Plant Breeding **123**, 235—240 (2004)

ISSN 0179-9541

Genetics, fertility behaviour and molecular marker analysis of a new TGMS line, TS6, in rice

R. Latha[1], K. Thiyagarajan[2] and S. Senthilvel[3]

[1] Department of Plant Breeding and Genetics, Pandit Jawaharlal Nehru College of Agriculture and Research Institute (PAJANCOA), Karaikal 609 607, Pondicherry, E-mail: latharamaiah@yahoo.co.in; [2] Centre for Plant Breeding and Genetics, Tamil Nadu Agricultural University, Coimbatore 641 003; [3] International Crops Research Institute for the Semi-Arid Tropics, Patancheru, India

With 3 figures and 4 tables

Received January 7, 2003/Accepted November 7, 2003
Communicated by Q. Zhang

Abstract

The thermosensitive genic male sterility (TGMS) system has great potential for revolutionizing hybrid rice production through simple, less expensive and more efficient seed production technology. For the successful utilization of this novel male sterility system, knowledge of the breeding and fertility behaviour of a TGMS line is essential. In this study, the fertility transformation behaviour, the critical fertility and sterility temperatures and the mode of inheritance of male sterility were studied for a new TGMS line, TS6, identified at Tamil Nadu Agricultural University, Coimbatore, India. The pollen and spikelet fertilities recorded on plants raised at fortnightly intervals revealed that this line was completely sterile for 78 consecutive days (35/22 to 32/23°C, maximum/minimum temperatures) and reverted to fertile when the temperature was 30/18°C. It remained fertile continuously for 69 days and the maximum pollen and spikelet fertilities recorded were 75 and 70%, respectively. The fertility was highly influenced by daily maximum temperature followed by average and minimum temperatures. It was not influenced by relative humidity, sunshine hours or photoperiod. The critical temperature inducing sterility and fertility was 26.7 and 25.5°C, respectively. The male sterility in TS6 was inherited as a monogenic recessive in the F_2 and BC_1 populations of TS6 × MRST9 as well as TS6 × IR68281B. Using bulked segregant analysis on an F_2 population of TS6 × MRST9, an RAPD marker, $OPC05_{2962}$, was identified to be associated with TGMS in TS6.

Key words: *Oryza sativa* — fertility behaviour — RAPD markers — TGMS

Today, rice is synonymous with food security in most parts of Asia, which produces more than 91% of the global harvest. Recent progress in plant breeding research indicated that a significant shift in the yield frontiers could be possible through hybrid rice. Many years of practice and experience has proved that three-line breeding utilizing a cytoplasmic genic male sterility system (CMS) is an effective way to develop rice hybrids and will continue to play an important role in heterosis breeding. But this system has some constraints such as a yield plateau in rice hybrids, dependency on a single CMS source (WA), restriction on choice of male parents due to problems associated with fertility restoration, complex seed production procedures and high seed cost (Yuan, 1997). The two-line system of hybrid breeding utilizing environment-sensitive genic male sterility (EGMS) is considered as an alternative to overcome the problems associated with three-line breeding and to exceed the yield plateau. For breeding two-line hybrids under tropical conditions, where day length differences are marginal, a temperature-sensitive genic male sterile (TGMS) system is considered more useful than the photoperiod-sensitive genic male sterile (PGMS) system (Virmani, 1996). After the identification of the TGMS mutant Annong S-1 (Tan *et al*., 1990), several TGMS lines have been developed in China, IRRI and elsewhere. For the successful exploitation of this novel male sterility system in heterosis breeding, more TGMS lines need to be developed and characterized for their sterile and fertile phases, critical stages and temperatures for fertility alteration. Studying the inheritance of TGMS would help in breeding new TGMS lines with diverse genetic backgrounds. In this context, the present study was undertaken to characterize the new TGMS line TS6 for its fertility behaviour and to study the inheritance pattern of TGMS. In addition, an attempt was made to identify RAPD markers associated with TGMS in TS6, which may facilitate and simplify the breeding process.

Materials and Methods

Plant materials: The new TGMS line of rice, *Oryza sativa* L., TS6, is a spontaneous mutant of RP 2161-106-1-1 identified at Paddy Breeding Station (PBS), Tamil Nadu Agricultural University (TNAU), India. Two non-TGMS lines, viz. MRST9 and IR68281B, were used to generate F_2 and back cross-populations to study the inheritance of TGMS.

Fertility behaviour: TS6 was sown at fortnightly intervals from July 1999 to June 2000 in the experimental field of PBS, TNAU, Coimbatore (36/22°C, maximum/minimum temperatures), India, and all the necessary management practices were carried out. The pollen and spikelet fertilities were recorded on 10 random plants. The pollen grains were stained with 1% IKI stain. The panicles that emerged from the primary tiller were bagged before anthesis and the number of well-filled grains and chaffs in the panicle were counted at the time of maturity. The ratio of well-filled grains to the total number of spikelets was expressed as spikelet fertility percentage.

From the 24 sets of data on pollen fertility, the fertile and sterile phases, the duration of each phase and the fertility transition phase were identified. The periods of flowering during which the plants recorded 100% pollen sterility and more than 50% pollen fertility were considered as sterile and fertile phases, respectively. The period of partial sterility was considered as the phase of fertility transition from male sterility to fertility and vice versa.

The association of weather factors, viz. maximum temperature, minimum temperature, mean temperature, relative humidity, sunshine

Table 1: Panicle development stages and their durations

S. no.	Stages	Duration (days)	Days before heading
1	Differentiation of first bract primordium (S1)	2	25–26
2	Differentiation of primary branch primordium (S2)	3	22–24
3	Differentiation of secondary branch primordium (S3)	3	19–21
4	Differentiation of stamen and pistil primordium (S4)	4	15–18
5	Pollen mother cell formation (S5)	3	12–14
6	Meiotic division of pollen mother cell (S6)	3	9–11
7	Pollen filling stage (S7)	6	3–8
8	Pollen ripening (S8)	2	1–2

Source: Rangaswamy et al. (1993).

hours and photoperiod of each day from day 26 to 1 day before heading, average of each factor at different stages of panicle development and the overall mean of each factor throughout the panicle development stages with pollen sterility was assessed by simple correlation analysis. The correlation coefficients between sterility and the mean of daily mean temperatures at differentiation of the first bract primordium (S1) to pollen ripening (S8) stages (Table 1) were calculated to determine the thermosensitive stage of fertility alteration. The panicle development stages exhibiting significant correlation between temperature and pollen sterility were considered as the critical stages for fertility alteration.

The critical sterility temperature (CST) (the temperature at which the line becomes sterile from the fertile condition) and the critical fertility temperature (CFT) (the temperature at which the line becomes fertile from the sterile condition) were worked out from the dissemination figure charted between pollen sterility and mean temperature. The lowest mean temperature among the temperatures inducing sterility was the CST and the highest mean temperature inducing fertility was the CFT.

Inheritance of TGMS: The inheritance pattern of sterility in TS6 was studied by crossing it with two non-TGMS lines, viz. IR68281B and MRST 9, during the high-temperature season. The F_1s were raised along with their parents and the pollen and spikelet fertilities were recorded on 10 random plants. The F_1s were selfed to obtain an F_2 population. Backcross populations were also generated by crossing the F_1s with TS6. The F_2 and backcross populations were evaluated for their pollen and spikelet fertilities under high-temperature conditions. The individuals were grouped into sterile and fertile and the goodness of fit to a Mendelian segregation of fertile and sterile in the segregating population was tested by Chi-square.

Tagging the TGMS gene: The F_2 individuals of the cross TS6 × MRST9 were used for tagging the TGMS gene. A subset of 50 homozygous sterile and fertile individuals was selected from the original population of 400 individuals based on the fertility behaviour and F_3 segregation pattern. DNA from 50 selected F_2 individuals and the parents was extracted following the protocol described by McCouch et al. (1988). To identify putative markers linked to TGMS genes, DNA bulks were made from individuals of two phenotypic extremes as suggested by Michelmore et al. (1991). DNA from 15 sterile and 15 fertile individuals was aliquoted to constitute fertile and sterile bulks, respectively.

A set of 160 arbitrary decamer primers (A, B, C, E, F, G, S and T series of OPERON Technologies, Inc., California, USA) was used for bulked segregant analysis. Amplification reactions were in volumes of 20 µl containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, dATP, dCTP, dGTP and dTTPs each at 0.1 mM, 0.2 mM primer, 25–30 ng of genomic DNA and 0.5 unit of Taq DNA polymerase (Bangalore Genei Pvt. Ltd., Bangalore, India). Amplification was performed in 0.2-ml thin-walled PCR tubes in a thermocycler (Perkin Elmer Gene Amp PCR System 2400, Foster City, USA) programmed for 35 cycles. After the initial denaturation for 2 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 35 cycles were followed by a 10-min final extension at 72°C. PCR amplified products were subjected to electrophoresis on 1.5% agarose gel in 1X TBE buffer at 120 V for 3 h 30 min. The electronic images of ethidium bromide stained gels were captured using a digital camera.

Results

Fertility behaviour

The sterile and fertile phases, critical stages of fertility alteration and critical sterility and fertility temperature of TS6 were determined based on the pollen fertility at various dates of heading (from September 1999 to September 2000) at Coimbatore.

In this study, TS6 was completely sterile with 100% pollen sterility when headed during the first week of April to the last week of June (32/23°C) for a period of 78 days and converted into fertile during the third week of July (31/23°C) with 70% pollen fertility. It remained fertile for a period of 14 days only and was in the transformation stage with partial fertility till the third week of November. The second fertile phase of TS6 started during the last week of November (29/20°C) and continued till the first week of February (30/18°C). The maximum pollen fertility (75%) was recorded when the plants headed during the last week of November (Fig. 1).

The relative influence of environmental factors, viz. maximum temperature, minimum temperature, mean temperature, sun shine hours, relative humidity and photoperiod on the fertility of TS6, was studied by simple correlation analysis between these factors and pollen sterility percentage. The results of correlation analysis revealed that the fertility of TS6 was influenced only by temperature factors, viz. maximum, minimum and mean temperatures, and no influence of sunshine hours, relative humidity and photoperiod was observed. The effect of maximum temperature was the highest followed by mean and minimum temperatures.

The stages of panicle development, which were sensitive to temperature, were determined from simple correlation analysis between pollen sterility and the mean temperature at each stage of panicle development (S1–S8). In TS6 all panicle developmental stages from differentiation of the primary bract primordium (S1) to pollen ripening (S8) were sensitive to temperature (Table 2).

The mean temperature from 25.9 to 30.7°C during the sensitive stages of panicle development induced complete sterility in TS6. It was fertile with maximum fertility at 21.8–26.4°C. From the dissemination figure (Fig. 2), it is noted that the temperatures 26.3 and 26.4°C occurred only twice during fertile conditions and the temperature 25.9°C was observed once in fertile as well as in sterile conditions (Table 3). By taking this into consideration, approximately 25.5°C was recorded as the CFT and 26.7°C was considered as the CST.

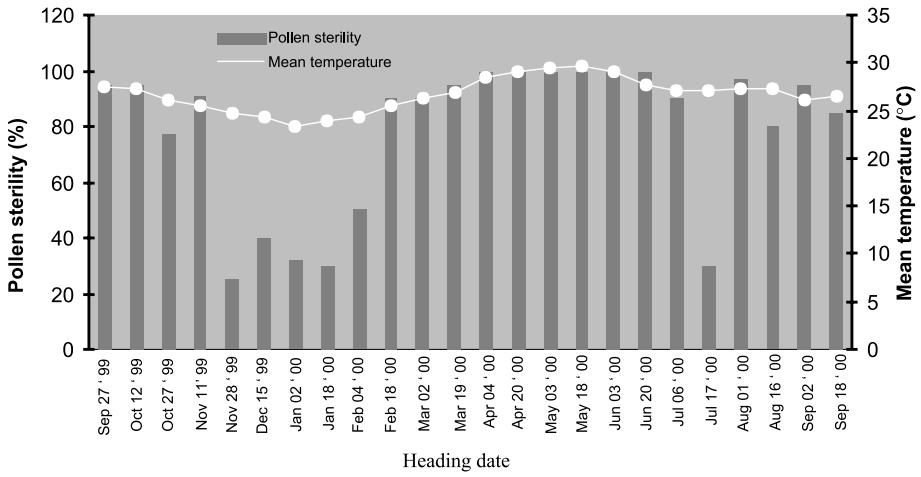

Fig. 1: Fertility behaviour of TS6 in field conditions

Table 2: Correlation coefficient of pollen sterility with weather parameters at different stages of panicle development

Panicle developmental stages	Maximum temperature	Minimum temperature	Mean temperature	Relative humidity	Sun shine hours
S_1	0.649**	0.278	0.624**	−0.600**	0.386
S_2	0.584**	0.570**	0.655**	−0.535**	0.389
S_3	0.593**	0.675**	0.730**	−0.292	−0.037
S_4	0.766**	0.424*	0.691**	−0.403	0.333
S_5	0.654**	0.487*	0.770**	−0.236	0.091
S_6	0.706**	0.597**	0.742**	−0.382	0.233
S_7	0.671**	0.546**	0.727**	−0.232	0.322
S_8	0.639**	0.403	0.514*	−0.251	0.095
Over all mean	0.597**	0.456*	0.568**	−0.375	0.378

*, ** Significant at P = 0.05 and P = 0.01, respectively.

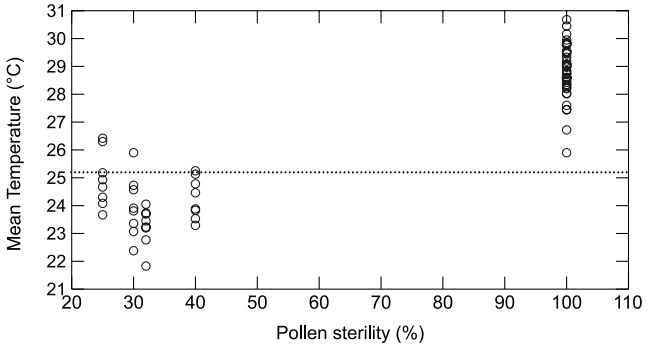

Fig. 2: Dissemination figure showing the probable critical sterility and fertility temperatures for TS6

Inheritance of TGMS

The inheritance pattern of TGMS in TS6 was studied by crossing it with two male parents, viz. IR68281B and MRST9. The F_1 was fertile and the F_2 and backcross populations of the crosses involving TS6 showed a segregation of 3 fertile : 1 sterile and 1 fertile : 1 sterile, respectively, under high-temperature condition (Table 4). The F_3 progeny from fertile F_2 plants of TS6 × MRST9 segregated in the ratio of 1 true breeding fertile : 2 segregating. These results clearly indicated that the TGMS trait in TS6 was controlled by single recessive gene.

RAPD marker associated with TGMS

In the present study, 160 random oligo-nucleotide primers were surveyed for potential polymorphism between the DNA bulks of sterile and fertile lines and the parents. Approximately 85% of these produced DNA amplification in both parents and bulks. In total, 119 primers produced polymorphic DNA fragments between the parents. Out of the 119 polymorphic primers, three, viz. OPC 05, OPE 15 and OPE 17, yielded specific fragments, viz. OPC 05_{2962}, OPE 15_{2776} and OPE 17_{2869}, respectively, in the sterile parent and the sterile bulk, which were absent in the fertile parent as well as the fertile bulk. Absence of two fragments, viz. OPC 14_{1636} and OPG 08_{3309}, generated by the primers OPC 14 and OPG 08 was observed in the sterile parent and the sterile bulk and these two fragments were present in the fertile parent as well as the fertile bulk. The five putative markers were screened in 50 F_2 individuals (homozygous sterile and fertile in equal numbers) of the cross TS6 × MRST9, with selection based on the F_3 segregation pattern. Out of the five primers, only one, OPC 05, produced a fragment of 2962 bp in size, which cosegregated with the phenotype (Fig. 3) in the F_2 individuals tested. This confirmed the association of OPC 05_{2962} with the TGMS gene in TS6.

Discussion

The multiplication of TGMS lines and the seed production of two-line hybrids are not much more difficult than with CMS lines and three-line hybrids. The key point is to determine the stable sterile and fertile phases of TGMS lines at different locations through sequential sowing so that the proper seasons and locations for the propagation of sterile lines and hybrid seed production can be recommended (Liu et al., 1997a). In this study, TS6 was completely sterile for more than 75 consecutive days during the sterile phase (from the first week

Table 3: Pollen sterility and mean temperatures at different stages of panicle development in TS 6

Heading date	Sep 27	Oct 12	Oct 27	Nov 11	Nov 28	Dec 15	Jan 02	Jan 18	Feb 04	Feb 18	Mar 02	Mar 19
Pollen sterility (%)	95.00	95.00	77.22	90.62	**25.00**	**40.00**	**32.00**	**30.00**	**50.00**	90.00	90.00	95.00
S_8	28.75	26.60	26.13	23.68	26.30	23.88	23.73	**25.90**	23.88	25.00	24.40	28.73
S_7	28.13	25.83	25.62	25.78	24.08	23.29	23.23	23.91	24.05	26.83	25.73	28.25
S_6	27.60	27.03	25.35	24.55	24.67	23.53	22.77	24.73	**25.50**	26.75	26.80	27.77
S_5	26.82	27.42	26.97	25.97	23.67	25.13	21.83	24.57	23.42	26.90	26.50	25.85
S_4	26.81	28.83	26.36	25.81	24.30	24.78	24.05	23.36	24.63	23.80	25.39	24.98
S_3	27.59	27.28	25.92	25.15	24.93	24.46	23.45	23.81	24.38	25.91	26.58	27.18
S_2	28.18	27.38	25.95	25.42	26.42	23.83	23.20	23.07	23.75	25.08	26.77	25.60
S_1	26.75	27.70	27.15	25.53	25.18	25.25	23.70	22.38	25.13	23.75	27.60	27.33
Heading date	Apr 04	Apr 20	May 03	May 18	Jun 03	Jun 20	Jul 06	Jul 17	Aug 01	Aug 16	Sep 02	Sep 18
Pollen sterility (%)	**100.00**	**100.00**	**100.00**	**100.00**	**100.00**	**100.00**	90.00	30.00	97.00	80.00	95.00	85.00
S_8	28.23	28.60	30.45	30.68	27.45	28.20	26.33	27.38	27.73	27.50	26.38	27.98
S_7	28.85	29.45	29.82	29.80	28.60	27.44	26.40	27.44	27.29	26.26	25.33	27.11
S_6	28.03	29.53	29.55	28.82	29.48	**26.72**	27.67	26.33	27.68	27.27	25.57	27.40
S_5	28.50	29.07	29.08	29.08	29.07	**25.90**	27.07	26.38	27.55	27.62	26.40	26.97
S_4	28.59	28.75	28.99	30.16	29.86	27.60	28.10	26.06	27.34	27.78	26.59	26.65
S_3	28.72	29.33	29.77	29.80	29.19	28.29	27.84	27.21	26.79	26.88	26.30	25.73
S_2	28.02	28.60	29.53	29.95	29.83	28.55	27.20	27.12	27.52	28.05	26.28	24.97
S_1	28.35	28.43	28.98	29.03	28.60	28.35	26.43	27.88	26.63	27.30	26.80	24.93

Values in bold in the pollen sterility row show the sterile (100%) and fertile (25–50%) phases of TS6. The temperature 26.72°C is considered as the CST and 25.50°C is considered as the CFT (in bold).

Table 4: Pollen and spikelet fertility of F_2 and backcross (BC) generations of TS6 × MRST9 and TS6 × IR68281B

	Frequency of plants with pollen and spikelet fertility												
Generation	0–10	11–20	21–30	31–40	41–50	51–60	61–70	71–80	81–90	91–100	Total	Fertile : sterile	λ^2
Pollen fertility													
TS6 × MRST9													
F_2	89	5	–	–	2	11	48	119	80	46	400	306 : 94	0.48 (3 : 1)
BC	81	4	–	–	–	6	19	22	22	12	166	81 : 85	0.10 (1 : 1)
TS6 × IR68281B													
F_2	85	–	–	–	–	6	43	100	112	37	383	298 : 85	1.61 (3 : 1)
BC	48	4	–	–	–	4	8	17	19	7	107	55 : 52	0.08 (1 : 1)
Spikelet fertility													
TS6 × MRST9													
F_2	94	–	–	–	8	82	125	58	18	15	400	306 : 94	0.48 (3 : 1)
BC	84	1	–	–	8	17	35	12	6	3	166	81 : 85	0.10 (1 : 1)
TS6 × IR68281B													
F_2	85	–	–	2	–	86	115	63	14	18	383	298 : 85	1.61 (3 : 1)
BC	52	–	–	–	4	6	14	16	8	7	107	55 : 52	0.08 (1 : 1)

of April to the last week of June). Hybrid seed production involving TS6 can be undertaken in areas with a mean temperature of more than 26°C by raising the line in such a way that flowering coincides with the sterile phase. Similarly, seed production of TS6 could be carried out by raising the line during the fertile phase. For successful utilization of any TGMS line, the sterile and fertile phases should last for at least 30 consecutive days (Lu et al., 1998). Since TS6 had stable and long sterile and fertile phases, it could be effectively exploited in developing two-line rice hybrids.

In the present investigation, TS6 was completely sterile at temperatures exceeding 30/20°C and fertile when the temperature was less than 30/20°C. But, occasionally, sterility and fertility were observed at temperatures below and above this limit, which required the influence of other weather factors on fertility alteration of TS6 to be investigated. The results of correlation analysis revealed that the fertility of TS6 was influenced only by temperature factors, viz. maximum, minimum and mean temperatures, and no influence of sunshine hours, relative humidity and photoperiod was observed. But, sunshine hours and relative humidity influenced the fertility transformation in 5460S (Liu et al., 1997b). Hence, it is understood that various primary and secondary weather factors can influence the fertility of TGMS and this influence will vary among different lines due to different sources of male sterile genes and genetic backgrounds (Wu et al., 1991; Zhang et al., 1991).

Knowledge about the critical thermosensitive stage of fertility alteration of TGMS lines is useful to determine the most suitable time of sowing. There is a certain amount of risk in exploiting rice heterosis by means of TGMS if temperature fluctuation occurs at critical stages of panicle development (Wu, 1997). The stages of panicle development sensitive to environmental factors vary among TGMS lines. In TS6, all panicle developmental stages from differentiation of primary bract primordium (S1) to pollen ripening (S8) were found to be sensitive to temperature.

The critical sterility and fertility temperatures are also important factors causing fertility alteration in TGMS lines. Yuan (1998) suggested that the critical temperature inducing sterility must be relatively low, i.e. 23°C in temperate zones and 24°C in the subtropics. The lines with more critical

temperatures (>27°C) are not favourable for commercial exploitation since even a brief fall in temperature during the summer months may cause fertility reversion in TGMS lines that may lead to self seed set. In this study, the critical temperature for fertility alteration of TS6 was around 26°C, so this could be commercially exploited without any problem.

Though TS6 had a desirable critical temperature, the difference between CST and CFT was very narrow, which is considered as undesirable since there is a possibility of fertility reversion due to the unusual occurrence of a low temperature during hybrid seed production, which may lead to a mixture of selfed and hybrid seeds. Hence, it would have been advantageous if the CFT was still lower and the difference between CST and CFT was wider. However, Wu and Yin (1992) opined that low temperatures that would transform TGMS lines into fertile ones will not occur frequently during high-temperature seasons. Even if it occurs, it would last for only a few days, thus the purity of hybrid seed will not be affected. In the present study also, the same trend was observed. However, the results obtained on the fertility behaviour of this new line needs to be confirmed under controlled environmental conditions using a phytotron.

In this study, TGMS in TS6 was found to be inherited as a single recessive gene. A single recessive gene has been reported to control male sterility in 5460S (Sun et al., 1989), R59TS (Yang et al., 1990) and Norin PL 12 (Maruyama et al., 1991). The recessive nature of the TGMS lines facilitates their deployment in hybrid breeding programmes, because any fertile line can be used as a male parent to develop commercial rice hybrids. Single gene control also facilitates its transfer from one genotype to the other. Studies are in progress to determine the allelic relationship between the TGMS gene of TS6 with other known sources.

The incorporation of the TGMS gene through conventional breeding procedures is quite cumbersome and involves the identification of TGMS plants in segregating generations and induction of fertility by ratooning under an appropriate fertility-inducing temperature regime. The screening for sterility can be done only during a limited period, which coincides with the occurrence of the required temperature to induce sterility. Hence, 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 and in TGMS gene isolation by means of map-based cloning. This would also help in distinguishing different sources of TGMS genes without allelism tests. There have been several attempts to tag/map different TGMS genes (Wang et al., 1995, 2003; Subudhi et al., 1997; Lang et al., 1999; Reddy et al., 2000; Dong et al., 2000). In this study, a preliminary attempt was made to tag the TGMS gene through RAPD markers using an F_2 population of TS6 × MRST9 through bulked segregant analysis. This approach provides information simultaneously on polymorphism of the parents and possible linkage between the marker and the targeted gene using only two bulks and the parents, thereby significantly reducing the cost and work load. This has been well proven as an efficient technique to tag the major genes when near-isogenic lines are not available.

While considering a screening strategy based on molecular markers, tight linkage between the marker and the phenotype is necessary, since the weaker the linkage the less reliable will be the screening. In this context, the identified marker, $OPC05_{2962}$, would be more reliable to screen the TGMS gene of TS 6. The result obtained from this study proved that RAPD in combination with bulked segregant analysis of F_2 populations provides a highly efficient strategy to tag the genes of interest. RAPD and bulked segregant analysis can be used as the initial method for tagging the gene of interest and then the polymorphic RAPD products obtained can be mapped relative to the molecular markers with known map positions. This will permit the rapid mapping of the target gene as reported by Wang et al. (1995) and Subudhi et al. (1997). Thus, the identified RAPD marker may form a basis for further exploration of TGMS through molecular marker technology in future studies.

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