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Molecular mapping of a rice gene conditioning thermosensitive genic male sterility using AFLP, RFLP and SSR techniques

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Abstract The discovery and application of the thermosensitive genic male sterility (TGMS) system has great potential for revolutionizing hybrid seed production technology in rice. Use of the TGMS system in two-line breeding is simple, inexpensive, efficient, and eliminates the limitations associated with the cytoplasmic-genetic male sterility (CMS) system. An F_2 population developed from a cross between a TGMS *indica* mutant, TGMS–VN1, and a fertile *indica* line, CH1, was used to identify molecular markers linked to the TGMS gene and to subsequently determine its chromosomal location on the linkage map of rice. Bulk segregant analysis was performed using the AFLP technique. From the survey of 200 AFLP primer combinations, four AFLP markers (E2/M5–600, E3/M16–400, E5/M12–600, and E5/M12–200) linked to the TGMS gene were identified. All the markers were linked to the gene in the coupling phase. All except E2/M5–200 were found to be low-copy sequences. However, the marker E5/M12–600 showed polymorphism in RFLP analysis and was closely linked to the TGMS gene at a distance of 3.3 cM. This marker was subsequently mapped on chromosome 2 using doubled-haploid mapping populations derived from the crosses IR64×Azucena and CT9993×IR62666, available at IRRI, Philippines, and Texas Tech University, respectively. Linkage of microsatellite marker RM27 with the TGMS gene further confirmed its location on chromosome 2. The closest marker, E5/M12–600, was sequenced so that a PCR marker can be developed for the marker-assisted transfer of this gene to different genetic backgrounds. The new TGMS gene is tentatively designated as tms4(t).

Key words AFLP · Bulked segregant analysis · Gene tagging · Marker-assisted selection · Rice TGMS gene

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Introduction

The development of hybrids in rice and many other food crops has proved to be an effective way of improving crop productivity in today's agriculture. Hybrid rices show 15–20% higher yields than the best semi-dwarf inbred varieties in China and other countries (Virmani et al. 1994). The three-line breeding system using cytoplasmic-genetic male sterility (CMS) lines is the most common method for hybrid seed production. Although effective, the system is expensive and cumbersome. Maintaining the CMS lines and choosing an appropriate restorer line for developing the fertile hybrids are major limitations. Additionally, the availability of only a few CMS lines for hybrid development narrows the genetic base and increases the risk of genetic vulnerability.

The application of genetic male sterility in hybrid rice production has great potential to revolutionize hybrid seed production methodology in rice. In rice, both photoperiod and temperature-sensitive genetic male sterility (PGMS and TGMS) have been discovered and successfully developed. The TGMS system is considered to be an efficient alternative to the CMS system for hybrid seed production (Maruyama et al. 1991).

Male sterility expression in TGMS lines is influenced by temperature. The temperature occurring just after panicle initiation is the most critical in the expression of sterility and fertility. Most TGMS lines are sterile at high temperatures (>25°C), but fertile at lower temperatures (Sun et al. 1989). Thus, TGMS plants can be used not only as male-sterile lines but also as a maintainer lines,

providing the opportunity to produce hybrid seeds in rice by the two-line system. Secondly, in the TGMS system, almost all of the *indica* rices and most of the *japonica* rices can be used as male parents to produce fertile F_1 hybrids, thus eliminating the search for the restorer lines required in the traditional three-line system. Thirdly, the potential genetic vulnerability due to the restricted sterile cytoplasm base is greatly reduced, since this trait is relatively easy to transfer to diverse genetic backgrounds. The identification and exploitation of different TGMS genes in rice will not only broaden the genetic base of the hybrids, but will provide hybrid rice breeders with more choices to develop two-line rice hybrids with desirable traits.

Several TGMS genes in rice have been reported from China (Sun et al. 1989), Japan (Maruyama et al. 1991), and the International Rice Research Institute (IRRI) in the Philippines (Virmani and Voc 1991). More recently, some TGMS mutants derived from *indica* lines have been developed and characterized by morphological and genetic analysis (Dong et al. 1995a, b). The transfer of TGMS genes to different backgrounds can be greatly facilitated through the use of marker-assisted selection in hybrid rice breeding programs. With the development of molecular marker technology, it has been possible to map the genes of agronomic importance in segregating generations. Bulked segregant analysis (Michelmore et al. 1991) has been very effective in mapping genes of interest for hybrid rice breeders (Zhang et al. 1994, 1997; Wang et al. 1995; Subudhi et al. 1997; Yao et al. 1997). So far, only two TGMS genes of rice, *tms1* and *tms3(t)*, have been mapped on chromosomes 8 and 6, respectively (Wang et al. 1995; Subudhi et al. 1997); another TGMS gene, *tms2* from NPL12 (Maruyama et al. 1991), has yet to be mapped. This trait is reported to be controlled by a single recessive gene (Yang et al. 1992; Dong et al.1995a, b; Borkakati and Virmani 1996). Similarly, two PGMS (photoperiod-sensitive genetic male sterility) genes have been mapped on chromosomes 3 and 7 by Zhang et al. (1994), and two restorer genes have been located on chromosomes 1 and 10 (Yao et al. 1997; Zhang et al. 1997). Most of these genes have been mapped by using bulked segregant analysis in conjunction with RAPD and RFLP techniques. We have employed the AFLP (amplified fragment length polymorphism) technique (Vos et al. 1995), which allows rapid inspection of a large array of reproducible markers. The reproducibility of these markers is ensured by the use of restriction site-specific adaptors, adaptor-specific primers, and stringent amplification conditions (Vos et al. 1995). After identifying AFLP markers linked to the gene, RFLP and SSR (simple sequence repeat) techniques were employed to determine and confirm the gene location on the linkage map of rice.

In this paper, we report the molecular mapping of a new TGMS gene from a mutant rice line, TGMS–VN1, developed in Vietnam. An attempt was also made to develop PCR markers that will facilitate the transfer of this new TGMS allele to different genetic backgrounds through marker-assisted selection in two-line hybrid rice breeding programs.

Materials and methods

Development and characterization of the TGMS-VN1 mutant line

The TGMS-VN1 mutant line was obtained by gamma irradiation on a Vietnamese *indica* rice cultivar "Chiem Bau" at the Institute of Agricultural Genetics in Hanoi, Vietnam. In order to determine the critical temperature of this mutant line for TGMS expression, four lots of 100 plants of this mutant line were subjected to four different temperature regimes (20–22°C, 22–24°C, 24–26°C, 26–28°C) in a temperature-controlled chamber during panicle initiation stage. Twenty plants were sampled from each lot and were evaluated for pollen and spikelet fertility. Twenty to thirty florets were sampled at anthesis from primary panicles and fixed in 70% ethanol for examination of pollen fertility using a 1% iodine potassium iodide (IKI) solution. All round, dark brown-stained pollen was scored as normal fertile, and irregular-shaped, yellowish or light brown colored pollen grains were scored as sterile. Two hundred to three hundred pollen grains from three randomly chosen fields on each slide were evaluated. Two panicles per plant were evaluated for spikelet fertility. Panicles emerging from the leaf sheath were bagged with glassine paper bags prior to anthesis to prevent cross-pollination. Bagged panicles were harvested 25–30 days after anthesis. The filled and unfilled spikelets were counted and spikelet fertility was calculated as number of filled grains divided by the total number of grains. Plants with less then 5% stained pollen and/or seed set were considered sterile, whereas plants having more than 50% stained pollen and/or seed set were classified as fertile.

The TGMS-VN1 mutant was crossed with three fertile doubled-haploid lines: Line 11, Line 21 and Line 24 developed from the native Vietnamese varieties such as 'Chiem Bau', 'CR203' and 'D84–1' respectively. Forty F_1 plants in each cross combination were subjected to two different temperature ranges, 22–24°C and 26–28°C. All the F_1 plants in both the temperature ranges were found to be fertile based on the pollen and spikelet fertility result, indicating that the *tgms-vn1* gene is recessive in nature. In each of the above cross combinations, a large F_2 population was developed and evaluated for pollen and spikelet fertility analysis in the same two-temperature regime as described above.

Mapping population and phenotyping experiment

An F_2 population consisting of 114 lines developed from a cross between the TGMS mutant line, TGMS–VN1, and a fertile line, CH1, was used for the mapping of this *tgms–vn1* gene. The pollen and spikelet fertility of the mapping population and the parents were evaluated in the same way as described above at the Institute of Genetics, Hanoi, Vietnam, during 1997. The temperature in the greenhouse ranged from 25 to 35°C during the day and from 22 to 25°C at night. The sterile and fertile individuals were identified based on pollen and spikelet fertility. After observations on fertility and seed set were obtained, the main tiller was cut and allowed to develop secondary tillers. When the tillers developed new panicles, phenotypic characterization was repeated as above. This process was repeated three times for each individual plant from the ratooned panicles. A chi-square test was performed to determine the goodness of fit to a 3:1 ratio.

DNA extraction and AFLP analysis

DNA from each F_2 individual, along with both parents, was extracted following the method of McCouch et al. (1988). For each sample, the DNA concentration was determined using a spectrophotometer-160 U (SHIMADZU Corporation, Japan) in combina-

tion with running on a 0.8% agarose gel along with genomic DNA samples of known concentrations.

Bulked segregant analysis (Michelmore et al. 1991) was performed in conjunction with the AFLP technique following Vos et al. (1995). The *Eco*RI and *Mse*I primers used for pre-amplification were 5'–GAC TGC GTA CCA ATT C–3' and 5'–GAT GAG TCC TGA GTA A–3', respectively. Equal amounts of pre-amplified DNA obtained from 20 fertile plants and 20 sterile plants, respectively, of the mapping population were pooled to constitute the fertile and sterile bulks (Bulk-F and Bulk-S). Two bulk samples along with the parents were then subjected to AFLP analysis. A total of 200 primer combinations (Operon Technologies, Inc., Alameda, Calif., USA) with different numbers of selective nucleotides were used to amplify AFLP bands. The *Eco*RI primer was end-labeled using [γ-^{33}P] ATP and T4 polynucleotide kinase. The AFLP reactions were performed in 12-µl reaction volume. The AFLP reaction products were mixed with an equal volume of formamide dye (98% formamide, 10 mM EDTA, 0.005% each of bromophenol blue and xylene cyanol). The mixture was denatured for 10 min at 94°C and then quickly cooled in ice. All the PCR reactions were carried out in a PE–9600 thermocycler (Perkin Elmer Corp., Norwalk, Conn., USA). Three microliters of each sample were loaded on a 4.5% denaturing polyacrylamide gel, run in 1×Tris-borate EDTA electrophoresis buffer, and then the gel was dried for 2 h before subjecting it to autoradiography for 1–3 days at –70°C depending on the signal intensity. The whole mapping population was surveyed with the primer combinations showing putatively linked polymorphic AFLP fragments. These primers were E2: 5'–GAC TGC GTA CCA ATT C **AGT**–3', E3: GAC TGC GTA CCA ATT C **AAC**–3', E5: GAC TGC GRA CCA ATT C **ACA**–3', E10: GAC TGC GTA CCA ATT C **AGG**–3', M5: 5'–GAT GAG TCC TGA GTA A **TGC**–3', M12: 5'–GAT GAG TCC TGA GTA A **CAA**–3', and M16: 5'–GAT GAG TCC TGA GTA A **CTA**–3' (E and M stands for *Eco*RI and *Mse*I primers, respectively, and letters in bold indicate the selective nucleotides). The AFLP markers were named by using the name of the primer combination and the molecular weight of the polymorphic AFLP fragment (For example: E1/M1–500 indicates the 500-bp fragment amplified using the combination of *Eco*RI primer 1 and *Mse*l primer 1).

Isolation, cloning, and sequencing of AFLP fragments

In order to isolate the putatively linked AFLP fragments, the position of these fragments was determined on the dried polyacrylamide gel first by aligning all the corners of the dried gels on the autoradiogram with needles. Then the AFLP fragment of interest was excised and eluted in 100 µl of sterile water by heating at 100°C for 15 min. Five microliters of the elute were re-amplified using the same primer combinations and reaction conditions that had revealed the putative AFLP fragments linked to the *tgms–vn1* gene, and separated on 2% agarose. The fragments with the correct size were then excised from the agarose gel and purified using a Qiagen gel extraction kit (Qiagen, Hilder, Germany) and cloned into pGEM–T Easy Vector Systems (Promega, Madison). Each selected clone with the expected insert size was checked by Southern hybridization to confirm that the clones corresponded to the fragments of interest.

To develop sequence characterized amplified region (SCAR) markers (Paran and Michelmore 1993) from the cloned AFLP marker closely linked to the *tgms* gene, the DNA sequence was determined by the dideoxy chain-termination method using the sequenase cycle sequencing kit (Amersham Life Science Inc., Illinois).

RFLP analysis

The AFLP fragments were labeled by using the redi-prime labeling system (Amersham) and hybridized with AFLP blots of F_2 individuals and parents to confirm their linkage with the *tgms–vn1* gene. The AFLP blots were prepared from selective amplification products using the same primer combination and reaction conditions that had revealed AFLP markers putatively linked to the *tgms–vn1* gene. All AFLP fragments linked to the *tgms–vn1* gene were also hybridized to genomic DNA blots of parents after digesting with the 17 restriction enzymes *Eco*RI, *Eco*RV, *Hind*III, *Dra*I, *Xba*I, *Pst*I, *Apa*I, *Kpn*I, *Xho*I, *Bam*H1, *Bgl*I, *Bgl*lI, *Pvu*I, *Sma*I, *Sac*I, *Sca*I, and *Mlu*I) following standard procedures (Sambrook et al. 1989). Cosegregation in the F_2 population was confirmed with enzymes that showed polymorphism between the two parents.

Populations used for mapping AFLPs

A doubled-haploid (DH) population consisting of 135 lines developed at IRRI from the cross IR64/Azucena was used as a mapping population. A RFLP framework map has been developed for this population (Huang et al. 1994). Parental survey blots were made, digesting with the same 17 restriction enzymes described above for probing with the AFLP markers linked to the *tgms–vn1* gene. AFLP markers showing polymorphism between parents were used to survey the available 70 lines of the DH population. In the same way, another DH mapping population from the cross CT9993/IR62266 (Zheng et al. 1998) was also used for mapping the AFLPs.

Linkage analysis

After the AFLP markers were mapped on the chromosome, a number of RFLP markers and microsatellite markers from this region were selected for a parental polymorphism survey. SSR analysis was performed for the five microsatellite loci RM8, RM27, RM29, RM53 and RM262 using 50 ng of genomic DNA (Chen et al. 1997). Subsequently, the whole F_2 population was surveyed for microsatellite and RFLP markers showing polymorphism between parents. The phenotype, RFLP, AFLP, and SSR data were combined for linkage analysis using the MAPMAKER program (Lander et al. 1987) and a partial linkage map of the region of the chromosomes surrounding the *tgms–vn1* gene was constructed using the Kosambi mapping function (Kosambi 1944).

Results

Characterization of the *tgms-vn1* gene and its inheritance pattern

To determine the critical temperature for expression of this trait, the TGMS-VN1 mutant line was subjected to different temperature regimes, ranging from 20°C to 28°C. The results of this experiment are given in Table 1. This clearly shows that the critical temperature for TGMS-VN1 sterility is 24–25°C. The inheritance pattern of this gene was studied in three different cross combinations at two different temperature regimes, namely 22–24°C and 26–28°C (Table 2). All the F_1 plants expressed fertility in both the temperature regimes. At a temperature range of 22–24°C, all the F_2 plants were fertile (Table 2a), whereas clear segregation of fertile and sterile types were observed at the 26–28°C treatment (Table 2b). The segregation of fertile to sterile plants in the F_2 generation in all three cross combinations followed a 3:1 ratio, indicating the monogenic recessive nature of this gene. The results of the phenotypic analysis

of the F_2 individuals, the parents and F_1 of the mapping population are given in Table 3. The difference for pollen and spikelet fertility between the parents was clear. The F_1 plants were fertile like CH1, the fertile parent. In the whole mapping population, we found only four plants with an intermediate level (around 30–40%) of pollen and spikelet fertility. Repetition of the phenotypic evaluation helped us to confirm the TGMS and fertile plants accurately. The observation on pollen and spikelet fertility, taken at different times from ratooned plants, was highly consistent. The segregation of fertile to sterile plants in the F_2 generation followed a 3:1 ratio. This further confirmed the observation of Dong et al. (1995a, b) that *tgms–vn1* is a recessive gene.

Identification of AFLP markers linked to the *tgms–vn1* gene

Bulked segregant analysis using 200 AFLP primer combinations revealed four markers (E2/M5–200, E3/M16–400, E5/M12–600, and E10/M12–200) to be polymorphic between both the bulks as well as the parents. All four polymorphic markers were highly reproducible and were amplified from the fertile parent and fertile bulk. The AFLP amplification pattern showing cosegregation with the fertility allele in the E5/M12 primer combination is given in Fig. 1. To facilitate later analysis, all four AFLP markers linked to the *tgms–vn1* gene were cloned. The identities of the cloned AFLPs were verified by Southern hybridization of the cloned fragments to the selective amplification product of individuals that segregated for that particular AFLP band (Fig. 2a). This hybridization verification confirmed that the fragment cloned was correct and was linked to the *tgms–vn1* gene.

Table 1 Mean pollen and spikelet fertility of the TGMS-VN1 mutant line at different temperatures

Temperature range	Pollen fertility (%)	Spikelet fertility (%)
20–22°C	68.4±2.72	65.7±2.01
22–24°C	72.6±3.08	77.2±2.90
24–26°C	0.25±0.02	0
26–28°C	0	0

Chromosomal location of the *tgms–vn1* gene

Southern hybridization indicated that E3/M16–400, E5/M12–600, and E10/M12–200 were low-copy sequences and that E2/M5–200 contained repetitive sequences. Only E5/M12–600 showed dominant polymorphism between parents with the restriction enzyme *Apa*l (Fig. 2b). The distance between the *tgms–vn1* gene and E5/M12–600 was estimated to be 3.3 cM based on the segregation data of this F_2 population (Fig. 3b). The linkage distance of the other three linked AFLP markers was determined based on AFLP analysis (Fig. 3b). These markers were found to be linked to the *tgms–vn1* gene at more than 20 cM.

To determine the chromosome location of the *tgms–vn1* gene, RFLP analysis was conducted using the

Table 2 Pollen and spikelet fertility of the F_2 population of three different cross combinations, evaluated at two different temperature regimes

Cross	Number of F_2 plants with pollen and/or spikelet fertility			
	<5% (Sterile)	>50% (Fertile)	Total	
(a) 22–24°C Temperature regime				
TGMS-VN1×Line 11	0	100	100	
TGMS-VN1×Line 21	0	100	100	
TGMS-VN1×Line 24	0	100	100	
Cross[a]	Number of F_2 plants with pollen and/or spikelet fertiliy			
	<5% (Sterile)	>5% (Fertile)	χ^2 (3:1)	*P*-value
(b) 26–28°C Temperature regime				
TGMS-VN1×Line 11	112	310	0.53	0.2–0.5
TGMS-VN1×Line 21	107	350	0.60	0.2–0.5
TGMS-VN1×Line 24	102	294	0.12	0.5–0.8

[a] Line 11, Line 21 and Line 24 are fertile doubled-haploid lines developed from native Vietnamese cultivars Chiem Bau, CR203 and H84-1 respectively

Table 3 Pollen and spikelet fertility of the parents (P_1 and P_2), F_1 and F_2 generations of the cross (TGMS-VN1×CH1), used for mapping the *tgms-vn1* gene

Generation	No. plants with pollen and/or spikelet fertility			Observed segregation (fertile:sterile)	χ^2 (3:1)
	<5% (Sterile)	>50% (Fertile)	Total		
P_1 (CH1)	–	15	15	15:0	–
P_2 (TGMS-VN1)	15	–	15	0:15	–
F_1	–	15	15	15:0	–
F_2	31	83	114	83:31	0.292

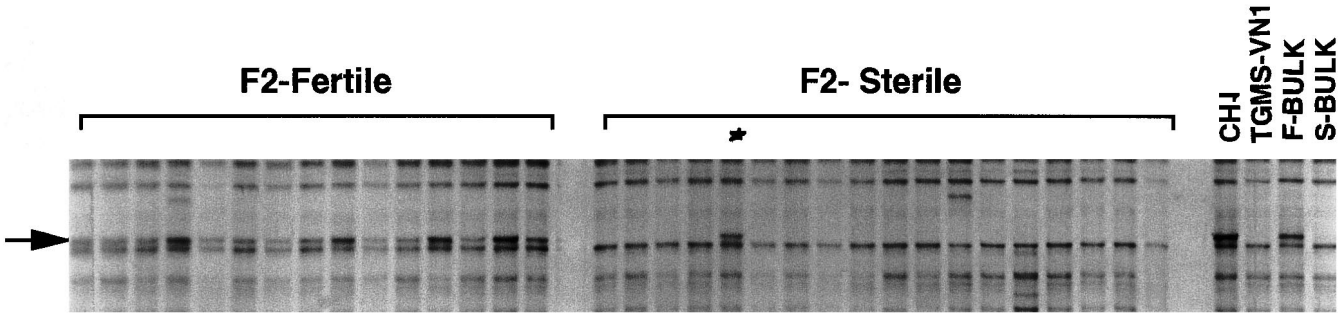

Fig. 1 An autoradiogram showing an AFLP marker linked to the *tgms–vn1* gene derived from the selective amplification of parents, fertile bulk, sterile bulk, and the individuals constituting the bulks, by primer combination E5/M12. The *arrow* indicates the polymorphic fragment E5/M12–600. An *asterisk* marks a putatively recombinant individual. The AFLP marker is amplified in fertile parents, fertile bulks, and fertile individuals of the F_2 population, indicating linkage. CH1 and TGMS–VN1 are the fertile and sterile parents, respectively

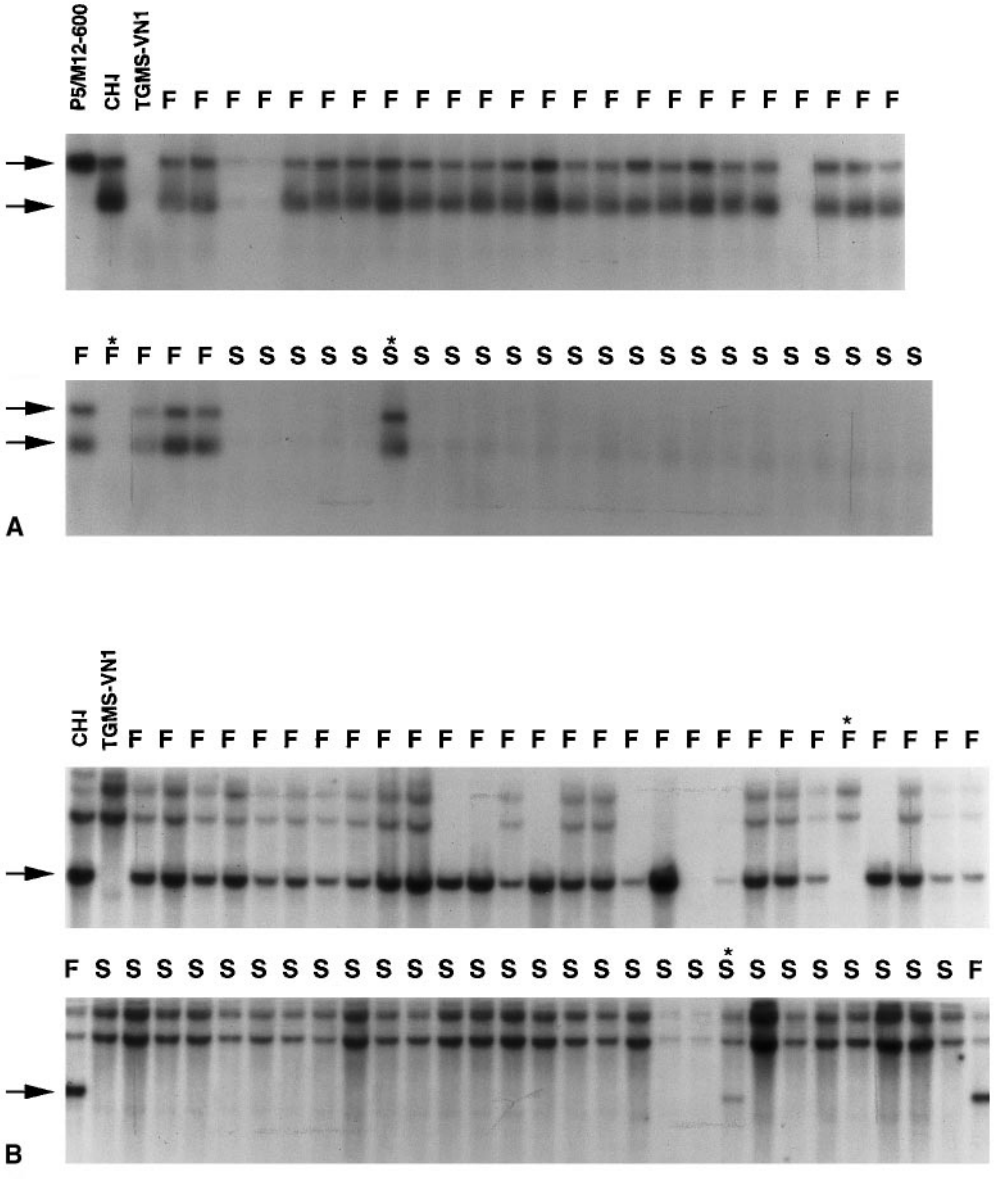

Fig. 2 **A** An autoradiogram showing hybridization patterns of E5/M12–600 with DNA fragments selectively amplified by E5/M12 primer combinations from the parents and F_2 fertile and sterile individuals. E5/M12–600 hybridizes to two selectively amplified fragments of fertile parents CH1 and F_2 fertile individuals, but not with sterile parents and the F_2 sterile individuals. In the case of *lane numbers 6, 7, and 24*, the faint signal is due to poor selective amplification. **B** Confirmation of linkage of E5/M12–600 to the *tgms–vn1* gene by Southern analysis of parents and F_2 progeny (both fertile and sterile) digested with *Apa*l. Dominant polymorphism is visible with the appearance of a hybridizing fragment in the fertile parent CH1 and the F_2 fertile individual but not in the sterile individual and the sterile parent TGMS–VN1. In both **A** and **B**, *F* and *S* refer to fertile and sterile F_2 individuals and *asterisks* mark the putatively recombinant individuals

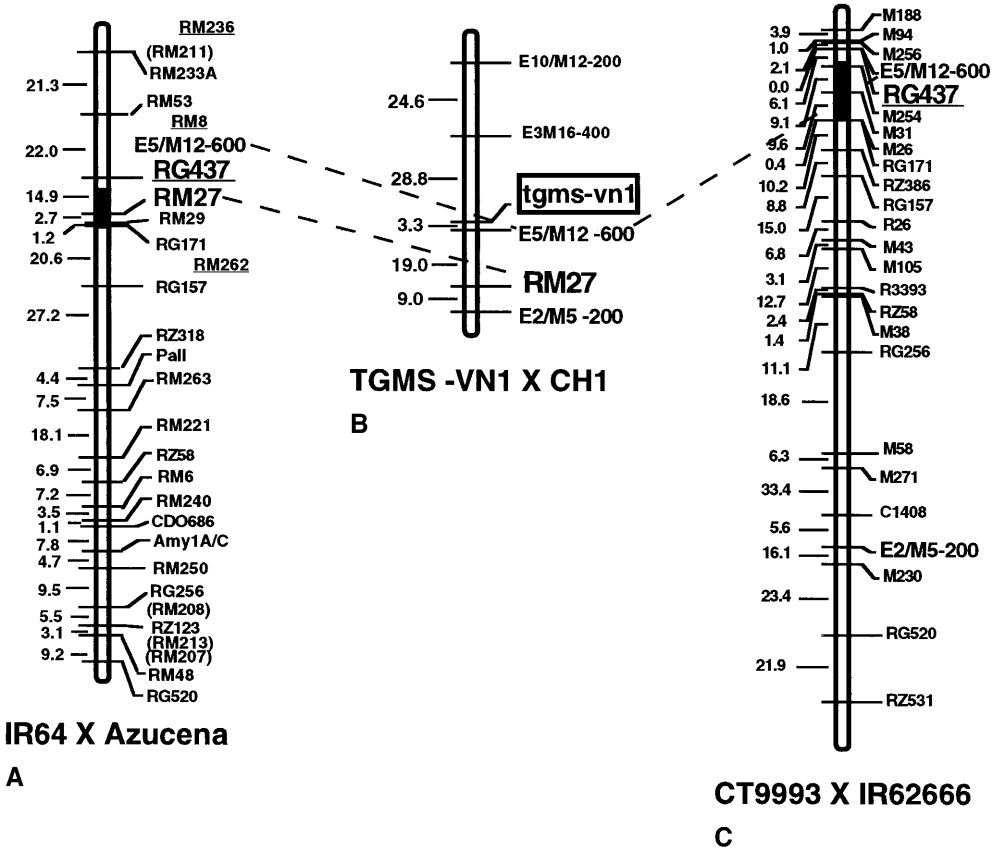

Fig. 3 **A** A linkage map of chromosome 2, showing the location of E5/M12–600 near RG437, integrated with microsatellite markers (Chen et al. 1997). The RFLP framework map was constructed from a DH population developed at IRRI, Philippines, from the cross IR64×Azucena (Huang et al. 1994). **B** A partial linkage map of the region surrounding the *tgms–vn1* gene on chromosome 2 derived from the cross TGMS–VN1×CH1. Out of the five surrounding markers, four are AFLP markers, except for RM27, a microsatellite marker. **C** Linkage map of chromosome 2 constructed from another DH population developed from the cross CT9993×IR62666, where E5/M12–600 cosegregated perfectly with the RFLP marker RG437. A *solid bar* in **A** and **C** indicates the approximate centromere location (Singh et al. 1996). *Dotted lines* indicate the alignment of the AFLP marker E5/M12–600, linked to *tgms-vn1*, with RG437 and RM27 in the linkage map of the above described three different populations

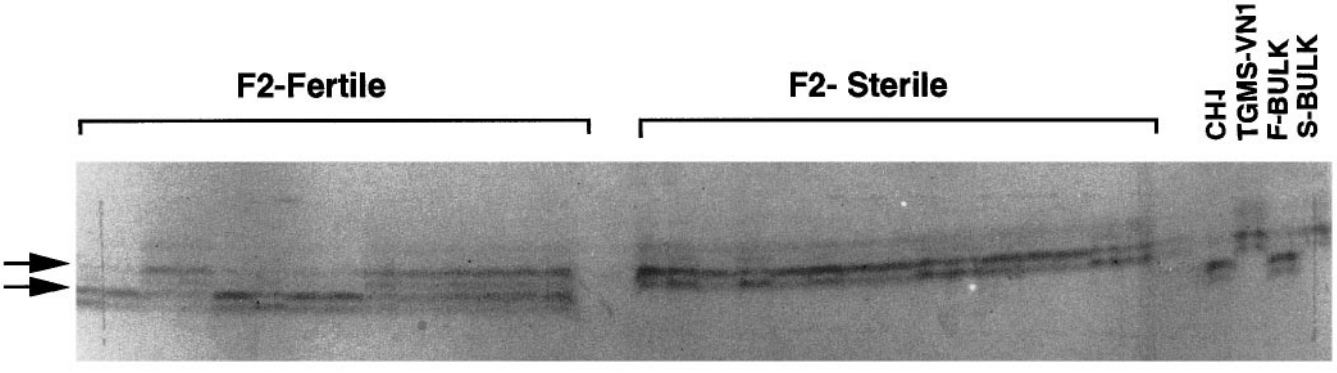

Fig. 4 Linkage between the microsatellite marker RM27 and the *tgms–vn1* gene confirmed its location on chromosome 2. Codominant polymorphism is visible between parents, fertile, and sterile bulks. The fertile F_2 individuals consist of both homozygous and heterozygous fertile individuals

three cloned polymorphic low-copy AFLP fragments. Only E5/M12–600 detected polymorphism between IR64 and Azucena with the restriction enzymes *Pst*I and *Eco*RI. When the doubled haploid lines of this mapping population were surveyed, Mendelian segregation was observed. The E5/M12–600 fragment was mapped next to RFLP marker RG437 on chromosome 2 at a distance of 9.1 cM (Fig. 3a). In the CT9993×IR62266DH mapping population, for which an AFLP/RFLP map has been constructed (Zheng et al. 1998), the same marker showed polymorphism with *Xba*I and *Pst*I and cosegregated perfectly with RG437 in RFLP analysis (Fig. 3c). In both populations, no polymorphism was detected for E3/M16–400 or E10/M12–200, even with the use of 17 restriction enzymes. The marker E2/E5–200, which contained repetitive sequences, was also mapped onto chromosome 2 along with E5/M12–600 using AFLP segregation data in CT9993×IR62666 population (Fig. 3c).

To further confirm the map location of the *tgms–vn1* gene and to identify more-closely linked markers for marker-assisted selection purposes, six RFLP markers (RG437, RZ643, RG157, RZ599, RG171 and RG83; Causse et al. 1994) and five microsatellite markers (RM8, RM27, RM29, RM53 and RM262; Chen et al. 1997) were selected from the region surrounding the *tgms* gene on chromosome 2. However, none of the RFLP markers

showed polymorphism after using even 17 restriction enzymes. Only one microsatellite marker, RM27, showed linkage with the *tgms–vn1* gene (Figs. 3b, 4). The distance between E5/M12–600 and RM27 was comparable between the partial linkage map constructed from TGMS–VN1×CH1 and the microsatellite integrated linkage map of IR64×Azucena respectively (Fig. 3a, b). All the evidence described above led to the conclusion that the *tgms–vn1* gene is located on chromosome 2.

Sequencing of AFLP marker E5/M12–600

The AFLP marker E5/M12–600, which is closely linked to the *tgms–vn1* gene, was sequenced. The sequence data revealed the presence of a selective amplification primer sequence, thereby confirming the correctness of the fragment. The DNA sequence of this marker has been deposited in GenBank under Accession No. AF070954.

Discussion

The objective of developing hybrids in rice is not only to increase yield but also to improve several other desirable traits, such as resistance to key biotic and abiotic stresses and grain quality traits that make them stable in unfavorable environments and acceptable by farmers. Genetic improvement of these traits is a function of the parental lines involved in the hybrid combination. In order to achieve this, TGMS lines must be developed in different genetic backgrounds that will be suitable for use in commercial two-line hybrids. Since the identification of TGMS individuals in segregating populations under field conditions is very cumbersome, the identification of closely linked molecular markers will greatly facilitate the transfer of the TGMS allele to desirable genetic backgrounds via marker-assisted selection. This will also help in the selection of TGMS segregants with greater accuracy in early generations. Therefore, the identification of new sources of TGMS genes and molecular mapping of these genes will have a significant impact on hybrid rice breeding.

With advances in molecular marker technology and the development of well-saturated molecular maps, mapping genes of agronomic importance and subsequently practicing marker-assisted selection (MAS) for these genes has become a reality. In this report, we identified four AFLP markers linked to the *tgms–vn1* gene using bulk segregant analysis. One marker, E5/M12–600, was found to be tightly linked, with a genetic distance of 3.3 cM. Since this marker also detected RFLPs, it can be used for marker-assisted selection. However, since this marker is linked to the fertility allele and is dominant in nature, it will also help in the indirect selection of TGMS individuals in a segregating population by eliminating fertile individuals, which constitute both the homozygous and heterozygous individuals. We are now developing a larger population from distantly related varieties to fine map the *tgms–vn1* gene with more-closely linked markers, which will be helpful for increasing selection accuracy in MAS and ultimately for isolation of this gene by map-based gene cloning. Since PCR markers are robust, practical, cost effective and efficient, we also determined the DNA sequence of marker E5/M12–600, closely linked to the gene, so that this PCR marker can be developed for marker-assisted selection.

Even though the TGMS system is simple and efficient for producing hybrids in rice, very little is known regarding the molecular basis of the TGMS trait, which limits its wide application in hybrid rice breeding. Pollen fertility seems to be a complex trait that is the end product of complex pathways or processes controlled by numerous loci, and mutation in any of these loci may have an influence on pollen fertility. Most of the TGMS lines were developed by mutagenesis, and all the genes were reported to be monogenic recessive (Maruyama et al. 1991; Wang et al. 1995; Borkakati and Virmani 1996). The fact that all three TGMS genes mapped so far, including the one in this report, are located on different chromosomes substantiates the complexity involved in the manifestation of this phenotype. It may be speculated that the mapped TGMS loci might be mutations on those loci controlling the component steps of the complex biochemical pathways leading to pollen sterility. Cloning, isolation, and characterization of those genes in the future will improve our understanding regarding the molecular basis of this trait.

In this investigation, we bulked the pre-amplified DNA of selected fertile and sterile individuals constituting the bulks instead of bulking the DNA first, as is usually done by many workers, and then conducted the AFLP analysis. This eliminated the problems associated with poor-quality DNA, partial digestion, which subsequently makes it difficult to identify the putatively linked fragments in bulked segregant analysis. In order to identify the desirable cloned AFLP fragment by Southern hybridization, we used blots of AFLP products of the progeny and parents. This step quickly and precisely identified the correct clone by revealing the linkage between the gene of interest and the putative AFLP marker.

The results reported in this paper clearly demonstrate the power of bulked segregant analysis coupled with AFLP technology, which allowed us to identify the locus governing thermosensitive genic male sterility and to determine the chromosomal location of this locus on the molecular linkage map of rice. The chromosomal location of AFLP fragment E5/M12–600, closely linked to the *tgms–vn1* gene next to RG437, in two different DH mapping populations provides unambiguous evidence that the *tgms–vn1* gene is located on the short arm of chromosome 2 (Singh et al. 1996). This conclusion is further strengthened by our observation of linkage of a microsatellite marker, RM27, located on the same chromosome. Failure to obtain any RFLP markers linked to this gene can be attributed not only to the *indica* genetic background of both parents, but also the location of this gene proximal to the centromere (Singh et al. 1996). Pre-

viously, two TGMS genes, *tms1* from the 5460 S TGMS line of China and *tms3(t)* of IR32364TGMS from the International Rice Research Institute in the Philippines, were mapped on chromosomes 8 and 6, respectively (Wang et al. 1995; Subudhi et al. 1997). Therefore, the *tgms–vn1* gene is nonallelic to the above two TGMS genes. However, it will be interesting to determine the allelic relationship of this gene with *tms2* of NPL12 (Maruyama et al. 1991). Through the present investigation, we determined the chromosomal location of a new TGMS gene, which we propose to designate as *tms4(t)*. The addition of this new gene to the array of TGMS genes will help broaden the genetic base of two-line hybrids, if the TGMS alleles are transferred to a desirable genetic background via marker-assisted selection.

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