Theor Appl Genet (1995) 91:1111–1114

B. Wang · W. W. Xu · J. Z. Wang · W. Wu
H. G. Zheng · Z. Y. Yang · J. D. Ray · H. T. Nguyen

Tagging and mapping the thermo-sensitive genic male-sterile gene in rice (*Oryza sativa* L.) with molecular markers

Received: 25 February 1995 / Accepted: 17 March 1995

Abstract The thermo-sensititve genic male-sterile (TGMS) gene in rice can alter fertility in response to temperature and is useful in the two-line system of hybrid rice production. However, little is known about the TGMS gene at the molecular level. The objective of this study was to identify molecular markers tightly linked with the TGMS gene and to map the gene onto a specific rice chromosome. Bulked segregant analysis of an F_2 population from 5460s (a TGMS mutant line) × 'Hong Wan 52' was used to identify RAPD markers linked to the rice TGMS gene. Four hundred RAPD primers were screened for polymorphisms between the parents and between two bulks representing fertile and sterile plants; of these, 4 primers produced polymorphic products. Most of the polymorphic fragments contained repetitive sequences. Only one single-copy sequence fragment was found, a 1.2-kb fragment amplified by primer OPB-19 and subsequently named TGMS1.2. TGMS1.2 was mapped on chromosome 8 with a RIL population and confirmed by remapping with a DHL population. Segregation analysis using TGMS1.2 as a probe indicated that TGMS1.2 both consegregated and was lined with the TGMS gene in this population. It is located about 6.7 cM from the TGMS gene. As TGMS1.2 is linked to the TGMS gene, the TGMS gene must be located on chromosome 8.

Key words Rice TGMS gene · RAPD · RFLP · Molecular markers · Gene tagging

This research was supported by the Rockefeller Foundation and China National High-Tech Research and Development Program. The first author is a Rockefeller Career Fellow at Texas Tech University

Communicated by F. Salamini

B. Wang (✉) · J. Z. Wang · W. Wu · H. G. Zheng · Z. Y. Yang[1]
Institute of Genetics, Chinese Academy of Sciences, Beijing 100101, China

W. W. Xu · J. D. Ray · H. T. Nguyen (✉)
Plant Molecular Genetics Laboratory, Mail Stop 2122, Department of Plant and Soil Sciences and the Institute for Biotechnology, Texas Tech University, Lubbock, TX 79409, USA

Permanent address:
[1] Crop Breeding Institute, Fujian Agricultural University, Fuzhou 350002, China

Introduction

Rice is a staple food for about half the world's population. The rapidly increasing demand for rice and the continuous decrease in rice-growing areas have resulted in searches to improve rice production. The use of hybrid rice has proved to be an effective and economical way to increase rice production output. It is easy to obtain about a 10% higher yield just by growing hybrid rice instead of the common rice varieties. As the widely used three-line system for producing hybrid rice is extremely time-consuming and costly, a two-line system using either photoperiod-sensitive genic male-sterile (PGMS) or thermo-sensitive genic male-sterile (TGMS) rices has been developed. This system has become a simple and efficient breeding method for producing hybrid rice (Yuan 1993). However, little is known about these two genes at the molecular level, which subsequently limits their widely application.

The pollen fertility of TGMS rice is regulated by temperature. It is completely sterile at a high temperature, but fertile at lower temperatures (Sun et al. 1989). Therefore, TGMS plants can be used to produce hybrid seeds by interplanting them with normal fertile lines under high temperatures and allowing them to reproduce by selfing under low temperatures. Thus, it can be used not only as a male-sterile line, but also as a maintainer line, thereby providing the opportunity to produce hybrid rice by the two-line system. There are many advantages to using the two-line system developed with TGMS rice rather than the commonly used three-line system. First, because TGMS rice can be used as both a sterile line and a maintainer line, the expressly developed maintainer line is no longer needed.

Second, TGMS rice has a broad spectrum of fertility restoration; almost all of the *Indica* rice and most of the *Japonica* rice can be used as its pollen-donor to produce fertile F_1 hybrids. A restorer line can easily be obtained among the wide range of germ plasms available, therefore, the deliberative efforts of developing the restorer lines in the traditional three-lines method are also eliminated. Third, the performance of TGMS hybrid rice does not suffer from the adverse effects of male-sterile cytoplasm such as that commonly happens in the three-line hybrids. These advantages make the two-line system developed from TGMS rice simpler and more economical than the widely used three-line system. Its application in hybrid rice production will greatly reduce the cost in labour, time and resources. As a result, great economic benefits will be achieved.

Rice has a relatively small genome and well-developed linkage maps (Tanksley et al. 1992; Kurata et al. 1994). In addition, the rice transformation system is well-developed and successful. Previous research has indicated that the fertility of TGMS rice is controlled by one Mendelian locus (Yang et al. 1992).Therefore, it should not be very difficult to develop new TGMS lines by transferring the TGMS gene to selected desirable cultivars.

If the rice TGMS gene is cloned and transferred into other crops, it will greatly advance the theoretical research and practical application of hybrid rice production for even the entire field of crop breeding. But we have to map the TGMS gene onto a specific chromosome before it can be cloned.

The objective of the research presented here was to tag the rice TGMS gene with random amplified polymorphic DNA (RAPD) and restriction fragment length polymorphism (RFLP) markers using bulked segregant analysis and to map the TGMS gene onto a specific rice chromosome.

Materials and methods

Plant materials

In order to identify RAPD markers linked to the TGMS gene, an F_2 population was developed from a single cross between 5460s (TGMS mutant line) and 'Hong Wan 52' (normal fertile line). The original F_2 population consisted of 200 individuals. Fertility determination results indicated that the fertility segregation in this F_2 population was controlled by one major locus, with fertility segregation fitting the Mendelian 3:1 ratio as a recessive gene.

Bulk generation

Bulk generation was based on the method described by Michelmore et al. (1991). Rice genomic DNA was isolated from fresh leaves frozen in liquid nitrogen according to McCouch et al. (1988). Two DNA bulks, each containing equal amounts of genomic DNA from each of the 15 individuals, were used for RAPD analysis to identify polymorphisms. These two DNA bulks were constructed on the basis of their fertility. The character differentiating the two bulks was that the individuals in one bulk were all extremely fertile, while those in the other were all extremely sterile. In the fertile bulk, each individuals had less than 40% abortive pollen and above 50% seed set. In the sterile bulk, the corresponding percentages were above 95% and below 4%.

RAPD analysis

Genomic DNAs were used as templates for polymerase chain reaction (PCR) amplification as described by Williams et al. (1991) and modified specially for rice in our laboratory (Li et al. 1993). A single 10-mer oligonucleotide primer (Operon Technologies, Almeda, Calif.) was used in each PCR amplification. The PCR amplification was performed in 25-µl volumes containing 25 ng of template DNA, 10 m*M* Tris HCl, pH 8.3, 50 m*M* KCl, 2.0 m*M* $MgCl_2$, 0.01% gelatin, 100 µ*M* of each dNTP, 15 ng of primer and 1 unit of Taq DNA polymerase (Perkin Elmer Cetus). The reaction mixture was overlaid with 25 µl of mineral oil. Amplification was carried out in a Perkin Elmer Cetus DNA thermal cycler for 40 cycles after predenaturing for 3 min at 94 °C. Each cycle consisted of 1 min at 94 °C, 1 min at 36 °C and 2 min at 72 °C. The 40 cycles were followed by a 10 min final extension at 72 °C. Amplification products were resolved by electrophoresis in 1.4% agarose gel and visualized by ethidium bromide staining.

Cloning of polymorphic fragments

Polymorphic DNA bands of interest resolved by agarose gel electrophoresis were purified with a GeneClean™ kit (Bio-101, La Jolla, Calif.) as recommended by the manufacturer. The purified products were then cloned with a Sureclone™ ligation kit from Pharmacia (Piscataway, N.J.) according to the provided instructions.

Linkage analysis

The RAPD marker TGMS1.2 was mapped in the F_7 RIL population from the cross of 'CO39' × 'Moroberekan' using the programme MAPMAKER (Lander et al. 1987). Previously mapped loci (Wang et al. 1994) were used as anchor loci to determine chromosomal location. The mapping result was reconfirmed with a doubled haploid line (DHL) mapping population from the cross 'Azucena' × 'IR64'.

Results and discussion

Screening RAPD markers linked to the TGMS gene

Bulked segregant analysis was employed to identify RAPD markers linked to the rice TGMS gene. A total of 400 primers were screened for their polymorphism between the two bulked and two parental DNA samples ('Hang Wan 52' and 5460s). Of these, 4 primers amplified polymorphic products that were present in one bulk but not in the other. Figure 1a shows the amplification results with primer OPB-19. Results of other primers were not shown because their amplification products proved to be repetitive sequences. The polymorphic product amplified by OPB-19 was present in the sterile bulk and sterile parent 5460s, but not in the fertile bulk and fertile parent 'Hong Wan 52'. The polymorphic product amplified by OPB-19 was named as OPB-19_{2200}.

The polymorphism identified with OPB-19 by bulked segregant analysis was further confirmed by amplification on the F_2 individuals template DNAs. OPB-19_{2200} was present in 13 and absent in 2 of the 15 sterile F_2 plants. In some sterile F_2 plants it was very weak. It was absent in all of the fertile F_2 plants (Fig. 1b).

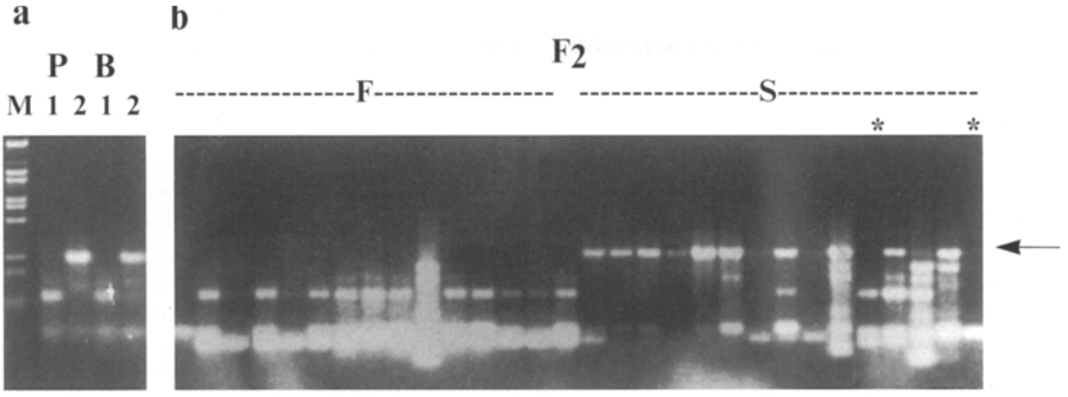

Fig. 1a, b Polymorphisms detected with OPB-19 between the two DNA bulks (**a**) and in the F_2 population (**b**) *M* Molecular weight marker (*Bst*EII digested Lambda DNA), *P1* normal fertile parent 'Hong Wan 52', *P2* male-sterile parent 5460s, *B1* bulked DNAs from fertile (*F*) F_2 inidividuals, *B2* bulked DNAs from sterile (*S*) F_2 individuals. Putative recombinant invididuals are indicated by an *asterisk*. *Arrow* indicates the amplified polymorphic product

Copy-number determination and cloning of the polymorphic amplification product

In order to find single-copy sequence fragments, OPB-19_{2200} was radiolabeled and hybridized to filters containing the two parental DNAs digested with different restriction enzymes. Hybridization results indicated that OPB-19_{2200} was a low-copy sequence fragment; all of the other polymorphic amplification products contained repetitive sequences. OPB-19_{2200} was cloned and subcloned into pUC18. The subcloned 1.2-kb insert DNA proved to be a single-copy sequence fragment and was named TGMS1.2. Further, it was used as probe in TGMS gene mapping.

Cosegregation between the TGMS1.2 marker and the TGMS gene

In order to confirm the putative linkage between the TGMS gene and TGMS1.2, and to convert it to RFLP marker, we further analysed its parents by Southern blotting. More than 30 restriction enzymes were tested; only 1 RFLP was found when the parental DNAs were digested with *Sac*I. Then, cosegregation analysis was performed. TGMS1.2 was found to be cosegregated with and linked to the TGMS gene. Cosegregation data for TGMS1.2 is shown in Fig. 2. Two recombinants were found. The genetic distance between the TGMS1.2 and TGMS genes was estimated to be 6.7 cM based on the recombination data observed from this F_2 population. This result was in agreement with that obtained from RAPD analysis in the F_2 population. The two putative recombinant individuals detected in RAPD analysis and in cosegregation analysis were the same ones. The F_2 population used in this research was developed from populations showing extreme characters, which is a more effective and efficient method for finding linked molecular markers than the use of ordinary F_2 individuals. However, it may not ideal for calculating the recombination ratios between the TGMS gene and the linked molecular markers. Therefore, a larger F_2 population developed from ordinary F_2 individuals is still necessary for a further accurate calculation of the recombination distance. It is still not yet clear on which side of TGMS1.2 the TGMS gene is located. Through F_2 segregation analysis with more flanking markers of TGMS1.2 will give us a satisfactory answer to this question. This is being performed now.

Mapping *TGMS1.2* onto a specific chromosome

To map the rice TGMS gene onto a specific chromosome, our strategy was to use the anchor loci to map the

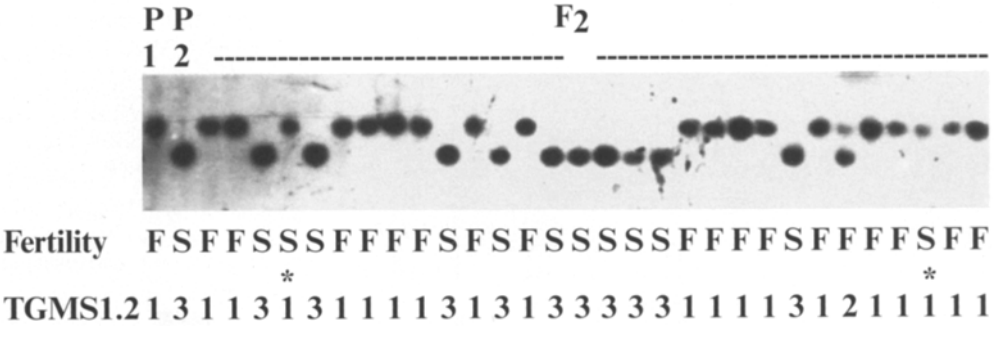

Fig. 2 Hybridization of labeled *TGMS1.2* to a Southern blot containing F_2 individual DNAs digested with *Sac*I. *P1* 'Hong Wan 52', *P2* 5460s, *F* fertile, *S* sterile, *1* 'Hong Wan 52' genotype, *2* heterozygote, *3* 5460s genotype. Putative recombinant individuals are indicated by an *asterisk*

RAPD markers onto a specific rice RFLP linkage group with an F_7-RIL population of 'CO39' × 'Moroberekan'. The previously mapped RFLP loci in the population serve as the anchor loci. The dataset consisted of 147 RFLP loci mapped with 282 RI lines of the cross 'CO39' × 'Moroberekan' developed by Wang et al. (1994). Because this population is skewed toward the female parent 'CO39', we started with a subset of RI lines of this population. A total of 55 RI lines were selected, all of which have roughly an equal percentage of alleles from each parent at most of the 147 loci.

TGMS1.2 was labeled with [^{32}P] by the random primer labeling procedure and hybridized to the *Bam*HI- or *Eco*RV-digested DNAs from the 55 RI lines. Data from TGMS1.2 was added to the dataset of 147 loci from 55 RI lines and analysed with MAPMAKER/EXP (IBM v.3.0) with the linkage criteria of minimum LOD score = 3.0 and maximum recombination fraction = 40%. TGMS1.2 was grouped with RG333, RZ562 and RG978. TGMS1.2 was located between RZ562 and RG978, linked to RZ562 by 12.6 cM and linked to RG978 by 12.7 cM. The hybridization data of TGMS1.2 was further analysed with MAPMAKER (Mac v.2.0) with the linkage criteria of minimum LOD score = 5.0 and max recombination fraction = 40%; TGMS1.2 was also grouped with RG333, RZ562 and RG978. Both analyses showed that the best order was RG333-RZ562-TGMS1.2-RG978. It has been reported that RG333, RZ562 and RG978 are on linkage group 8 (chromosome 8) (Wang et al. 1994), therefore, the TGMS gene must be located on chromosome 8. Later, TGMS1.2 was remapped at the International Rice Research Institute with a DHL mapping population from the cross 'Azucena' × 'IR64'. It was confirmed that TGMS1.2 was located on the same region of chromosome 8 (Fig. 3).

This is the first report on rice TGMS gene mapping. The screened molecular markers will be very useful for monitoring the TGMS gene transfer in rice breeding and in TGMS gene isolation by means of map-based cloning.

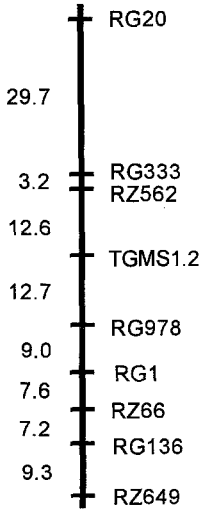

Fig. 3 RFLP map of rice chromosome 8 showing the location of *TGMS1.2* and its linked markers. The genetic distance between the loci are shown in cM

Many studies have indicated that RAPD analysis is a powerful approach by which to find DNA polymorphisms. Our results indicate that RAPD analysis in combination with bulked segregant analysis of F_2 populations provides a highly efficient strategy to tag target genes. RAPD and bulked segregant analysis can be used as the initial method for tagging the interested gene, and then the obtained polymorphic RAPD products will be mapped relative to the molecular markers with known map position. This will permit the rapid mapping of a target gene. Because higher plants contain significant amounts of repeat sequences, the difficulty in this kind of research is to find single-copy sequence markers from the identified polymorphic products.

Acknowledgements The authors thank Dr. N. Huang for helping us to check the result of chromosomal location of the RAPD markers with the DHL mapping population; Dr. S. R. McCouch, Dr. Qifa Zhang and Dr. L. Liu for helpful discussions; Dr. H.E. Shashidhar and Mr. M.S. Pathan for providing rice seeds of the RI lines. We also thank Ms. Erin Eisen for technical assistance.

References

Kurata N, Nagamura Y, Yamamoto K, Harushima Y, Sue N, Wu J, Antonio BA, Shomura A, Shimizu T, Lin SY, Inoue T, Fukuda A, Shimano T, Kuboki Y, Toyama T, Miyamoto Y, Kirihara T, Hayasaka K, Miyao A, Monna L, Zhong HS, Tamura Y, Wang ZX, Momma T, Umehara Y, Yano M, Sasaki T, Minobe Y et al. (1994) A 300-kilobase interval genetic map of rice including 883 expressed sequences. Nat Genet 8:365–372

Lander ES, Green P, Abrahamson J, Barlow A, Daly MJ, Lincoln SE (1987) Mapmaker: an interactive compupter package for constructing primary genetic linkage maps of experimental and natural populations. Genomics 1:174–181

Li ST, Zhang ZT, Wang B, Bao XM, Huang BQ (1993) The identification of two Agropyron intermedium translocation lines in wheat by RAPD. Chin J Genet 20:51–58

McCouch SR, Kochert G, Yu ZH, Wang ZY, Khush GS, Coffman WR, Tanklsley SD (1988) Molecular mapping of rice chromosomes. Theor Appl Genet 76:815–829

Michelmore RW, Paran I, Kesseli V (1991) Identification of markers linked to disease resistance genes by bulked segregant analysis: a rapid method to detect markers in specific genomic region is by using segregating populations. Proc Natl Acad Sci USA 88:9828–9832

Sun ZX, Min SK, Xiong ZM (1989) A temperature-sensitive male-sterile line found in rice. Rice Genet Newsl 6:116–117

Tanksley SD, Fulton TM, McCouch SR (1993) Linkage map of rice (Oryza sativa) (2n = 24). In: O'Brien SJ (ed) Genetic maps, vol. 6. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., pp. 6.61–6.76

Wang GL, Mackill DJ, Bonman JM, McCouch SR, Champoux MC, Nelson RJ (1994) RFLP mapping of genes conferring complete and partial resistance to blast in a durably resistant rice cultivar. Genetics 136:1421–1434

Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res 18:6531–6535

Yang RC, Liang KJ, Wang NY, Chen SH (1992) A recessive gene in Indica rice 5460s for thermosensitive genic male sterility. Rice Genet Newsl 9:56–67

Yuan LP (1993) Progress of two-line system hybrid rice breeding. In: Yuan LP (ed) Current status of two-lines hybrid rice research. Agricultural Publ House, Beijing, China pp 1–12