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Y. G. Wang · Q. H. Xing · Q. Y. Deng · F. S. Liang · L. P. Yuan · M. L. Weng · B. Wang

Fine mapping of the rice thermo-sensitive genic male-sterile gene *tms5*

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Abstract AnnongS-1, a thermo-sensitive genic male-sterile (TGMS) rice line, has a new TGMS gene. Genetic analysis indicated that the sterility of AnnongS-1 was controlled by a single resessive gene named *tms5*. In our previous studies based on an F_2 population from the cross between AnnongS-1 and Nanjing11, *tms5* was mapped on chromosome 2. Recently, a RIL (recombinant inbred line) population from the same cross was developed and used for the fine mapping of the *tms5* gene. Molecular marker techniques combined with BSA (bulked segregant analysis) were used. As a result, two AFLP markers (AF10, AF8), one RAPD marker (RA4), one STS marker (C365-1), one CAPs marker (G227-1) and four SSR markers (RM279, RM492, RM327, RM324) were found to be closely linked to *tms5* gene. The DNA sequences of the RFLP marker of C365 and G227 were found in GenBank, and on the basis of these sequences, many primers were designed to amplify the two parents and their RIL population plants. Finally, the *tms5* gene was mapped between STS marker C365-1 and CAPs marker G227-1 at a distance of 1.04 cM from C365-1 and 2.08 cM from G227-1.

Keywords Rice · TGMS · Fine mapping · CAPs

Introduction

The application of thermo-sensitive genic male-sterile (TGMS) lines has made a great contribution to hybrid rice production by providing the two-line system instead of the commonly used three-line system. To date many TGMS rice lines have been developed and applied in hybrid rice production. Various 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 chromosome 8, 7, 6, 2, 2 and 9, respectively (Wang et al. 1995; Yamagushi et al. 1997; Subudhi et al. 1999; Dong et al. 2000; Jia et al. 2000; Wang 2001; Koh et al. 1999).

AnnongS-1 is the first *indica* TGMS line in rice to be developed in the world. Its sterility is controlled by a recessive gene named *tms5* (Jia et al. 2000; Wang 2001). None of the TGMS genes has yet been cloned. In order to isolate and clone the *tms5* gene, it is necessary to develop a fine map of the *tms5* gene-encompassing region and to find the nearest linked markers. In the investigation reported here, the *tms5* gene was fine-mapped with a newly developed recombinant inbred line (RIL) population.

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Y.G. Wang and Q.H. Xing contributed equally to this contribution.

Y. G. Wang · Q. H. Xing · F. S. Liang · M. L. Weng · B. Wang (✉)
Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing 100101, China
e-mail: bwang@genetics.ac.cn
Fax: +86-10-64873428

Q. Y. Deng · L. P. Yuan
China National Hybrid Rice Research and Development Center, Changsha 410125, China

Materials and methods

Development of rice RIL population and fertility characterization

AnnongS-1 was obtained from the spontaneous mutation of AnnongF. A RIL F_8 population consisting of 96 sterile individuals and 97 fertile individuals from the cross between AnnongS-1 and Nanjing11 was developed from 1997 to 2001; two generations were performed per year.

Pollen and spikelet fertility were determined at the anthesis stage using a 1% iodine-potassium iodide (I-KI) solution. Plants with no stained pollen were considered to be completely male-sterile, whereas plants having more than 95% darkly stained pollen were classified as fertile. At the same time, self-pollination was tested to confirm the sterility and fertility.

DNA preparation and establishment of the bulks

Total genomic DNA from both parents and each individual of the RIL population was extracted from leaves using the method of McCouch et al. (1988). Fifteen individual DNAs of the sterile population were mixed with the same amount to form a sterile bulk,

Table 1 Designed primer sequences which were used for the fine mapping of the *tms5* gene (*F* forward, *R* reverse)

Primers	Sequence (5′–3′)	Size of PCR products	T_m (°C)	GenBank accession no.
C365-1	F: ATTTTGGTTGCGCATTAGAGG R: GAAATATGCCAAGTACGGAGGAT	800 bp	56	AP004181
G227-1	F: ACACATCAGCAACAATTCATCTAC R: AACAGCATTTCCCCCTACTACA	500 bp	58	AP004030

and 15 individual DNAs of the fertile population were mixed with the same amount to form a fertile bulk.

In order to confirm the linkage relationship, we further assayed the potential bands linked to the *tms5* gene detected by bulked segregant analysis (BSA) with a large number of the RIL population.

Polymerase chain reaction (PCR) analysis

Simple sequence repeat (SSR) analysis

All PCR reactions were performed in a MJ PTC-100 thermocycler (Waltham, Mass.). The basic SSR procedure was as follows: 1× reaction buffer, 2.0 m*M* Mg^{2+}, 0.1 m*M* of each dNTP, 2.0 U *Taq* polymerase, 50 ng template DNA, 30 ng each primer, fixation to 20 μl with distilled water, overlay with one drop of mineral oil. The amplification reactions were carried out using the following profile: 94 °C for 4 min, 35 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min, with a final extension step of 72 °C for 10 min. The amplification products were separated on a 6% PAGE sequencing gel run at 100 W for 1.5 h following a pre-electrophoresis for 30 min. The gel was removed from the apparatus and stained by the silver-staining method (Xu et al. 2002).

Amplified fragment length polymorphism (AFLP) analysis

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

Randomly amplified polymorphic DNA (RAPD) analysis

The basic procedure of the RAPD reaction was as follows: 1× reaction buffer, 0.1 m*M* of each dNTP, 2 m*M* Mg^{2+}, 1 U *Taq* polymerase, 25 ng template DNA, 25 ng primer; fixation to 25 μl with distilled water, finally overlay with one drop of mineral oil. The RAPD primers were purchased from Operon Technology (Alameda, Calif.). The amplification reactions were carried out using the following profile: 94 °C for 5 min, 40 cycles of 94 °C for 1 min, 37 °C for 1 min, 72 °C for 2 min, with a final extention step of 72 °C for 5 min. The amplification products were analyzed by electrophoresis on 1.0% agarose gels, followed by 0.1 μg/ml ethidium bromide staining and visualizing under UV light.

Sequence-tagged site (STS) marker and cleaved amplified polymorphic sequence (CAPs) marker analysis

Based on the closely linked flanking RFLP (restriction fragment length polymorphism) markers, the DNA sequences of the *tms5*-encompassing region were found from the Web (http://www.genome.arizona.edu/shotgun/rice/status/seqSum2.html). Primers were designed and synthesized according to the sequence (Table 1). PCR reactions were then performed among the two parents and the two bulks. The PCR reaction was performed in 20 μl of reaction system containing 25 ng of template DNA, 15 m*M* $MgCl_2$, 1× PCR buffer, 2.5 m*M* dNTP, 15 ng primer, 1 U *Taq* DNA polymerase. After an initial heat denaturation at 94 °C for 4 min, the reaction mixture was subjected to amplification for 30 cycles of 94 °C for 1 min, 56 °C or 58 °C for 1 min, 72 °C for 1.5 min. A final extension was performed for 5 min at 72 °C. Amplication products were separated on 3.0% agarose gels, stained with ethidium bromide and visualized under UV light, then photographed or scanned with a Bio-Rad DOC-1000 scanner (Herculer, Calif.).

Following the PCR reaction, 2.5 μl 10× PCR buffer H, 2.5 μl bovine serum albumin (1%) and 1 μl *Eco*RI were added into each reaction tube and the contents digested at 37 °C for 2–3 h.

Results

Identification of molecular markers linked to *tms5* gene

SSR marker

Based previous results on the F_2 population, 30 SSR primer pairs flanking the *tms5* gene on chromosome 2 were selected to fine map the *tms5* gene using the RIL population. As a result, 11 primer pairs (RM492, RM279, RM327, RM324, RM341, RM6, RM263, RM262, RM166, RM208, OSR28) amplified polymorphism between the two parents. Following identification with a large number of the RIL plants, it was found that RM492, on the short arm of Chromosome 2 near the centromere region, was located apart from the *tms5* gene at a distance of 5.4 cM (Fig. 1), and RM279 was located on the other side of *tms5* gene at a distance of 19 cM.

AFLP marker

Sixty-four AFLP primer combinations were used to amplify the two parents and the two bulks; three [E:AA-M:CTG_{300} (AF10), E:AT-M:CAC_{400} (AF8) and E:AC-M:CTC_{350} (AF6)] were found to show significant linkage to the *tms5* gene at a distance of 2.8 cM (Fig. 2A), 3.4 cM (Fig. 2B) and 15.8 cM, respectively.

RAPD markers

Among the 410 RAPD primers examined, four primers (OPA07, OPB01, OPE16, OPP08) amplified polymorphism between the two parents and two bulks, but only $OPE16_{350}$ (RA4) was linked closely to the *tms5* gene at a distance of 7.7 cM (Fig. 3).

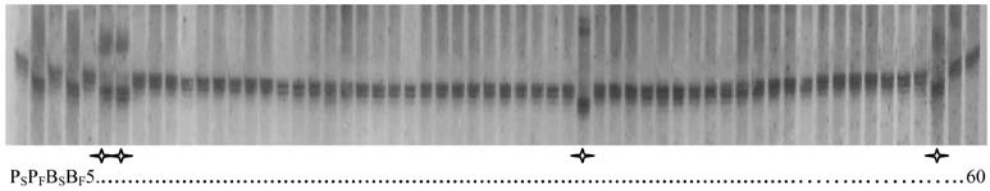

Fig. 1 The amplification results of the SSR marker RM492. Lanes: P_S AnnongS-1, P_F Nanjing11, B_S sterile bulk, B_F fertile bulk, *5–60* sterile plants, +recombinant type

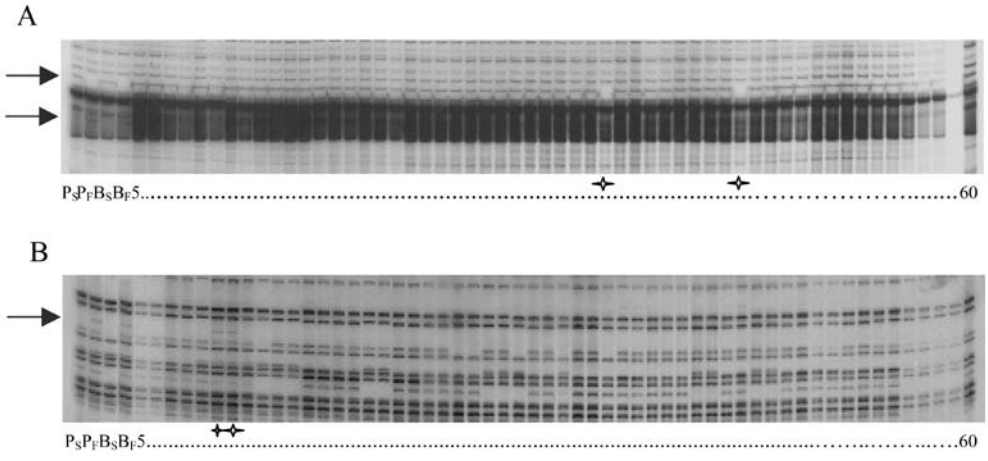

Fig. 2 The amplification results of AFLP markers of E:AA/M:CTG (**A**) and E:AT/M:CAC (**B**). Lanes: P_S AnnongS-1, P_F Nanjing11, B_S sterile bulk, B_F fertile bulk, *5–60* sterile plants, +recombinant type

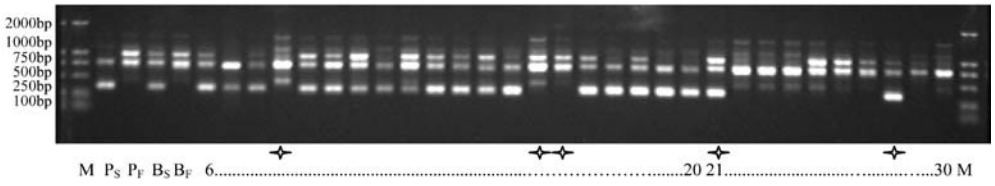

Fig. 3 The amplification results of RAPD marker OPE16. Lanes: *M* DNA molecular-weight marker 2000, P_S AnnongS-1, P_F Nanjing11, B_S sterile bulk, B_F fertile bulk, *6–20* sterile individuals, *21–30* fertile individuals, +recombinant type

STS marker and CAPs markers

In order to find more markers closely linked to the *tms5* gene, we designed 15 PCR primers according to the sequence of the RFLP markers around the *tms5* gene published in the GenBank. Among them, S14115 and C365-1 showed a linkage relation to the *tms5* gene at a distance of 20 cM and 1.04 cM, respectively (Fig. 4). There was no polymorphism between the two parents and two bulks using primer G227-1, but when the PCR products were digested with *Eco*RI, the polymorphism was shown (Fig. 5). The genetic distance of G227-1 from the *tms5* gene was 2.08 cM (Fig. 6).

Construction of the fine map of the *tms5* gene-encompassing region

Based on our results of SSR, AFLP, RAPD, STS and CAPs marker analyses, a local linkage map of the *tms5* gene-encompassing region was constructed (Fig. 7). Flanking the *tms5* gene, G227-1, AF9 and C365-1 are the most closely linked markers. CAPs marker G227-1 and AFLP marker AF9 are in the same loci, approximatly 2 cM from the *tms5* gene. On the other side of the *tms5* gene, marker C365-1 is about 1 cM from *tms5* gene. STS marker C365-1 and RFLP markers of R394 and C630 are in the same loci.

Discussion

The application of the TGMS line has greatly facilitated the application of a two-line system in hybrid rice production. The molecular markers closely linked to the *tms5* gene, such as C365-1 and G227-1, can be used for isolating the *tms5* gene by means of map-based cloning strategy. Fine-mapping and cloning of the *tms5* gene in rice will promote the molecular study of the thermo-sensitive genic male sterility in rice. In this study, a RIL

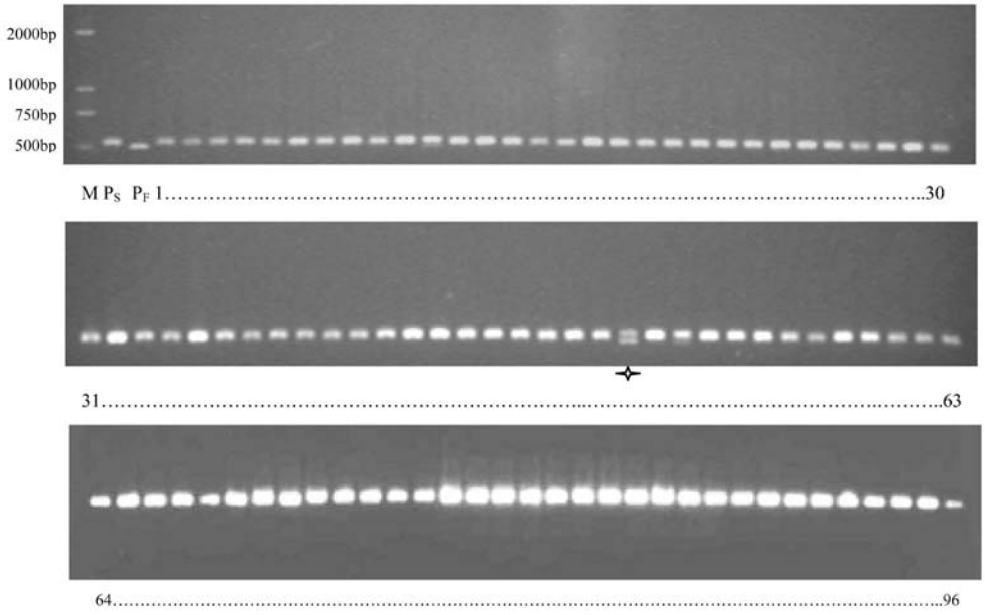

Fig. 4 The amplification results of C365-1 in 96 sterile individuals of the RIL population. Lanes: *M* DNA molecular-weight marker 2000, P_S AnnongS-1, P_F Nanjing 11, *1–96* sterile plants

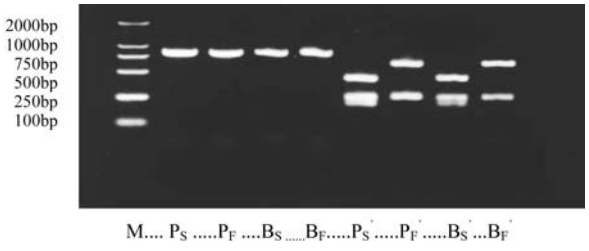

Fig. 5 The amplification of G227-1 in two parents and bulks before and after *Eco*RI digestion. Lanes: *M* DNA molecular weight marker 2000, P_S AnnongS-1, P_F Nanjing11, B_S sterile bulk, B_F fertile bulk, P_S', P_F', B_S', B_F', the pattern after *Eco*RI digestion

F_8 population was used to map the gene of interest. It is a suitable population for fine-mapping the *tms5* gene, especially as the markers linked to the target gene in the sterile individuals are stable after eight self-pollinations, and the extreme recessive class (sterile plants) were used to perform the co-segregating analysis (Zhang et al. 1994). We are at present constructing an integrated BAC clone contig according to the positive clones screened from the AnnongS-1 BAC library constructed by our Laboratory. The next steps are to find more closely linked markers using a larger population and further to clone the *tms5* gene via map-based cloning.

The rice genome has now been sequenced (Yu et al. 2002), thereby providing a convenient method for finding molecular markers linked to the target gene. In this study, the RFLP technique was performed once to detect polymorphism but failed to find polymorphism between the two parents. We then attempted to design primers based on the DNA sequence of clones flanking the target

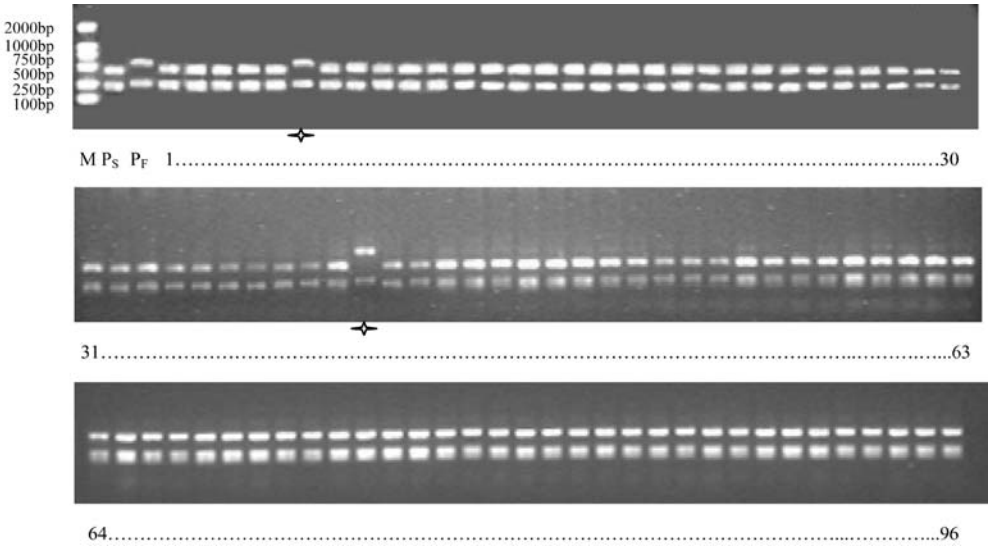

Fig. 6 The CAPs analysis of G227-1 in 96 sterile individuals. Lanes: *M* DNA molecular-weight marker 2000, P_S AnnongS-1, P_F Nanjing11, *1–96* sterile plants, +recombinant type

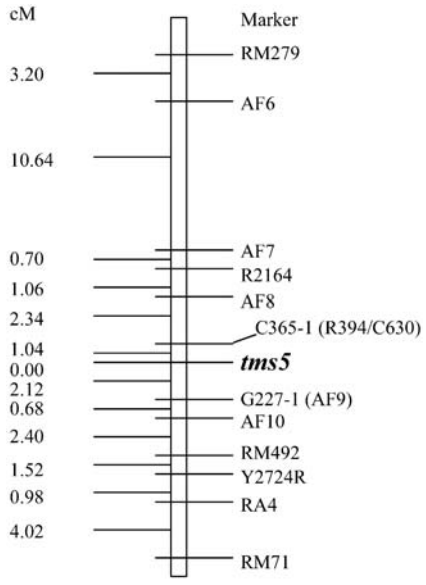

Fig. 7 Fine map showing *trm5* gene-encompassing region on the short arm of chromosome 2

gene and performed PCR. In this way we obtained a STS marker and a CAPs marker more closely linked to the *tms5* gene. This method has some advantages – it does not need much template DNA and is simple and rapid – so it is a better method for gene fine-mapping.

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