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MARKER ASSISTED CHARACTERIZATION OF TGMS GENE IN RICE CULTIGEN TS 29 (*ORYZA SATIVA* L.) TO DEVELOP TWO LINE HYBRIDS

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Abstract

The application of the thermosensitive genic male sterility (TGMS) system has great potential for revolutionizing hybrid rice production through simple and effective seed production technology. For the successful utilization of this novel male sterility system, knowledge of the fertility behaviour and genetic control of a TGMS line is essential. In this study, an investigation was carried out to characterize the TGMS gene present in the mapping population of TS 29 x CO(R)49. Through Bulk Segregant Analysis RM 3476 alone produced a polymorphic relationship between the parents and corresponding bulks and was used for surveying the individual plants of F_3/F_4 population. The marker RM 3476 produced contrasting fragments between sterile and fertile plants. The result was associated with the phenotypic observations on pollen fertility under the field conditions, confirming co-segregation of the marker with the gene of interest. Hence, the tight linkage of marker with TGMS gene was identified by RM 3476 which was already identified as a marker located near *tms 6* gene in chromosome 5. From this study four TGMS lines were also identified with desirable floral traits for high out crossing and medium slender grain type.

Introduction

Rice is one of the most ancient grain crops, both agronomically and nutritionally important, that remains the principal staple food of the majority of the population in developing countries. The rapid increase in rice consumption is mainly due to the expansion of the human population in developing countries. Facing the challenge of population growth and cropland reduction, it is obvious that the only way to solve this problem is to improve the yield of cereal crops, namely, rice, wheat, and corn, *etc.,* (Yuan and Peng 2005). Initially, the Green revolution used semi-dwarf genes that led to a huge increase in the yields of wheat and rice. Subsequent increase came through the use of heterosis following the approach of male sterility in rice. It contributes to the increase of the grain yield of 10 to 20 per cent over the improved inbred rice varieties. Hybrid rice breeding includes three- line and two-line systems that are developed via, cytoplasmic male sterility and Environment-Sensitive Genic Male Sterility respectively.

The two-line system of hybrid breeding utilizing environment-sensitive genic mal e sterility (EGMS) is considered as an alternative to overcome the problems associated with three-line breeding and to exceed the yield plateau of three line hybrids. Male sterility expression in a TGMS line is heritable but regulated by temperature. At certain temperatures occurring during panicle development stages, the male sterility is altered into partial to complete fertility. This characteristic of the TGMS system eliminates the need for a maintainer line for its multiplication.

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Also, the system does not require a restorer line. Since the cytoplasm is not involved in sterility expression, plant breeders can develop hybrids with diverse cytoplasmic backgrounds to reduce the risk of their potential genetic vulnerability. Virmani and Ilyas-Ahmed (2001) reported that in the tropics, where the photoperiod differences are minimal but wide temperature differences exist among different altitudes or different seasons, it is more practical to use the TGMS system.

The association of molecular markers with the TGMS gene may help in monitoring the TGMS gene transfer in rice breeding by early screening of the genotypes with relative ease. With the availability of high-density rice molecular maps and molecular markers, molecular marker assisted breeding is considered as a promising approach to identify genes governing various traits. Previous genetic studies and recent molecular investigations have revealed that a recessive nuclear gene regulates the expression TGMS in rice. Microsatellite markers associated with the TGMS genes were used to rapidly screen for their presence in the breeding population, thus facilitating the development of promising TGMS lines. DNA markers, which are absolutely heritable and environmentally insensitive, can be efficiently used to tag the TGMS gene, utilizing the tightly linked markers for indirect selection tools. Development of stable and agronomically superior PGMS and TGMS lines using promising gene sources, identification of an ideal location/season for hybrid seed production, and multiplication of parental TGMS or PGMS lines have facilitated the evolution of two-line hybrids of rice for commercial planting. Hence, this study was undertaken to characterize the stable TGMS line and also develop new TGMS lines using marker assisted selection in the early generation.

Materials and Methods

The most stable TGMS line of rice, TS 29, is a mutant developed through 250 Gy gamma ray irradiation of a local variety identified at the Department of Rice, Tamil Nadu Agricultural University (TNAU), India is used as female parent and crossed with the agronomically improved adapted high-yielding medium slender grain variety CO(R)49 for developing good grain quality TGMS lines.

The genomic DNA of F_2 progenies and corresponding parents was extracted by the CTAB method (Doyle and Doyle, 1987), and the nucleic acids were quantified by the methods of Spectrophotometric determination and agarose gel electrophoresis (0.8 per cent w/v). DNA concentration for PCR amplification was estimated by comparing the band intensity of a sample with the band intensities of known dilutions that gave good amplifications. The dilutions were carried out by dissolving the genomic DNA in an appropriate volume of TE buffer. A total of 50 SSR markers consisting of both random and markers reported to be linked to *tms* genes were used for the study. The reaction mixture (15 µl for PCR amplification was prepared by adding 2.0 µl template DNA (~10 ng /µl), 0.6 µl dNTPs (2.5mM), 1.0 µl forward primer (50 ng), 1.0 µl reverse primer (50 ng), 0.30 µl *Taq* DNA polymerase (Genei 3 IU / µl), 1.5 µl *Taq* buffer, and 8.6 µl Sterile distilled water respectively. The reaction mixture was given a momentary spin for thorough mixing of the cocktail components. Amplification was performed in 0.2 ml (each tube) PCR tubes loaded in Bio-Rad (MyCycler thermal cycler). The thermal cycler was programmed as follows: The samples were initially incubated at 94°C for 5 minutes and then subjected to 35 times of the following cycle: 94 °C for 1 min (Denaturation), 55°C for 1 min (Annealing) and 72°C for 2 mins (Extension). Final extension at 72 °C for 5 min. The amplified PCR products were on hold at 4°C until sample retrieval. PCR amplified products (15.0 µl) were subjected to electrophoresis in a 3.0 per cent agarose gel in 1X TBE buffer at 100 volts for 3.0 hours using a submarine electrophoresis unit. The ethidium bromide stained gels were documented using Alpha Imager TM 1200-

Documentation and Analysis System of the Alpha Infotech Corporation, USA. Sizes of the identified bands were determined relative to the 100 bp ladder (Fermentas, Germany).

The genomic DNA's were extracted from the two parental lines. A set of 50 simple sequence repeat (SSR) markers putatively located in the vicinity of any one of the six *tms* genes reported in rice was initially targeted (Table 1). Parental polymorphism was studied for these selected markers by using different annealing temperatures for each marker. After PCR amplification, the products were loaded in the 3% agarose gel and finally documented using the Gel-documentation systems. Band differences (base pairs) were observed between the parents were scored as polymorphic marker for corresponding parents and those markers were selected for further analysis (Fig. 1).

Table 1. List of Simple Sequence Repeat (SSR) markers utilized.

Genes	Chromosome	Markers surveyed for parental polymorphism
tms1	10 (20.00- 24.00 cM)	RM104, RM239
tms2	7 (37.00- 47.00 cM)	RM11, RM2, RM214
tms5	2 (3.00 - 8.00 cM)	RM5862, RM5897, RM7576, RM492, RM6378, RM257, RM174, RM7575, RM7355
tms6	5 (81.0-91.0 cM)	RM3351, RM440, RM10, RM289, RM 3476, RM6054
Neutral markers (for tgms genes)		RM244, RM7653, RM27, RM29, RM 341, RM411, RM132, RM570, RM401, RM584 RM470, RM70, RM435, RM216, RM147, RM266, RM536, RM144, RM20, RM17, RM260, RM125, RM312, RM523, RM248, RM7012, RM246, RM4674, RM24, RM9 and RM247

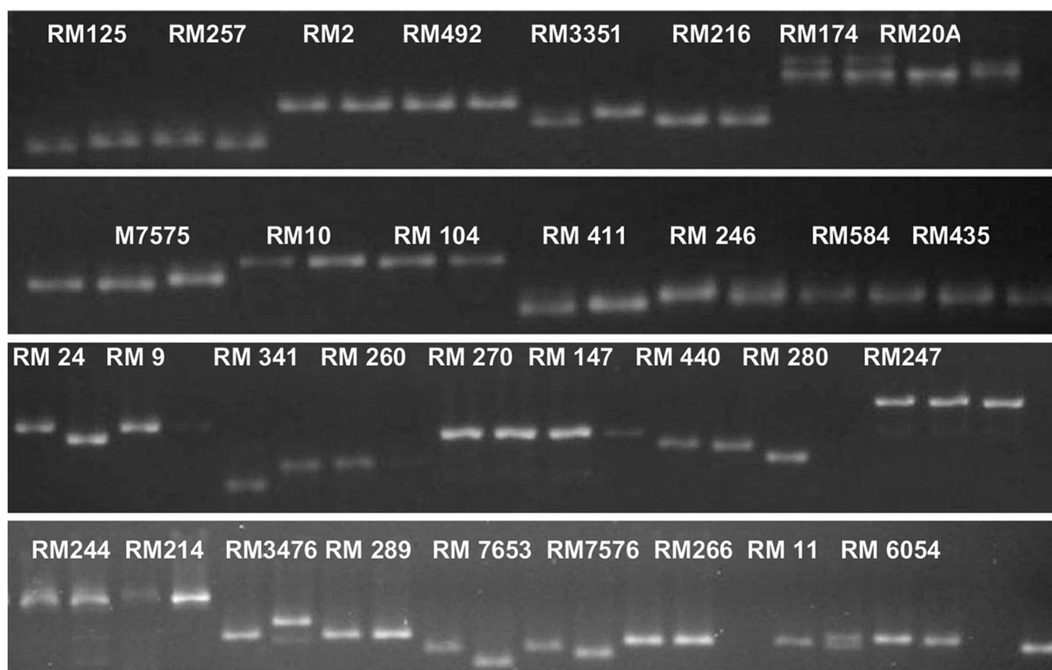


Fig. 1. Parental Polymorphic Survey with SSR markers.

The F_2 population was raised under a sterility-restrictive environment favouring fertility reversion, and around 400 individual plants were randomly selected for characterization of the TGMS gene. DNA was extracted from all the plants and preserved. The F_3 seeds collected from the individual plants were raised under a sterility-favouring environment in the plains (Coimbatore) in ear-to-rows. The plants in individual progeny were assessed for pollen fertility. Based on the F_3 family performance in homogeneity, the DNA bulks of fertile and sterile progenies were made from the corresponding F_2 plants. Bulk segregant analysis of the F_2 population was used to identify the SSR markers linked to the rice *tgms* gene (Fig. 2). Based on F_3 population out of 84 sterile homogeneous progeny rows identified, eight sterile bulks of 10 progenies each was constructed, and 10 homogeneous fertile progenies was pooled to make a fertile bulk from F_2 genomic DNA's. Based on marker polymorphism survey of the parents, already identified 19 markers were screened for polymorphisms among parents and bulks representing extremes of fertile and sterile plants. Desirable bulks were scored based on the bands present in the TGMS line TS 29 and the sterile bulk. These bulks and markers were selected for further analysis.

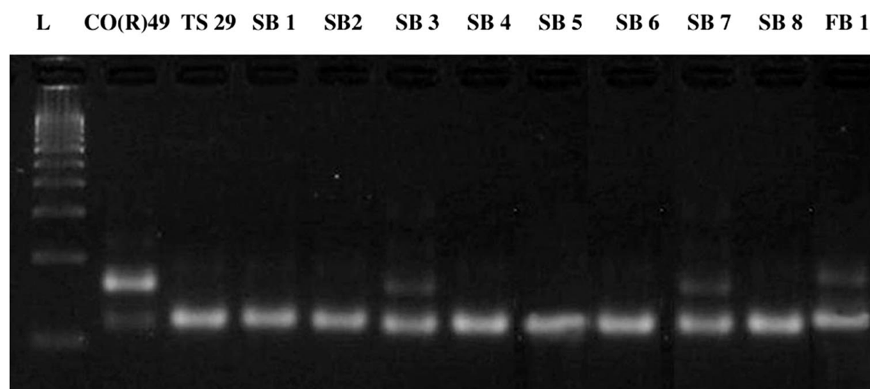


Fig. 2. Bulk segregant analysis using microsatellite marker RM 3476
L - 100-bp DNA ladder, SB_1- SB_8 - sterile bulks, FB - Fertile bulk

The primer pairs exhibiting marker loci putatively linked with the trait in the bulks then tested on all individual DNA samples along with the fertile and two concerned parents. The co-localization of the markers with already positioned rice *tms* genes were verified. The efficiency of the marker in co-segregating with sterility in populations derived from other crosses but involving the same male sterile parent also studied.

Results and Discussion

Molecular markers for trait selection have numerous advantages over morphological markers used in conventional plant breeding. The application of molecular markers in rice improvement has been reported by many scientists (Collard 2005, Jena and Mackill 2008). The identification and use of different TGMS alleles in rice will provide a wider genetic base for hybrid rice development. In order to exploit two line system, it is necessary to evaluate the lines that present such a behaviour, estimate the robustness of the expression of the trait in a range of temperatures, and understand the genetic control of the trait to initiate development in cultivars. Out of 50 primer pairs assayed for evolving polymorphism, 19 primer pairs produced polymorphic alleles between parents. Monomorphic amplification was observed in 20 primer pairs, and the rest of the

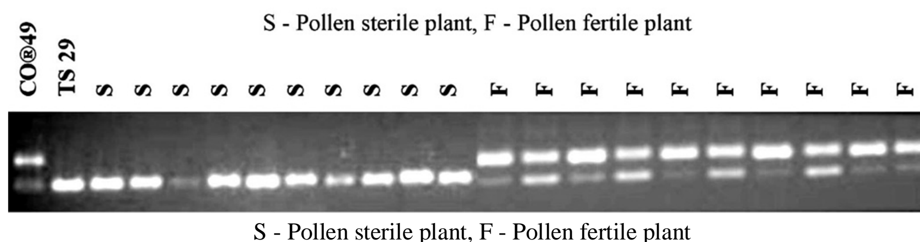
primers did not generate any amplification products (Table 2). The SSR markers revealed 38 percentage of polymorphism between TS 29 and CO(R) 49. The markers, *viz.,* RM 3476, RM 341,

Table 2. Parental polymorphism produced between CO(R)49 and TS 29.

Sl. No.	Marker	Polymorphic	Monomorphic	No Amplification
1	RM 216		✔	
2	RM 2		✔	
3	RM 174		✔	
4	RM 492		✔	
5	RM 5897	✔		
6	RM 24	✔		
7	RM 411	✔		
8	RM 132			✔
9	RM 104		✔	
10	RM 570		✔	
11	RM 10		✔	
12	RM 401			✔
13	RM 584		✔	
14	RM 470			✔
15	RM 270		✔	
16	RM 435		✔	
17	RM 20 A	✔		
18	RM 147			✔
19	RM 266		✔	
20	RM 536			✔
21	RM 144	✔		
22	RM 7575	✔		
23	RM 17			✔
24	RM 260		✔	
25	RM 125	✔		
26	RM 248	✔		
27	RM 6054		✔	
28	RM 246	✔		
29	RM 7355	✔		
30	RM 7012	✔		
31	RM 4674		✔	
32	RM 9		✔	
33	RM 247		✔	
34	RM 214		✔	
35	RM 244		✔	
36	RM 341	✔		
37	RM280		✔	
38	RM 7653		✔	
39	RM 3476	✔		
40	RM 7576	✔		
41	RM 11	✔		
42	RM 289		✔	
43	RM 29	✔		
44	RM 257	✔		
45	RM 27			✔
46	RM 6378	✔		
47	RM 440		✔	
48	RM 239			✔
49	RM 3351	✔		
50	RM 5862			✔

RM 248, RM 7575, RM 246, RM 9 and RM 411, produced good amplification. The low level of polymorphism was expected since both the parents were of *indica* types and of Coimbatore origin. The results also suggested that, with plentiful SSR markers available in the public domain, a workable polymorphism can be achieved within *indica* genotypes.

In hybrid rice breeding, screening for the TGMS trait is an intensive process influenced by the environment. Tagging TGMS genes from new donors with DNA markers would increase the speed and efficiency of developing two line hybrids. Selection for the TGMS trait, which is controlled by a single recessive gene, is difficult under field conditions by conventional breeding methods. The development of PCR based markers is useful in marker-assisted selection for thermosensitive genetic male sterility and to precisely identify genotypes in a segregating population in early generations, especially when selected plants are intended to be used in future crosses. Moreover, selection can be carried out independent of temperature conditions based on markers closely linked to a gene of interest.



S - Pollen sterile plant, F - Pollen fertile plant

Fig. 3. Marker (RM3476) co-segregation analysis in the F_4 generation

The difficulty of genotyping all the plants in a mapping population can be reduced through selective genotyping by Bulk Segregant Analysis (BSA). BSA involves the selection of extremely sterile and fertile lines and pooling their DNA into two bulks, viz., sterile and fertile bulks (Michelmore *et al.*1991 and Govindaraj *et al.* 2005). The efficiency of this strategy relies mainly on allele differences, the larger the difference, the more efficient the pooling strategy. Here, BSA was followed to identify the markers linked with the TGMS gene. This approach provides information simultaneously on the polymorphism of the parents and possible linkage between marker and tagged genes by using sterile and fertile bulks. This has been well proven as an efficient technique to tag the major genes when near isogenic lines are not available. The identified 19 primers were used for bulk segregant analysis. Among polymorphic primers, only one marker alone produced polymorphism between the parents and corresponding bulks. For studying the polymorphic survey, the already identified tagged markers for the TGMS trait, and some of the random primers were used. Bulk segregant analysis was done using already reported markers, *viz*., RM 239 for tms1 (Wang *et al.* 1995) in chromosome 8, RM 11 for *tms2* (Maruyama *et al*. 1990) in chromosome 7, RM 257 for *tms4* (Dong *et al.*2000) in chromosome 2, RM 3351, RM 3476, and RM 440 for *tms6* (Lee *et al.*2005) in chromosomes 5 and 3, respectively. The BSA revealed that the putative linkage of the TGMS trait with a marker, RM 3476, is closely associated with *tms6*.

The identified marker RM 3476 was used for surveying 10 individual F_4 of both fertile and sterile bulks. The progeny survey with RM 3476 produced the same size of amplification fragments in sterile plants, and the female parent and the fertile individuals produced homozygous and heterozygous fragments in all but one (fig.3). The homozygosity of the sterile individuals confirmed the recessive nature of TGMS trait. Same was confirmed with the phenotypic observations recorded in the field. Which have confirmed the co-segregation of RM 3476 with the

gene of interest. Some of the fertile plants also produced heterozygous bands. This may be due to the heterozygous nature of a trait. The pollen sterility (TGMS) trait co-segregated with the marker in all but one case. Fertile plants exhibited both heterozygous and homozygous dominant (CO(R) 49) alleles.

The result proved that SSR analysis in combination with bulked segregant analysis of F_3 population provides a highly efficient strategy to tag the gene of interest. SSR and bulked segregant analysis can be used as initial methods for tagging the TGMS trait, and the obtained polymorphic markers can be mapped to known map positions. This will permit rapid mapping of the target gene, as reported by many researchers (Michelmore *et al.*1991 and Govindaraj *et al.*2005) . The tagged marker RM3476 was already mapped in chromosome 5 of the rice genome, consisting of a gene *tms6* for inducing male sterility, as reported by Lee *et al.* (2005). Hence, the same marker was identified here also. Therefore, *tms6* might be the gene responsible for inducing sterility in the TGMS progenies derived from TS 29. However, a mismatch of one in 10 plants suggests recombination between the trait and marker, which can be overcome by additional screening for more markers in this region. The association of molecular markers with the TGMS gene may help in monitoring TGMS gene transfer in rice breeding through early screening of the genotypes with relative ease.

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