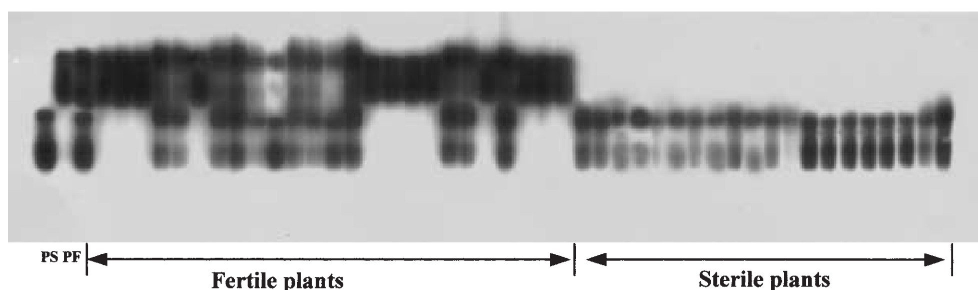

Fig. 4 Progeny analysis with the microsatelite marker RM239. The fertile F_2 individuals consist of both homologous and heterozygous fertile individuals. *PS* sterile parent J207S; *PF* fertile parent E921

Discussion

This is the first report on rice reverse TGMS gene-mapping. The discovery and application of the reverse TGMS line will facilitate the application of a two-line system in hybrid rice production. The mapping results described above showed that the *rtms1* gene was located on the short arm of chromosome 10. Therefore, *rtms1* is non-allelic to the above-discussed six TGMS genes (*tms1*, *tms2*, *tms3*, *tms4*, *tms5* and *ms-h*). The molecular markers closely linked to the *rtms1* gene, such as RG257 and RM239, can be used in *rtms1* gene isolation by means of the map-based cloning strategy. Mapping and cloning of the *rtms1* gene in rice will promote the molecular study of the thermo-sensitive genic male sterility in rice. Currently, we are developing a larger population and obtaining more RFLP probes between L169 and S1387 in chromosome 10 (according to Harushima et al. 1998) from the Japanese Rice Genome Research Program in order to perform the fine mapping of the region flanking the *rtms1* gene.

The results reported in this paper clearly demonstrated that the AFLP technique was a powerful, reliable, stable and rapid assay in molecular-marker screening (Ballvora et al. 1995; Thomas et al. 1995; Cervera et al. 1996). Coupled with bulked segregant analysis, it allowed us to identify the locus governing thermo-sensitive genic male sterili ty and to effectively determine the chromosome location of this locus on the molecular linkage map of rice. Eight polymorphic bands amplified from 64 primer combinations also showed the high efficiency of the AFLP technique in marker screening. Meanwhile, our results indicated that the 2+3 primer combinations were suitable for a small-genome plant like rice.

PCR is the most accurate, efficient, economical and simplest method in marker-assisted selection (Paran and Michelmore 1993). The SCAR marker obtained in this study will greatly facilitate the transfer of the reverse TGMS allele to desirable genetic backgrounds via marker-assisted selection. This will also help in the selection of reverse TGMS segregants with great accuracy in early generations. Our study also showed that AFLP markers were easier to be converted into SCAR markers because AFLPs were developed from RFLPs and RAPDs.

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