Theor Appl Genet (2000) 100:794–801

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Genetic analysis of temperature-sensitive male sterilty in rice

Received: 10 April 1999 / Accepted: 29 July 1999

Abstract The present study of genetic analysis is an attempt to precisely characterize diverse temperature-sensitive genic male-sterile (TGMS) lines so as to explore the possibilities of utilizing the most promising in large-scale hybrid seed production. Genetical studies revealed that the TGMS segregants derived from crosses involving TGMS lines ID24 and SA2 expressed differential fertility levels at low-temperature conditions. A majority of these progenies expressed transgressive segregation towards either sterility of fertility, causing instability of sterility and low reversibilty of fertility which may be due to large numbers of single-locus QTLs and their epistatic interactions. We identified two putative genes imparting temperature-sensitive male sterility after observing crosses involving diverse TGMS sources. To identify suitable molecular markers closely linked to the trait we used RAPD, AFLP and microsatellites which generated polymorphism through bulked segregant analysis. AFLP analysis using a smaller genome kit resulted in enormous polymorphism, out of which the combination EAA/MCAG amplified a 330-bp fragment, which closely segregated with the gene at a distance of 5.3 cM. This fragment was eluted for cloning and from the sequence a STS primer (TS200) was developed which produced a dominant polymorphism specific to TGMS. The microsatellite RM257, located earlier on chromosome 9, was linked with the TGMS trait in SA2 at a distance of 6.2 cM. RM257 produced a codominant polymorphism with 145-bp (sterile) and 132-bp (fertile) products. Both individually and collectively, the markers TS200 and RM257 located on either side of the TGMS locus are very useful for marker-assisted selection.

Key words TGMS · RAPD · AFLP · Microsatellites · STS · Marker-assisted selection · Bulked seg analysis

Communicated by G. Wenzel

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Introduction

One of the major anticipated constraints to the sustenance of heterosis breeding in rice is the availability of quality seed within an affordable price range. The widely employed cytoplasmic male sterility-fertility restoration system based on three-line breeding is often difficult to maintain. Problems with the maintenance of A lines, the lack of diversity in A and R lines, and the presence of minor fertility genes in B lines have to constantly be addressed in breeding programs, ultimately leading to high seed production costs. Following the landmark finding by Prof. Shi Ming Shang of the Hubei University that sensitive rice genotypes reversibly turn male fertile to male sterile and *vice versa* with changes in environmental factors, such as temperature and/or daylength during critical phases of plant growth, the concept of two-line breeding emerged as an alternative to the three-line approach (Yuan 1987). The main advantages of two-line heterosis breeding include the ability to use a wide range of genotypes as male parents, absence of negative effects associated with sterility-inducing cytoplasm, and no need for maintainer lines. The trait temperature-sensitive genic male sterility (TGMS) is known to be influenced by fertility differential genes (*Fd*), and epistatic interactions make the selection of appropriate TGMS lines under field or controlled conditions very difficult (He et al. 1999). The identification and use of suitable molecular markers closely linked to TGMS could be an easy and reliable approach to solving this problem. Gene pyramiding of non-allelic TGMS sources which are sensitive un-

Table 1 Relative stability of selected TGMS lines for sterility (S) fertility (F) under field conditions

TGMS lines	Year	Flowering months											Maximum seed set (%)
		June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.	April	May	
SA2	1994–95	S	S	F	S/F	F/S	F/S	S	S	S	S	S	S	11.20
	1995–96	S	S/F[a]	F/S	F	F	F/S	S	S	S	S	S	S	
ID24	1994–95	S	S	F	F/S	F	F	F	F	S/F	S	S	S	25.00
	1995–96	S	S	S	F/S	F	F	F/S	S	S	S	S	S	
IR68945	1994–95	S	F/S	F	F	F	F	F	S/F	F	S/F	S	S	70.23
	1995–96	S	F	F	F	F	F	S	S	F	F/S	S/F	S	

[a] S/F, Predominantly sterile; F/S, predominantly fertile

der a wide range of temperature conditions might help increase the stability of the sterile phase during hybrid seed production, even under conditions of unpredicted low temperature. As the manifestation of all sources of TGMS genes in terms of fertility alteration can not be strictly demarcated, closely linked markers for various TGMS sources are a must for pyramiding in appropriate backgrounds. Genetic and molecular characterization of all available TGMS sources through tests of allelism aids in identifying their diversity. There are few reports on the allelic relationships of TGMS sources. Zhang et al. (1994) mapped two photoperiod-sensitive male-sterility genes to chromosomes 3 and 7. Kinoshita (1992) has designated the TGMS gene of NorinPL12 as *tms*2 since the TGMS gene of 5460S is already designated as *tms*1 by Yang and Wang (1988). Wang et al. (1995) mapped *tms*1 on chromosome 8, whereas the locus of *tms*2 was estimated to be on chromosome 7 (Yamaguchi et al. 1997). Subudhi et al. (1997) reported that the TGMS gene in NorinPL12 and IR32364 are non-allelic and identified linked random amplified polymorphic DNA (RAPD) markers for the TGMS trait in IR32364 and designated the TGMS gene in IR32364 as *tms*3.

An effort was made in India to identify/isolate suitable TGMS sources through various approaches such as the selection of spontaneous or induced mutants and the introduction of exotic TGMS sources. The result was as many as three promising lines which could be used (Ali et al. 1995). In the study reported here, we attempted to gain an understanding of the genetics of the TGMS trait from different sources.

Materials and methods

Plant materials

Two TGMS lines, SA2 and ID24, made available by the Directorate of Rice Research, Hyderabad and line IR68945, a cross derivative of the TGMS Norin PL12, from the International Rice Research Institute, The Philippines were used for the study. SA2 and ID24 show complete sterility above 28°C and high fertility between 20°C and 26°C. However, these lines are predominantly temperature-sensitive, interacting strongly with photoperiod. Photoperiodic range for temperature activity (PRTA) is very wide (12–13.5 h) for SA2 and ID24, indicating that these lines can be used in a wide range of areas with lower temperatures. In all these lines seed set is very low (10–47%) under field conditions, as opposed to as high as 50–75% under controlled conditions. Therefore, suitable areas have to be identified for their multiplication, while they can be used for hybrid seed production at any place since they remain totally sterile from March to June (Table 1). IR68945 is characterized by the absence of photoperiod interaction and remains sterile within a temperature range of 30°C to 35°C. This line remains highly fertile with as high as 75% seed seeting under field conditions.

Pollen/spikelet fertility

At flowering a minimum of five plants were observed for pollen fertility, and at maturity spikelet fertility was recorded. At least three spikelets were sampled from each plant during anthesis, and their anthers were smeared with a 1% IKI solution. Darkly stained pollen grains of normal shape were scored as fertile, brown or yellowish colored or irregular and shriveled types, as sterile. Pollen fertility averaged over five plants was expressed as a percentage. At maturity, panicles covered with bags were taken for recording spikelet fertility.

Stages of panicle development

All of the lines were labeled for their panicle development using the method of Matsushima and Manaka (1956) which correlates the growth of the flag leaf to that of its preceding leaf and stage of panicle development. The following five different treatments were adopted in the study. (1) All the TGMS lines were continuously subjected to 20°C until the flag leaf (n) reached half the length of its preceding leaf (n-1); they were then immediately transferred to 32°C until heading. (2) Lines were kept at 20°C until the collar of the nth leaf reached the full length of its n-1th leaf and further subjected to 32°C until heading. (3) Plants were kept at 32°C until the collar of the nth leaf reached half the length of its n-1th leaf and then shifted to 20°C until heading. (4) Subjecting the plants to 32°C until the collar of the nth leaf reached the complete length of its n-1th leaf and then transferring them back to 20°C till heading (5) Plants were kept at 32°C until the nth leaf legth reached half the length of the n-1th leaf, then transferred to 20°C until the collar of the nth leaf reached an equal length of the n-1th leaf and again brought back to 32°C until heading. The differential response between different tillers in a plant as well as within panicles were expressed as mean ± standard error.

Inheritance of TGMS

F_1s, F_2s, and F_3s of crosses of TGMS lines with normal parents, IR64446, BPI 176P, Dular, N22, IR58025B, Vikramarya, 9310, 9311, 9314, Jaya, IR46, IR64, IR10198 and Pakisan, along with the parental lines constituted the experimental material. The segregation pattern was tested for significance by the χ^2 test. F_2 plants from the crosses ID24/N22 (72 plants), SA2/N22 (50 plants) and IR 68945/IR58025B (70 plants) were evaluated under a controlled

environmental to understand the inheritance patterns of varying fertility levels. Allelic relationships among various TGMS lines were assigned using progeny of crosses of the homozygote for TGMS (*tms tms*) with a heterozygote of another source (i.e., *tms*1 *tms*1 *Tms*2 *Tms*2/*Tms*1 *Tms*1 *Tms*2 *tms*2), the BC_1F_1, as experimental material. Based on the pattern of fertility changes over the seasons, i.e., *rabi* 1995 (Oct–Nov) in which the temperatures were above 30–38°C during the critical periods for fertility alteration where all the TGMS lines turn sterile and *kharif* 1996 (June–July) where the temperature and photoperiod combinations (20°-26°C with 10–12 h of daylength) were the most favorable for turning fertile, we determined the allelic relationships among diverse TGMS. The non-allelic BC_1F_2 families (ID 24//SA2/N22 and IR 68945//SA2/N22) were further tested during rabi, 1996 with the expectation of finding segregation for fertility and sterility in accordance with either 3:1 or 9:7 to confirm the non-allelic relation that was observed earlier.

Mapping population

The cross SA2/N22 was selected for identifying molecular markers. Initially 150 F_2 progeny were phenotyped by screening under both controlled and field conditions. The segregation pattern for fertility in the F_3s enabled us to distinguish the homozygous from the heterozygous fertile F_2 individuals. DNA was isolated following the protocol developed by Dellaporta et al. (1983). The concentration of DNA was determined using Hoechst dye 33258 in a TKO mini-fluorometer (Hoefer Scientific). Identification of molecular markers was done using bulk segregant analysis (BSA) (Michelmore et al. 1991). DNA bulks F(fertile) and TG(TGMS) were made by mixing equal amounts of DNA of each of ten plants. The bulks and parents were subjected to RAPD, amplified fragment length polymorphism (AFLP), microsatellite, and sequence-tagged site (STS) analyses. The primers showing putatively linked fragments were then tested on all individuals.

RAPD

A standard 15-µl reaction mixture contained 9.6 ng of template DNA, 3 µ*M* of primer (10 mers obtained from Operon Technology Inc.), 0.7 U Ampl*Taq* DNA polymerase Stoffel fragment (Perkin Elmer Cetus), 1.5 µl 10 × buffer, 2.5 m*M* $MgCl_2$, 11.1×10^4 Bq [^{32}P]-dCTP and a final concentration of 100 µ*M* dATP, dGTP, dTTP and 6 µ*M* dCTP. Since the radioactive protocol gives 40% more bands in RAPD, [^{32}P] was used to obtain more amplifications by using a PCR-9600 thermocycler (Perkin Elmer Cetus) with the following conditions: denaturation of 92°C for 1 min followed by 30 cycles of 1 min at 92°C; annealing at 37°C for 40 s; extension at 72°C for 1.15 min with a ramp of 15 s; a final extension at 72°C for 15 min. A total of 100 primers were tested.

AFLP

The AFLP analysis system of GIBCOBRL (Life Technologies) was used according to the manufacturer's instructions with some modifications. DNA (100 ng) was digested with 0.625 U *Eco*RI and *Mse*I restriction enzyme mix for 2 h at 37°C. After heat inactivation of the enzyme mixture, *Eco*RI/*Mse*I adapters (6 µl of adapters containing 0.4 m*M* ATP, 10 m*M* TRIS-HCl, pH 7.5, 10 m*M* Mg-acetate, 50 m*M* K-acetate) were ligated using 0.25 U T4 DNA ligase for 2 h at room temperature. The adapter-ligated DNA template was diluted with 18.5 µl of TE buffer (10 m*M* TRIS-HCl, pH 8.0, and 0.1 m*M* EDTA). Preamplification of restriction-ligated and diluted DNA was carried out in 12.75-µl total volume containing 10 µl of preamplification primer mix, 1.25 µl 10 × polymerase chain reaction (PCR) buffer for AFLP (200) m*M* TRIS-HCl, pH 8.4, 15 m*M* $MgCl_2$, 500 m*M* KCl), 2.5 U *Taq* DNA polymerase and 1.25 µl template DNA. Amplification was carried out for 20 cycles, each consisting of 94°C for 30 s, 56°C for 1 min, and 72°C for 1 min. A part of the preamplified product was tested on a 1.2–1.5% agarose gel, and the remaining sample was diluted 50 times with TE buffer, if digestion was intense enough. TGMS and fertile bulks were made by mixing equal amounts of preamplified diluted template DNA. For selective amplification, 2.5 ng of *Eco*RI primer was labeled with [^{32}P]-ATP (0.1 µl of 3000 µCi/mole) using 0.1 units of T4 polynucleotide kinase and its appropriate buffer for 1 h at 37°C. The enzyme was heat-inactivated. Selective amplification was carried out in a 10-µl volume containing 0.25 µl labeled *Eco*RI primer, 2.25 µl *Mse*I primer (6.7 ng/µl and dNTPs), 3.95 µl of H_2O, 1 µl 10 × PCR buffer for AFLP, 0.5 U *Taq* polymerase, and 2.5 µl preamplified template DNA. *Eco*RI primers meant for both larger (3 extra nucleotides at 3′ end) and smaller (2 extra nucleotides at 3′ end) genomes were used in all combinations with eight *Mse*I primers in order to detect polymorphism.

Gel electroporation and visualization for RAPD and AFLP

An equal volume (10 µl) of sequencing loading dye (98% deionized formamide, 10 m*M* EDTA, pH 8.0, 0.025% xylene cyanol, 0.025 bromophenol blue) was added to the PCR product. The samples were heated at 94°C for 5 min, chilled on ice, and immediately loaded on a 5% acrylamide gel containing 8 *M* urea (0.4-mm thickness). Electrophoresis was done using a 0.5 × TBE buffer. After approximately 2 h of electrophoresis time, the gels were transferred to 3MW blotting paper and dried. They were then exposed to Kodak X-omat film for 48 h at –85°C.

Cloning and sequencing

A polymorphic AFLP fragment linked to the TGMS trait in SA2 was excised from the gel and the eluted DNA was purified, cloned into the PCR2.1 vector (Invitrogen), and sequenced using an ABI Dye-Terminator sequencing kit (Perkin Elmer, USA). Forward and reverse primers (TS 200) were designed from the sequenced fragment.

Microsatellite and STS analyses using hot-start PCR

A total of 120 microsatellite primer pairs (Research Genetics, Huntsville, Ariz., USA) were used for the study. Amplification reactions were performed in 20-µl volumes containing 2.0 µl 10 × PCR buffer with $MgCl_2$, 1 µl BSA, 0.2 µl 25 m*M* of a dATP, dGTP, dTTP, and dCTP mix, 50 ng of each reverse and forward primer, and 50 ng template DNA; 0.5 U *Taq* DNA polymerase (Promega) was added to the reaction at the annealing temperature of first cycle. Amplifications were carried out with a first denaturation at 94°C for 3 min followed by 45 cycles of 94°C for 30 s, either 50°C, 55°C, or 60°C (depending on the microsatellite) for 1 min (annealing) and 72°C for 2 min (extension). A final 15-min extension at 72°C was then performed. For separation of microsatellite and STS alleles a 4% gel made with a mix of metaphor and low-melting-point agarose in proportions of 1.2:0.8 was used and run at high voltage in prechilled 0.5 × TBE buffer by connecting a pump to the buffer tank to circulate the chilled buffer. Gels were stained with ethidium bromide and photographed on a UV transilluminator using poloroid film (667,3000 ASA).

Data scoring and analysis

Polymorphic RAPD, AFLP and SSR markers were scored and mapped using the MAPMAKER (Lander et al. 1987) program. A critical LOD score threshold of 3.0 was used for estimating linkage.

Table 2 Allelic relationship of the gene(s) governing TGMS among BC_1F_1 with respect to different sources

TGMS line	Season	IR68945/IR58025B		SA2/N22		ID24/Dular	
		F:S[a]	χ^2 1:1	F:S	χ^2 1:1	F:S	χ^2 1:1
IR68945	*Rabi*	22:23	0.02 (P = 0.9)	30:0	–	37:23	3.27 (P < 0.01)
	Kharif	59:0	–	30:0	–	41:5	28.17** (*P* < 0.001)
SA2	*Rabi*	20:0	–	19:18	0.03	39:0	
	Kharif	20:0	–	25:7	10.12** (P < 0.001)	39:0	–
ID24	*Rabi*	30:23	0.92 (P > 0.3)	40:9	19.61** (P < 0.001)	30:29	0.02 (P = 0.9)
	Kharif	53:0	–	36:3	27.92** (P < 0.001)	59:0	–

** Significant at 1% level
[a] F:S, Fertile:sterile

Table 3 Pollen fertility among F_2 progenies of TGMS/N22 crosses grown at 24°C (12.5-h daylength)

Cross	Total No. of plants	Fertile pollen (%)						
		0–10	10–20	20–30	30–40	40–50	50–60	60–70
ID24/N22	72	38	15	7	2	5	3	2
SA2/N22	50	18	16	10	3	2	1	0
IR68945/N22	70	0	0	0	0	20	25	15

Results and discussion

Inheritance of TGMS

A basic knowledge of the mode of inheritance and the number of genes governing the trait are important in planning a result-oriented breeding program involving a TGMS system. The crosses of the TGMS lines IR68945, SA2 and ID24 with a set of normal pollen parents were observed for segregation of fertility in the F_2s and subsequent F_3s. They segregated in accordance with the expected ratio of 3 fertile : 1 sterile in most cases with a deviation from the expected ratio of 3:1 in some of the combinations, IR68945/BPI 176P (138:13), IR68945/Dular (51:39), SA2/N22 (200:47), SA2/9310 (210:51), and ID24/9313 (61:38) (Reddy et al. 1998). It appears that crosses involving normal parents, BPI 176P, IR10198, and IR56, segregate with a far greater excess of fertile plants distorting the expected ratio of 3:1. This may be due to the presence of a strong fertility modifier complex in the pollen parents which could mask the effect of the TGMS genes. The F_2 populations of crosses of various TGMS lines with pollen parents possessing wide compatibility gene(s), Dular, IR65598, 9310, 9314, and IR64446, have been found to produce more sterile progenies than the expected monogenic ratio. This skewness towards sterile progenies may be due to co-segregation of the wide compatibility gene(s) present in these parents with the TGMS gene. In the F_3 generation all the families except the cross IR32364/IR64446 were found to segregate in the ratio of 1:2 for true breeding and segregating families. Similarly, the various testcross (BC_1F_1) populations, namely, IR68945//IR68945/25B, SA2// SA2/N22, and ID24// ID24/Dular, were found to segregate in the ratio of 1 fertile to 1 sterile plant, thus confirming the simple inheritance of TGMS (Table 2). Nonconformity of the 1:1 ratio in some crosses is discussed later. Overall, the results of our study, however, confirm with those of earlier reports (Maruyama et al. 1990; Borkakati and Virmani 1996) that environment-induced male sterility is monogenically controlled.

The progenies of the crosses ID24/N22, SA2/N22, and IR68945/N22 when studied at 24°C under a 12.5-h daylength exhibited differential responses in terms of fertility levels (Table 3). In the F_2 of ID24/N22 and SA2/N22 trangressive segregants for both sterility and fertility along with the parental types were observed, whereas in the cross IR68945/N22 all the progenies were of the parental-type TGMS and no transgressive segregation for sterility was observed. Such distortions from this expected ratio of 3:1 are explained largely by the association of modifier gene(s) (Oard and Hu 1995; Wu and Yin 1996). The reason for transgressive segregation towards sterility/fertility could be due to large number of loci involving single-locus quantitative trait loci (QTLs) and the epistatic interactions of different fertility genes located on different chromosomes, for example, on chromosomes 2, 3, 5, 6, 7, 8, and 10 (He et al. 1999).

In the present study BC_1F_1 populations of crosses involving two different TGMS sources were studied during the seasons *rabi* (Oct–Nov) as well as *kharif* (June–July) for differential expression of fertility (Table 2). During these seasons the critical period for sensitivity of all the TGMS lines coincides with the most ideal temperature and daylength conditions to turn fertile into sterile and *vice-versa*. The segregation pattern for fertility: sterility in BC_1F_1s of crosses involving 3 F_1s (IR68945/IR58025B, SA2/N22, and ID24/Dular) with

Table 4 Segregation for fertility of BC_1F_2 populations of crosses involving non-allelic TGMS sources

Cross	Family code	Observed ratio	Theoretical ratio	χ^2	Probability (%)
ID24//SA2/N22	A	17:5	3:1	0.060	0.80
	B	14:9	9:7	0.200	0.70–0.50
	C	12:9	9:7	0.007	0.95–0.90
IR68945//SA2/N22	A	13:9	9:7	0.072	0.80–0.70
	B	17:5	3:1	0.060	0.80
	C	13:9	9:7	0.072	0.80–0.70
	D	19:3	3:1	1.510	0.30–0.20
	E	17:5	3:1	0.060	0.80

Table 5 Determination of stage of sensitivity for various TGMS gene sources

Treatment no.	Panicle development stage[a]			Fertile pollen (%) Mean ± SD		
	I	II	III	IR68945	SA2	ID24
1	Low[b]	High	High	44.8 ± 15.9	0.0	13.6 ± 9.6
2	Low	Low	High	50.3 ± 14.1	21.6 ± 14.7	25.8 ± 11.4
3	High	Low	Low	20.3 ± 5.9	31.0 ± 17.6	32.5 ± 20.1
4	High	High	Low	0.0	0.0	10.9 ± 12.4
5	High	Low	High	12.4± 8.9	36.2 ± 12.3	29.4 ± 16.3

[a] I, Flag leaf is half or less than the length of its preceding leaf; II, flag leaf attains a length equal to its preceding leaf; III, later stages till heading
[b] Low, 20°C; high, 32°C (daylength: 13 h)

TGMS lines (IR68945, SA2, and ID24) indicated that the TGMS lines ID24 and IR68945 are allelic to each other since the segregating pattern is in accordance with 1:1 for fertility: sterility during *rabi* and completely fertile during *kharif*. SA2, however, is not allelic to any as none of the BC_1F_1s involving this segregated in both the seasons. The non-allelic relations have been further confirmed by observing the segregation pattern in BC_1F_2 (Table 4). Interestingly, ID24, the TGMS line which shows strong interaction with varying photoperiod conditions, is allelic to IR68945, which is a strictly temperature-sensitive sterile.

TGMS lines from various sources differ in their stage of sensitivity to temperature as well as panicle development stage. The development process of the young panicle of rice and its identification can be correlated with growth of flag leaf (n) and its preceding (n-1) leaf lengths (Matsushima and Manaka 1956). In the present study three morphological stages (1) flag leaf length is half or less than the length of its preceding leaf; (2) the flag leaf attains a length equal to that of its preceding leaf; (3) later stages till heading encompasses all the panicle developmental stages: stamen and pistil primordia, meiotic (PMC formation) and post-meiotic (uninucleate), respectively. IR68945 was relatively more sensitive to temperature before the flag leaf reached half the length of its preceding leaf, exhibiting high pollen fertility as high as 44.8% ± 15.9% and 50.3% ± 14.1% in the first and second treatments (Table 5). In the third treatment it exhibited 20.3% ± 5.9% pollen fertility, while complete sterility was reached in the fourth. The fifth treatment, however resulted in a pollen fertility of 12.4% ± 8.9%, indicating that about 68.8% response is during stamen-pistil primordia and 31.2% from PMC formation to the end of meiosis. SA2 was completely sterile in the first and fourth treatments and fertile in second, third, and fifth treatments; hence this line is 100% sensitive when flag and preceding leaf lengths are equal where the respective panicle development stage is predicted to be at PMC formation. ID24 remained fertile during all the treatments, although the levels varied from treatment to treatment with the first and fourth treatments showing a lower percentage fertility (13.6% ±9.6% and 23.9% ± 12.4%, respectively) than the second, third, and fifth treatments (25.8% ± 11.4%, 32.5% ± 20.1%, and 29.4% ± 16.3%, respectively). In spite of a high standard error (SE) due to the classification of panicle developmental stages set by Matsushima and Manaka (1956) in relation to the growth and development of flag leaf versus its preceding leaf, which is only relative in its nature and there are developmental differences within the top, bottom, and middle portions of a panicle at any given stage, it was possible to classify various TGMS lines based on the stage of sensitivity. It can be concluded that ID24 is unique in exhibiting sensitivity during all the panicle developmental stages, with more emphasis during meiotic stage. These results further indicate that the TGMS line ID24 possesses additional genetic factor(s) other than the IR68945 TGMS genes which influence expression of its fertility/sterility.

Mapping TGMS

Bulk segregant analysis using the RAPD technique indicated that the primers OPS1 and OPA12 have specific polymorphism with bulks. However, when tested for co-segregation with the entire F_2 population, the primer

OPA12 alone produced a 0.74-kb amplicon specific to the trait in repulsive phase. This marker was found to be 23.1 cM from the loci with a recombination frequency of 21.6% (Fig. 1). Similar to our finding, Wang et al. (1995) identified a 1.2-kb product of OPB19 that is linked to TGMS in 5460S at a distance of 6.7 cM, mapping to chromosome 8. Subudhi et al. (1997), from their study of the mapping population of the cross IR32364 TGMS/IR68, identified five RAPD markers generated by five primers, namely, OPF18, OPB19, OPAC1, OPAC3 and OPAA7, to be putatively linked to the TGMS gene. Out of these the nearest marker is the 0.64-kb OPAC3, which is linked to the TGMS gene at a the distance of 7.7 cM and mapped to chromosome 6. None of these primers used in above studies have shown any polymorphism with TGMS SA2.

AFLP maps are currently being developed in a variety of plant species (Becker et al. 1995; Maheswaran et al. 1997). This approach to molecular mapping promises great efficiency because of the ability to screen large numbers of DNA fragments in a single lane of polyacrylamide gels, thus increasing the possibility of identifying polymorphisms and expediting the construction of high-density linkage maps. Results of AFLP analysis using the larger genome kit revealed that the primer combinations EACG/MCTC, EACC/MCAA, and EACT/MCTA generated polymorphism in repulsive phase; however, EACT/MCAG amplified a 350-bp product linked in coupling phase at the distance of 17.3 cM and recombination frequency of 16.7%. Other mentioned combinations were not completely polymorphic in the individuals of the bulks. However, AFLP analysis using the smaller genome kit resulted in enormous polymorphism. The primer combinations ETG/MCTA, ETG/MCAA, EAC/MCAA, and EAA/MCAG showed polymorphism in coupling phase and the combinations EAA/MCAT, ETC/MCTG, ETC/MCTC, ETC/MCTT, ETA/MCTT, ETA/MCTG, EAT/MCAA, and EAT/MCAT in repulsive phase. Out of these, the combination EAA/MCAG alone amplied a 330-bp fragment (Fig. 2) which was closely linked at a distance of 5.3 cM. This fragment was eluted and cloned, and the sequence was obtained by sequencing from both ends of the cloned product. The terminal bases of the sequence matched exactly with primer sequences. A STS primer (TS200) was developed from the above sequence taking compatible sequences from each end to design forward (5′CGGAATGTAATTCACATGC3′) and reverse (5′CAGAGAAACATCAGTTGTGG3′) primers. The primer TS200 produced a dominant kind of polymorphism specific to the TGMS trait (Fig. 3). A single DNA fragment of 150 bp was resolved on 4% metaphor agarose gel and placed at a distance of 1.8 cM from EAA/MCAG$_{0.3}$ and 23.1 cM from OPA12$_{0.7}$.

The current status of the microsatellite map in rice has already enabled the identification of microsatellite markers near many genes, and studies previously mentioned have demonstrated that microsatellite markers are reliable in tracing the ancestry of target genes and chromosomal regions. In the present study, out of 120 primer pairs tested 45 were polymorphic between the parents. However, only the microsatellite RM257 was linked with the TGMS trait in SA2 with a linkage of 6.2 cM and recombination frequency of 6.1%. RM257 produced codominant polymorphism for both alleles with 145-bp (sterile) and 132-bp (fertile) products (Fig. 3). McCouch et al. (1997) developed a microsatellite framework map providing genome-wide coverage in all 12 chromosomes

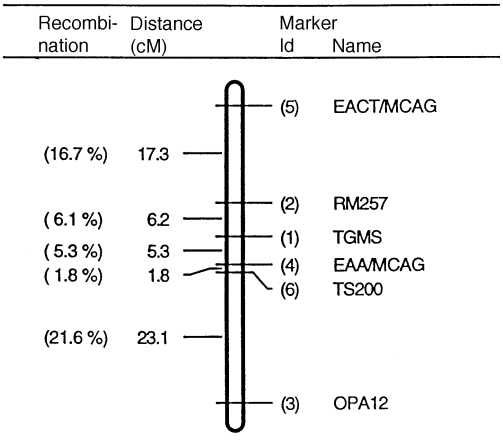

Fig. 1 Map of linked markers flanking the TGMS locus

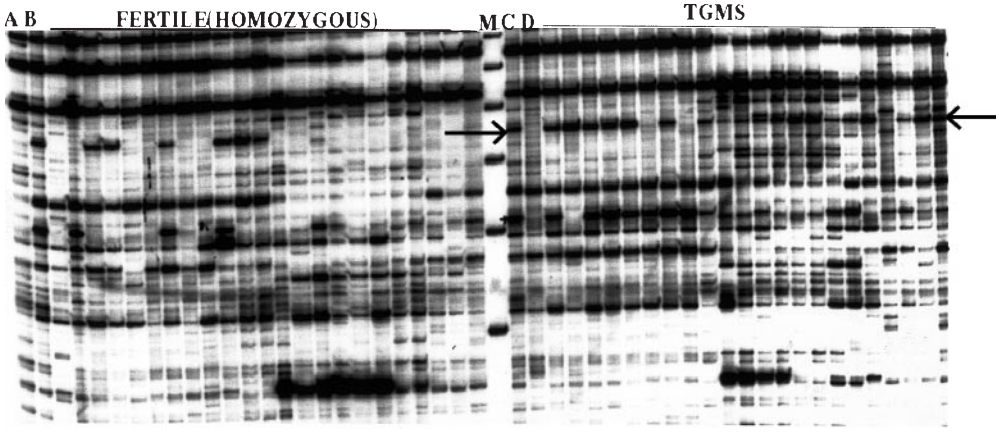

Fig. 2 A portion of the AFLPs obtained with the EAA/MCAG primer combination of homozygous-fertile and TGMS F_2 individuals along with parents and bulks. **A**, **B** Parents, fertile (N22) and TGMS (SA2), respectively, *M* 50-bp ladder, *C*, *D* TGMS and fertile bulks. *Arrow* indicates the 330-bp amplicon specific to TGMS

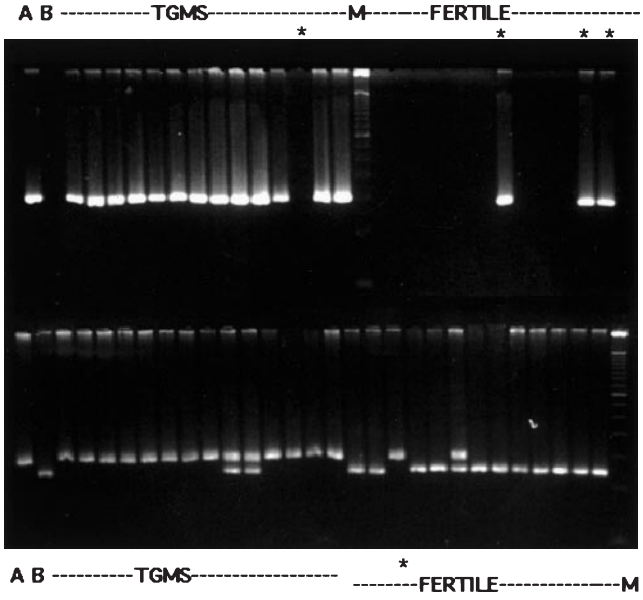

Fig. 3 STS (*top*) and microsatellite (*bottom*) analyses of TGMS using TS 200 and RM 257. *A*, *B* Parents, TGMS and fertile, respectively, followed by TGMS and fertile F_2 individuals, *M* 50-bp ladder. The putative recombinants for different markers are indicated by an *asterisk*

of rice from a cross between IR64/Azucena. In this map, RM257 was mapped on chromosome 9 between RZ422 and RZ228 at distances of 8.9 cM and 7.7 cM, respectively. Based on this, we conclude that the gene governing TGMS in SA2 is on chromosome 9.

When the markers are mapped using MAPMAKER with a LOD score of 3.0, the markers EACT/MCAG and RM257 fall on one side of the locus and the EAA/MCAG, TS200, and OPA12 markers fall on the other side. After screening the large number of TGMS lines it was found that the markers RM257 and TS200 can be used as multiplex markers for selection, as these two markers are on either side of the TGMS locus (Fig. 1).

The results of allelic studies involving TGMS gene sources and expression of TGMS genes with respect to different developmental stages, coupled with the present molecular characterization enabled the TGMS gene in SA2 to be differentiated from other sources; it can be tentatively designated as *tms*4. However, gene nomenclature can be reviewed after thoroughly considering many more detailed studies which shows the relation between temperature and photoperiod influences for expression of sterility in rice. It appears that there is no strict temperature-sensitive or photoperiod-sensitive sterile types, and many studies have proven both factors to be interactive in different degrees based on the background of genotype in which the gene is transferred (Lu et al. 1992; Cheng et al. 1996). Two photoperiod-sensitive male-sterility genes, *pms*1 and *pms*2, have already been mapped from Nongken58S on chromosome 3 and 7, respectively. Several of Nongken58S derivatives have proven to be temperature-sensitive rather than photoperiod-sensitive (Zhang et al. 1994), which necessitates redesignation of the gene nomenclature of *pms* and *tms* as *ems*; i.e., referring to environmental male sterility.

Acknowledgements The first author grateful to the Rockefeller Foundation, New York, for financial assistance. The authors are thankful to Dr. K. Krishnaiah, Project Director, Directorate of Rice Research, Hyderabad, India for providing the facilities for the field and growth chamber studies. We also thank Dr. M.I. Ahmed of the hybrid rice section, Directorate of Rice Research, Hyderabad for his supervision and help in conducting the field experiments. We are grateful to Dr. S.S. Virmani, Hybrid Rice Breeder, International Rce Research Institute, Phillippines for providing TGMS lines and technical help. The work is partly financed by UNDP-Hybrid Rice Programme. The experiments comply with current regulations in both India and the USA.

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