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ORIGINAL PAPER

Fine mapping of a gene for non-pollen type thermosensitive genic male sterility in rice (*Oryza sativa* L.)

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Abstract The thermo-sensitive genic male sterility (TGMS) lines play a crucial role in two-line hybrid rice production. For a practical TGMS line, the stability of male sterility is one of the most important technical indicators. In this study, XianS, a spontaneous mutant with stable male sterility from an *indica* rice cultivar Xianhuangzhan, was classified as a non-pollen type TGMS line. The critical non-pollen sterility point temperature of XianS was determined as 27°C. Genetic analysis demonstrated that the non-pollen sterility in XianS was controlled by a single recessive gene. Using SSR markers and bulked segregant analysis, the TGMS gene in XianS was fine mapped to a 183 kb interval between RMAN81 and RMX21 on chromosome 2. Two markers, 4039-1 and RMX14 completely cosegregated with this gene. Allelism test indicated that the non-pollen phenotype in seven non-pollen type TGMS lines from different sources, XianS, AnnongS-1, Q523S, Q524S, N28S, G421S, and Q527S is caused by the same TGMS gene. Although the location of TGMS gene in XianS is close to the gene *OsNAC6*, a previously identified candidate gene of *tms5* in AnnongS-1, the sequence of *OsNAC6* and its promoter region was identical in TGMS line XianS, AnnongS-1, and wild-type Xianhuangzhan. These results suggest that the non-pollen type TGMS trait probably be controlled by the same TGMS gene in different TGMS rice lines, but its real candidate gene still need to be further studied and identified.

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Introduction

Rice (*Oryza sativa* L.) is one of the most important crops. The development of hybrid rice has contributed tremendously to food security in China. The three-line breeding system consisting of one cytoplasmic genic male sterility (CMS) line, one maintainer line, and one restorer line has proved to be an effective method for the production of hybrid seeds. Although effective, the system is costly, cumbersome and limited in germplasm of maintainer and restorers. The discovery of photoperiod-sensitive genic male sterility (PGMS) and thermo-sensitive genic male sterility (TGMS) led to the development of a simple and highly efficient two-line breeding system in hybrid seed production. However, the male sterility of all developed PGMS and TGMS lines is influenced by temperature, thus resulting in the potential risk of seed production failure due to the variation of male fertility with temperature. Currently, the widely used PGMS and TGMS lines are male sterile under long day-length and/or high temperature, but male fertile under short day-length and/or low temperature. In order to reduce the risk of fertility instability caused by sudden drop in temperature, Yuan (1992) proposed that the practical PGMS and TGMS lines must possess low critical sterility point temperature (CSPT) and still express male

sterility below CSPT for a long time (i.e., ≤24°C in South China area or ≤23°C in Yangtze River Basin and its north area for 3 days or more).

The pollen abortion of male sterility in rice can be classified into five types: non-pollen, typical abortion, spherical abortion, stainable abortion, and nuclear proliferous type (Li 2000). Currently, most PGMS and TGMS lines express male sterility with typical pollen abortion, namely mature pollen grains are shriveled and do not accumulate starch. It is well known that the male sterility of non-pollen abortion is more thorough than that of typical abortion. Previous cytological and cytochemical studies indicated that anther abnormal changes appeared earlier in non-pollen abortion than typical abortion during pollen development in male sterile lines (Wang and Tong 1992; Li et al. 1993; Sun and Zhu 1995; Feng et al. 2000; Ku et al. 2003; Chen et al. 2005; Xia et al. 2005; Peng et al. 2006; Peng et al. 2009). This suggests that non-pollen abortion is more difficult to reverse from sterile to fertile phase than typical abortion. Under the same CSPT, the male sterility of non-pollen abortion is more stable than that of typical abortion, thus the PGMS and TGMS lines of non-pollen abortion could be safer than that of typical abortion in two-line hybrid seed production. Recently, some non-pollen type PGMS and TGMS lines were discovered and reported, such as 95850 ms (Ku et al. 2003), Zao25S (Guo et al. 2004), Meixiang851S (Guo et al. 2004), XianS (Peng et al. 2006), Lu18S (Yang et al. 2007a), Zhu1S (Yang et al. 2007a), HD9802S (Zhou et al. 2008), and N28S (Peng et al. 2009). XianS is a spontaneous mutant TGMS line from a conventional *indica* variety Xianhuangzhan in China. Our previous studies showed that the CSPT of XianS was about 23.5°C and XianS still remained male sterility at low temperature of daily mean temperature 21°C (25/19°C, day-time maximum/night-time minimum) for 7 days at fertility sensitive stage during panicle differentiation (Peng et al. 2006). Therefore, the characteristics of the TGMS in XianS can be used to reduce the potential risk of fertility fluctuation caused by exposure to unpredicted low temperature below CSPT for a short time in hybrid seed production.

To date, three PGMS genes from 32001S and Nongken58S, *pms1*, *pms2* and *pms3*, have been mapped to chromosomes 7, 3 and 12, respectively (Zhang et al. 1994; Mei et al. 1999). Two reverse PGMS genes, *rpms1* and *rpms2*, have been mapped to chromosomes 8 and 9, respectively (Peng et al. 2008). Seven TGMS genes, *tms1* from 5460S (Wang et al. 1995), *tms2* from PL-12 (Yamaguchi et al. 1997), *tms3* from IR32364 (Subudhi et al. 1997), *tms4* from TGMS-VN1 (Dong et al. 2000), *tms5* from AnnongS-1 (Wang et al. 2003), *tms6*(t) from 0A15-1 (Wang et al. 2004), and *TGMS* from SA2 (Reddy et al. 2000) have been mapped to the chromosomes 8, 7, 6, 2, 2, 3 and 9, respectively. One reverse TGMS gene, *rtms1* from J207S, has been mapped to chromosome 10 (Jia et al. 2001). The TGMS gene *tms6* in Sokcho-MS, which is not identical to either the TGMS gene or the reverse TGMS gene, has been mapped to chromosome 5 (Lee et al. 2005). Among these genes, *pms1* (Liu et al. 2001), *pms3* (Lu et al. 2005), *tms5* (Jiang et al. 2006; Yang et al. 2007b), *tms2* (Pitnjam et al. 2008), *rpms1* and *rpms2* (Peng et al. 2008) have been physically mapped to different chromosomal regions. Notably, for *tms5* from AnnongS-1, different physical locations were reported by Jiang et al. (2006) and Yang et al. (2007b). The distance between two reported locations is approximately 207 kb.

In this study, we aimed to map the TGMS gene in XianS and to analyze the allelic relationship of the TGMS genes in XianS and other non-pollen type TGMS lines from different sources. These results would be useful to reveal the molecular mechanism of non-pollen TGMS and develop practical TGMS lines with stable male sterility for hybrid seed production in rice.

Materials and methods

Plant materials

XianS, a new non-pollen type TGMS line, is a spontaneous mutant of the *indica* rice cultivar Xianhuangzhan in China. Male fertile parents used for genetic analysis in this study include three conventional *indica* rice cultivars (Xianhuangzhan, Jinhuazhan and B4) and three *indica*-compatible *japonica* lines (IC2-1-1-2-1-1, IC33-11-23-1-1-1 and IC38-200-1-1). Based on a relatively high level of polymorphism between the parents, two F2 populations "XianS × IC38-200-1-1" and "XianS × IC33-11-23-1-1-1" were selected for molecular mapping of the TGMS gene in XianS. In addition, seven non-pollen type TGMS lines from different sources, XianS, AnnongS-1, Q523S, Q524S, N28S, G421S, and Q527S (Table 1) were used for allelic test.

Phenotypic characterization of XianS

In order to determine the non-pollen CSPT in XianS, rice plants at panicle differentiation stage were grown in four phytotrons, in which daily mean temperatures were set at 25°C (26°C day and 24°C night), 26°C (27°C day and 25°C night), 27°C (28°C day and 26°C night), and 28°C (29°C day and 27°C night) under 12 h day-length, respectively. Six plants were sampled from each treatment to examine the type of pollen abortion. The plants of XianS, Xianhuangzhan, and F_1 of the cross XianS × Xianhuangzhan were photographed using a Sony digital camera to determine the phenotypic changes of XianS. Meanwhile, the mature spikelets of XianS and Xianhuangzhan were photographed with

Table 1 Origin of non-pollen type TGMS lines used to allelism test

TGMS line	Origin	Variability type
AnnongS-1	Natural mutation of AnnongN (Chao40/H285//6209-3)	Spontaneous mutation
XianS	Natural mutation of Xianhuangzhan	Spontaneous mutation
Q523S	Selective breeding from the selfs of *indica*-compatible *japonica* line J3	Spontaneous mutation
Q524S	Selective breeding from the progeny of Texianzhan25 induced by space radiation	Radiation-induced mutation
N28S	Selective breeding from the progeny of free cross-pollination of N18S (Mian6F_4/NR92//NR92///NR92)	Segregation of hybrid progeny
G421S	Selective breeding from filial generation of the male sterile plant from *indica* variety and *japonica* variety (Copslo17/Haogelao//Xin299///DS500) and N28S	Segregation of hybrid progeny
Q527S	Selective breeding from the progeny of free cross-pollination of YiD2S (B3/Hongjing)	Segregation of hybrid progeny

a Leica S8APO dissecting microscope. The intact anthers of XianS and Xianhuangzhan were squashed in 1% I_2-KI solution and photographed with a Leica DMLB light microscope.

Genetic analysis of TGMS in XianS

XianS and six male parents mentioned above were used to study the inheritance of TGMS in XianS. The parents, F_1s, and F_2 populations were evaluated for pollen fertility in July of 2003–2007 at Guangzhou, China. About 15 spikelets were sampled from three panicles per plant during anthesis and their anthers were squashed in 1% I_2-KI solution. Under a light microscope (Nikon YS2-H), all round and darkly stained pollens were scored as fertile, whereas unstained shriveled pollens, spherical pollens, light brown colored pollens, or no pollen were all scored as sterile. The average pollen fertility from three panicles was expressed as percentage. Plants with less than 5% stained pollens were considered as sterile, whereas all others were regarded as fertile. χ^2 test was applied to test the goodness of fit of F_2 segregation ratio.

Molecular mapping of the TGMS gene in XianS

The TGMS gene in XianS was mapped with bulked segregant analysis and recessive class approach. The polymorphism between the parents was first detected with SSR markers covering 12 rice chromosomes. Then, the polymorphic markers were further used to detect the polymorphism between the sterile and fertile DNA bulks to identify possible TGMS genes-containing chromosome regions. The two DNA bulks were constructed by selecting the extremely sterile and fertile individuals from each F_2 mapping population. The map location of the TGMS gene in XianS was finally determined by using the male sterile plants of the mapping population and the polymorphic markers between two DNA bulks. The recombination frequency (c) was calculated with the formula: $c = (N1 + N2/2)/N$, where N is the total numbers of sterile plants surveyed, $N1$ is the number of individuals with homozygous band from the fertile parent, and $N2$ is the number of individuals with heterozygous band from the two parents (Zhang et al. 1994). Then the recombination frequency was converted into genetic distance (centiMorgan, cM) using Kosambi function (Kosambi 1944). Linkage maps were obtained using software MapChart (Voorrips 2002).

SSR analysis

A total of 172 SSR markers were used to amplify DNA fragment according to PCR procedure previously described by Panaud et al. (1996). Among these SSR markers, RMX and PSM markers were designed by using sequence information of the TGMS gene region in rice genome (http://www.ncbi.nlm.nih.gov), simple sequence repeat identification tool (SSRIT) (http://www.gramene.org), and Primer Premier 5.0 software (Lalitha 2000). RMAN, 4039-1, and 4039-2 markers were used as described by Jiang et al. (2006) and Yang et al. (2007b). The amplified products were analyzed on the 6% PAGE gel and detected by silver staining.

Sequence analysis

Gene *OsNAC6* was previously identified as the candidate gene of *tms5* in AnnongS-1 (Yang et al. 2007b). Based on the BAC clone AP004039 sequence (http://www.ncbi.nlm.nih.gov), five specific PCR primers were designed to sequence *OsNAC6* and its promoter region (Nakasgima et al. 2007) in TGMS lines XianS, AnnongS-1, and wild-type line Xianhuangzhan. PCR-amplified products were purified and sequenced by Shanghai Invitrogen Biotechnology Co., China.

Allelism test

Seven non-pollen type TGMS lines from different sources, XianS, AnnongS-1, Q523S, Q524S, N28S, G421S, and Q527S were used to test allelism of TGMS genes (Table 1). Among these TGMS lines, AnnongS-1 was provided by Rice Research Institute of Guangdong Academy of Agricultural Sciences, other six TGMS lines were developed by our team. Plants of each TGMS line were divided into two groups. One group as male parents was cultured in phytotrons with the daily mean temperature at 21°C (25/19°C, day-time maximum/night-time minimum) and 11.5 h day-length to obtain male fertility. Another group as female parents was grown under natural high temperature condition in Guangzhou, China to obtain male sterility. Crosses were made between two different TGMS lines when the TGMS lines as female parent were completely sterile. Pollen fertility of F_1 was examined as described above.

Results

XianS is a non-pollen type TGMS line

XianS is a spontaneous mutant TGMS line from a conventional *indica* variety Xianhuangzhan (Fig. 1a), which is male sterile under high temperature (>23.5°C) but male fertile under low temperature (<23.5°C) (Peng et al. 2006). Pollen abortion type observations at 25, 26, 27 and 28°C (daily mean temperature) showed that XianS belonged to non-pollen type TGMS line, and its non-pollen CSPT was 27°C. When the temperature was higher than 27°C, which is suitable for rice to grow normally, anthers in the wild-type Xianhuangzhan were big and yellow (Fig. 1b), while those in the mutant XianS were small and white (Fig. 1c). In addition, mature pollens were normal in Xianhuangzhan anther (Fig. 1d), while no pollen was found in XianS anther (Fig. 1e).

Location of the TGMS gene in XianS

Six different cross combinations were used to investigate the inheritance pattern of the TGMS gene in XianS (Table 2). When XianS was completely sterile, the mean pollen fertility in F_1s ranged from 63.67 to 92.00% and the segregation ratio of sterile to fertile progenies in all F_2 populations fitted well to 1:3, which indicated the monogenic recessive nature of this gene. Considering that XianS was a new TGMS line, we tentatively designated the TGMS gene in XianS as *tmsX*.

To select the mapping populations for *tmsX* gene in XianS, the polymorphism between XianS and five male parents were analyzed using 136 SSR markers covering 12 rice chromosomes. The results showed that polymorphism percentage of XianS and Jinhuazhan, B4, IC2-1-1-2-1-1, IC33-11-23-1-1-1, and IC38-200-1-1 was 19.05, 30.61, 41.18, 61.03, and 60.29%, respectively. Thus, two F_2 populations from the crosses of XianS and *indica*-compatible *japonica* lines IC38-200-1-1 and IC33-11-23-1-1-1 were used for molecular mapping of the *tmsX* gene in XianS.

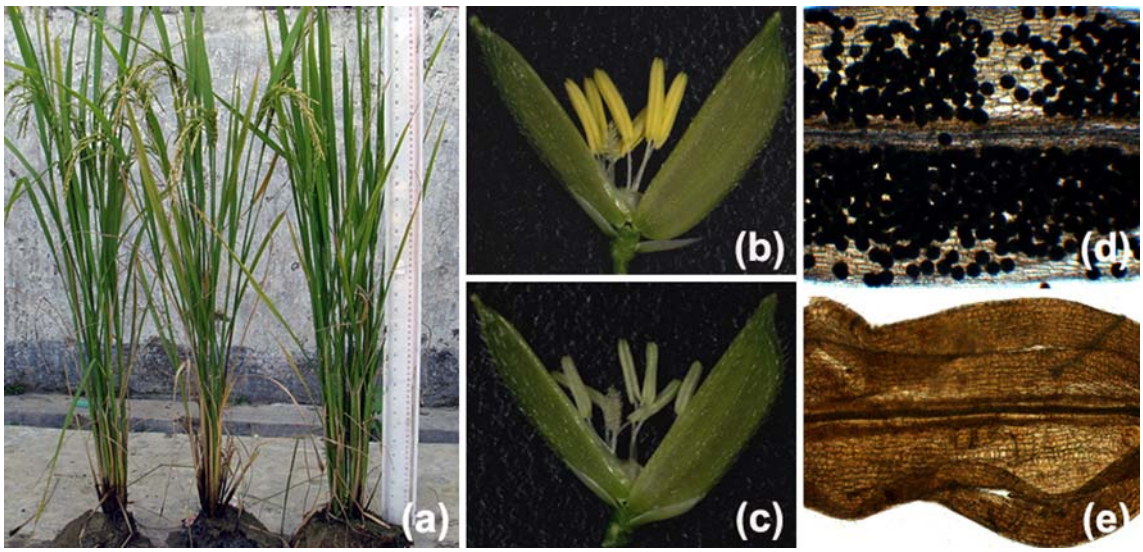

Fig. 1 Comparison of wild-type Xianhuangzhan and non-pollen type TGMS mutant line XianS. **a** Comparison of a wild-type Xianhuangzhan plant (*left*), a F_1 plant of the cross XianS × Xianhuangzhan (*middle*) and a non-pollen TGMS mutant XianS plant (*right*); **b** a spikelet of wild-type Xianhuangzhan with big and yellow anthers; **c** a spikelet of non-pollen type TGMS mutant XianS with small and white anthers; **d** a mature anther of wild-type Xianhuangzhan was squashed with 1% I_2-KI solution, in which pollens were round and darkly stained; **e** a mature anther of TGMS mutant XianS was squashed with 1% I_2-KI, in which no pollen was found

Table 2 Pollen fertility of the F_1 and F_2 populations from the cross of XianS and six different male parents

Cross combination	F_1	F_2			Observed segregation ratio (sterile:fertile)	χ^2 (1:3)
	Pollen fertility (%)	Number of sterile plant	Number of fertile plant	Number of total plant		
XianS × Xianhuangzhan	86.67 ± 7.64	113	342	455	1:3.03	0.0007
XianS × Jinhuazhan	63.67 ± 6.07	52	169	221	1:3.25	0.1825
XianS × B4	85.50 ± 0.91	84	254	338	1:3.02	0.0000
XianS × IC2-1-1-2-1-1	91.33 ± 1.15	68	212	280	1:3.12	0.0429
XianS × IC33-11-23-1-1-1	92.00 ± 5.29	63	194	257	1:3.08	0.0117
XianS × IC38-200-1-1	81.67 ± 2.89	85	280	365	1:3.29	0.4831

Subsequently, the polymorphic markers between the parents of two mapping populations were further used to screen polymorphism between the sterile and fertile DNA bulks. Three markers RM555, PSM116, and RM324 on chromosome 2 were polymorphic between both bulks of two mapping populations. The linkage of three polymorphic markers to the *tmsX* gene were further confirmed by using 81 complete sterile F_2 plants from the cross "XianS × IC38-200-1-1" and 58 complete sterile F_2 plants from the cross "XianS × IC33-11-23-1-1-1-1". As a result, the *tmsX* gene was mapped to the region between PSM116 and RM324 on chromosome 2. To further confirm the map location of the *tmsX* gene in XianS, 22 SSR markers from the region between PSM116 and RM324 on chromosome 2 were selected to screen polymorphism between the parents and both bulks of two mapping populations. Of these, four markers RMAN7, RMAN8, RMAN81, and RM7575 showed polymorphism and linkage with *tmsX*. Thus, the *tmsX* gene was further mapped to the interval between RM7575 and RMAN8 on chromosome 2.

For further fine mapping of the *tmsX* gene, 687 complete sterile plants from "XianS × IC38-200-1-1" F_2 population consisting of 3,000 plants and 227 complete sterile plants from "XianS × IC33-11-23-1-1-1" F_2 population consisting of 1,000 plants were used. Moreover, 14 SSR markers were selected from the region surrounding *tmsX* between RM7575 and RMAN8 on chromosome 2. Among these, four markers 4039-1, 4039-2, RMX14, and RMX21 showed polymorphism and linkage with *tmsX*. Meanwhile, the aforesaid RMAN81 was also used to detect recombinant in completely sterile plants of the two large F_2 mapping populations. Finally, the *tmsX* gene in XianS was mapped between RMAN81 and RMX21 with a genetic distance of 1.02 cM to RMAN81 and 0.15 cM to RMX21, respectively. Two SSR markers, 4039-1 and RMX14, completely cosegregated with *tmsX* and no recombinant was detected in all F_2 sterile plants. The partial linkage maps for the region around *tmsX* were presented in Fig. 2a. According to the physical mapping information from publicly available resources (http://www.ncbi.nlm.nih.gov), two

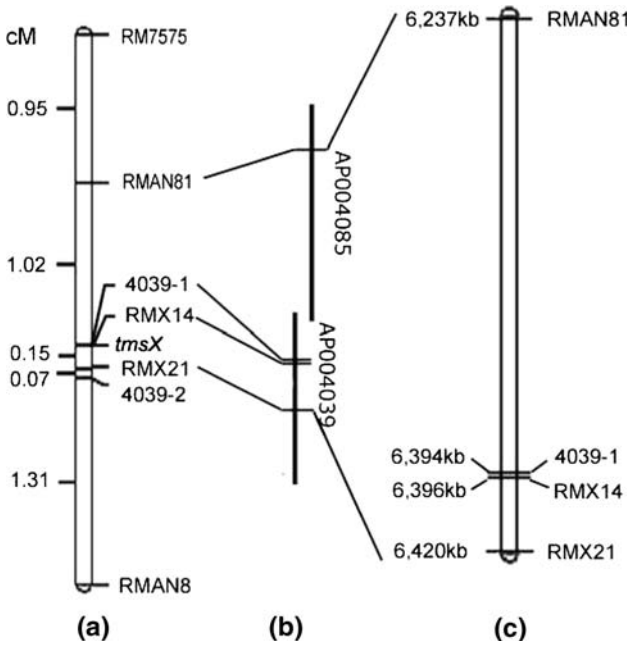

Fig. 2 Molecular mapping of the TGMS gene *tmsX* in XianS using two mapping populations F_2-1 (XianS × IC38-200-1-1) and F_2-2 (XianS × IC33-11-23-1-1-1). **a** Linkage map of the *tmsX* gene region on the short arm of chromosome 2. **b** The BAC contigs encompassing the *tmsX* gene region. **c** The physical position of the markers co-segregating with *tmsX* gene on chromosome 2

markers RMAN 81 and RMX21 on the BAC clone AP004085 and AP004039 (Fig. 2b) are located in the position of 6,237 and 6,420 kb on chromosome 2 (GenBank Accession No. AP008208), respectively. Thus, the *tmsX* locus of XianS has been mapped within the region of 183 kb (Fig. 2c), which locating at the 31.2 cM of chromosome 2 (http://rgp.dna.affrc.go.jp). The sequences of partial primers used for fine mapping the *tmsX* gene of XianS were showed in Table 3.

The region between RMAN 81 and RMX21 contains ONAC023 (*OsNAC6*, GenBank Accession No. AK107283), which was identified as the candidate gene of *tms5* from AnnongS-1 by Yang et al. (2007b). To determine the

Table 3 The sequences of primers used for fine mapping the TGMS gene of XianS

Molecular marker	Forward primer (5′–3′)	Reverse primer (5′–3′)
RMAN81	ACTGAAAGTGGTCTGTTAGC	TCTTACCAAAGCCGTGTGTC
4039-1	GTTAACCGGTTGAGGTGGTG	TCCGTCTGTTACAAAATGAA
RMX14	CGGTAATGTCGTAAGGAAATGC	CGATGACTTGCCGCTGTT
RMX21	CCGACGCCATGATTTCCA	CCGTACACCGCGATCCTTT
4039-2	TCTAGCGCCCCTACATGTCT	ACCGCAAAATATTCCACGAG

relationship of *tmsX* and *OsNAC6*, *OsNAC6* (1,843 bp) and its promoter region (about 1,500 bp) (Nakasgima et al. 2007) in XianS, AnnongS-1 and Xianhuangzhan were sequenced. Based on the BAC clone AP004039 sequence (http://www. ncbi.nlm.nih.gov), five specific primers covering *OsNAC6* and its promoter region were designed to obtain PCR-amplified products of XianS, AnnongS-1 and Xianhuangzhan (Table 4). About a 3,500 bp fragment was sequenced, completely covering the *OsNAC6* and its promoter regions. However, no difference was found in this region among XianS, AnnongS-1 and Xianhuangzhan. Blast analysis indicated that the 3,500 bp sequence is the same as the sequence from 16,788 to 20,290 bp of DQ448817 deposited by Yang et al. (2007b) in the GenBank database. The donor for the nucleotide sequences of DQ448817 is Annong.

Allelism of non-pollen TGMS genes in different TGMS lines

XianS, AnnongS-1, Q523S, Q524S, N28S, G421S, and Q527S were non-pollen type TGMS lines obtained from different germplasm resources (Table 1). The pollen fertility of F_1s of the crosses between different TGMS lines was examined. The results showed that all the F_1s were completely male sterile in the middle of June, 2007 or 2008. Therefore, the TGMS genes of seven non-pollen type TGMS lines were allelic.

Discussion

The male fertility of TGMS lines used in two-line system is mainly regulated by environmental temperature. Sudden drop in temperature could be disastrous during hybrid seed production because the reversion from sterile to fertile phase could result in self fertilization of the female parent. Therefore, the stable male sterility is critical for the application of TGMS lines. XianS is a novel non-pollen type TGMS line with stable male sterility. Identification of the corresponding mutated gene in XianS is valuable not only for revealing the developmental mechanism(s) of male organs in rice, but also for fully utilizing this gene in rice breeding. In this study, we mapped the genome localization of the mutated gene in XianS to a region of 183 kb between RMAN81 and RMX21 on chromosome 2 and discovered that two markers 4039-1 and RMX14 completely cosegregated with it.

Among the five mapped PGMS genes and nine mapped TGMS genes, only *tms4* from TGMS-VN1 and *tms5* from AnnongS-1 were mapped to chromosome 2 (Dong et al. 2000; Wang et al. 2003). Dong et al. (2000) mapped *tms4* to the region above the marker E5/M12-600 at a genetic distance of 3.3 cM. From publicly available resources, it can be inferred that E5/M12-600 locates within the region of 33.6–36.3 cM on chromosome 2 (http://rgp.dna.affrc.go.jp). Thus, the *tmsX* gene in XianS locating at the 31.2 cM should be within the region (30.3–33.0 cM) carrying *tms4* on chromosome 2, but it is still hard to determine allelic relationship of *tmsX* and *tms4* due to no further fine mapping information of *tms4* and seeds of TGMS-VN1. For *tms5* locating at the 31.2 cM of chromosome 2, Jiang et al. (2006) fine mapped *tms5* to the region of 181 kb between RMAN7 (6,006 kb) and RMAN54 (6,187 kb), whereas Yang et al. (2007b) fine mapped it to the region of 33 kb (*japonica*) or 19 kb (*indica*) between 4039-1 and 4039-2. The region carrying the TGMS gene *tmsX* in XianS almost contains the region of *tms5* in AnnongS-1 reported by Yang et al. (2007b), but it is about 50 kb apart from the

Table 4 Primer information used to sequence analysis of *OsNAC6* and its promoter region

Primer	Forward primer (5′–3′)	Reverse primer (5′–3′)	Position in AP004039	Fragment length (bp)
CX1	TGACCCTGATAGATGCGATAC	TAACAGCCAAACCAAACG	46,032–47,115	1084
CX2	ATGGCAATGACACCGCAGCTA	CTAGCCACCATGGTTTCTTTG	45,176–46,666	1490
CX3	CCAGGACATCCGTTTCTATC	AGCACCGTATGTAGTGAGGC	44,680–45,403	724
CX4	AGATAAAGACCACCCAGAT	CACAGGGATAAGATAGCA	43,782–45,033	1252
CX5	TATGGAGGATGAGATGGT	AGTGTAGCGGTAAGTGGT	43,306–44,279	974

region of *tms5* reported by Jiang et al. (2006). From the linkage distances between the markers and *tmsX* (Fig. 2), *tmsX* and *tms5* mapped by Yang et al. (2007b) locate in the region near 4039-1 and RMX14. The results of allelic test demonstrated their allelic relationship. Within this region, Yang et al. (2007b) identified a gene *OsNAC6* (GenBank Accession No. AK107283) as the candidate gene of *tms5* since a SNP mutation presents in the 19 kb region between 4039-1 and 4039-2 but the specific position of this SNP mutation was not reported. Nevertheless, our sequencing results indicate XianS does not carry any mutation in *OsNAC6* and its promoter region. Therefore, the real candidate gene of *tms5* may still need to be further studied and identified although its position was determined near 4039-1 and RMX14 on chromosome 2.

In addition, it is worth noting that this study discovered that the non-pollen phenotype in seven independent TGMS lines is caused by mutations of the same TGMS gene. The non-pollen phenotype in TGMS lines HD9802S, Zhu1S, and AnnongS-1 was also reported to be caused by an allelic TGMS gene (Xiang et al. 2002; Zhou et al. 2008). Why is non-pollen TGMS in so many independent lines caused by mutations of the same gene? A reasonable explanation is that the corresponding gene plays the most important role in the pollen development of rice and no other gene has overlapping function with this gene. It is confusing that the same TGMS gene was given different names. For simplicity, we propose to give the TGMS gene in these non-pollen type TGMS lines a fixed name, for example, *tms5*, in the further study. The expression of male sterility in TGMS lines is complicated. It is common that different plants with an identical TGMS gene show different pollen fertility due to differences in genetic background and/or temperature condition. However, *tms5* could express the male sterility of non-pollen abortion under various genetic backgrounds, so the transfer of *tms5* to desirable genetic background will be relatively easy. The isolation of *tms5* will be helpful to the revelation of molecular mechanism of non-pollen TGMS and the development of various practical non-pollen TGMS lines.

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