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ORIGINAL PAPER

Fine mapping and candidate gene analysis of the novel thermo-sensitive genic male sterility *tms9-1* gene in rice

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Abstract
Key message **Fine mapping of the novel thermo-sensitive genic male sterility locus *tms9-1* in the traditional TGMS line HengnongS-1 revealed that the *MALE STERILITY1* homolog *OsMS1* is the candidate gene.**
Abstract Photoperiod-thermo-sensitive genic male sterility (P/TGMS) has been widely used in the two-line hybrid rice breeding system. HengnongS-1 is one of the oldest TGMS lines and is often used in *indica* two-line breeding programs in China. In this study, our genetic analysis showed that the TGMS gene in HengnongS-1 was controlled by a single recessive gene that was non-allelic with the other TGMS loci identified, including C815S, Zhu1S and Y58S. Using SSR markers and bulked segregant analysis, we located the TGMS locus on chromosome 9 and named the gene *tms9-1*. Fine mapping further narrowed the *tms9-1* loci to a 162 kb interval between two dCAPS markers. Sequence analysis revealed that a T to C substitution results in an amino acid change in the *tms9-1* candidate gene (Os09g27620) in HengnongS-1 as compared to Minghui63. Sequencing of other rice accessions, including six P/TGMS lines, seven *indica* varieties and nine *japonica* varieties, showed that this SNP was exclusive to HengnongS-1. With multiple sequence alignment and expression pattern analyses, the rice *MALE STERILITY1* homolog *OsMS1* gene was identified as the candidate gene for *tms9-1*. Therefore, our study identified a novel TGMS locus and will facilitate the functional identification of the *tms9-1* gene. Moreover, the markers linked to the *tms9-1* gene will provide useful tools for the development of new TGMS lines by marker-assisted selection in two-line hybrid rice breeding programs.

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Introduction

Rice (*Oryza sativa* L.) is one of the most important food crops in the world. Hybrid rice has played a significant role in increasing rice productivity (Yuan 2004; Khush 2001). It has been demonstrated that hybrid rice has a 20 % yield increase over the conventional inbred varieties and has been successfully grown in many countries (Cheng et al. 2007). Hybrid rice is mainly produced using two systems: the two-line (photoperiod-thermo-sensitive genic male sterile line) and the three-line (cytoplasmic male sterility line, maintainer line and restorer line) systems. With the discovery and application of P/TGMS, the two-line system has made a tremendous contribution to the exploitation of rice heterosis (Si et al. 2011). The occurrence of male sterility in P/TGMS is environmentally dependent on temperature and/or photoperiod.

P/TGMS lines serve as the male sterile parents for hybrid seed production under high temperature and/or long-day conditions, and their self-propagation (male fertility) can be obtained under low temperature and/or short-day conditions. However, the reverse P/TGMS lines have an opposite phenotype as compared with the P/TGMS lines, which are male sterile under low temperature and/or short-day conditions, and self-propagate (male fertility) under high temperature and/or long-day conditions. Therefore, the two-line system has advantages for hybrid seed production in comparison with the conventional three-line system (Yuan 1990). Firstly, the P/TGMS lines can be self-propagated under a certain photoperiod and temperature without the need of a maintainer line. Secondly, they have a broad spectrum of genotypes as male parents, eliminating the necessity of a restorer line because almost all normal fertile varieties can restore the fertility of the F_1 hybrids derived using the P/TGMS lines. Furthermore, the application of P/TGMS hybrids can eliminate the potential adverse effects of male sterile cytoplasm on agronomic traits (Virmani et al. 2003).

Several P/TGMS genes have been identified on different rice chromosomes. Four PGMS genes, *pms1*, *pms2*, *pms3* and *p/tms12-1*, have been mapped to chromosomes 7, 3, 12 and 12, respectively (Zhang et al. 1994; Mei et al. 1999; Lu et al. 2005; Zhou et al. 2012). Three reverse PGMS genes, *rpms1*, *rpms2* and *CSA*, have been mapped to chromosomes 8, 9 and 1, respectively (Peng et al. 2008; Zhang et al. 2013). Nine TGMS genes, *tms1* (Wang et al. 1995), *tms2* (Yamaguchi et al. 1997; Lopez et al. 2003; Pitnjam et al. 2008), *tms3* (Subudhi et al. 1997), *tms4* (Dong et al. 2000), *tms5* (Wang et al. 2003; Yang et al. 2007a, b), *tms6* (Lee et al. 2005), *TGMS* gene from SA2 (Reddy et al. 2000), *ptgms2-1* (Xu et al. 2011) and *tms9* (Sheng et al. 2013), have been mapped to chromosomes 8, 7, 6, 2, 2, 5, 9, 2 and 2, respectively. One reverse TGMS gene, *rtms1* from J207S, has been mapped to chromosome 10 (Jia et al. 2001). Recently, studies of PGMS genes have made great progress as several PGMS genes have been cloned, and the underlying mechanisms have been recognized. For example, it was found that *pms3* and *p/tms12-1* are the same allele with a C to G substitution (Ding et al. 2012; Zhou et al. 2012).This gene encodes a long noncoding RNA (lncRNA) that may be involved in epigenetic modifications, and this SNP caused an unstable epigenetic state in Nongken58S; additionally, reduced expression of this lncRNA led to male sterility under long-day conditions (Ding et al. 2012). Alternatively, *p/tms12-1* encodes the precursor of a small RNA in the Peiai64 background, and the C to G substitution most likely leads to a loss-of-function of the small RNA, which might act as an important regulator of rice pollen development (Zhou et al. 2012). A reverse photoperiod dependent male sterile line, *carbon starved anther* (*csa*), resulted from a mutation in a R2R3 MYB transcription factor that participates in pollen development. This mutation was introduced into both *indica* and *japonica* rice lines, and could render the transformed lines male sterile under short-day conditions and male fertile under long-day conditions (Zhang et al. 2013).

However, gene exploration of TGMS loci is still underway. It was reported that *ptgms2-1* was mapped to a 50.4 kb interval on the AP004039 BAC, and a nuclear ribonuclease Z gene was identified as the candidate *ptgms2-1* gene (Xu et al. 2011). The *tms5* gene was also physically mapped to a 19 kb DNA fragment located on the AP004039 BAC. A member of the NAC (NAM-ATAFCUC-related) gene family, *ONAC023*, was identified as the candidate *tms5* gene (Yang et al. 2007a, b). All these TGMS genes have yet to be functionally identified.

In this study, we fine mapped a novel TGMS gene, designated as *tms9-1*, in the rice TGMS line HengnongS-1, which is an older line that is widely used in the two-line hybrid breeding system in China (Zhou et al. 1988). The *tms9-1* gene was physically mapped to an interval of 162 kb between two dCAPS markers, and a homolog of the *MS1* gene was identified as the *tms9-1* candidate. This study will provide essential information for the final isolation and functional characterization of a novel rice TGMS gene, as well as practical genetic tools for MAS of the TGMS lines to facilitate hybrid rice breeding.

Materials and methods

Plant materials

HengnongS-1, a thermo-sensitive male genic sterile line, has been widely used in two-line hybrid rice breeding since the 1990s in South China (Zhou et al. 1988). Five conventional *indica* varieties and lines (Minghui63, T92, T95, R96 and R98) were used for genetic analysis and fine mapping in this study. Three TGMS lines from different gene sources, C815S (Tang et al. 2007), Y58S (Deng. 2005) and Zhu1S (Yang et al. 2000), were crossed with HengnongS-1 for the allelic test. All plants were planted in the summer seasons in a paddy field at the experimental farm of the Zhejiang Academy of Agricultural Sciences, Hangzhou, China.

Phenotypic characterization of HengnongS-1

To determine the pollen fertility of HengnongS-1 under different temperatures and long-day (14 h) conditions, the plants were grown in four plant growth chambers with different temperatures; the daily temperatures were set to

23/21 °C (day/night), 25/23 °C, 26/24 °C and 27/25 °C. Six plants were included in each treatment to investigate pollen fertility. Anthers from flowering spikelets were collected, and the pollen was stained with 1 % I_2–KI solution. The pollen was photographed using a NIKON ECLIPSE E100 light microscope to assess the fertility of the pollen. The round and darkly stained pollen was classified as fertile, whereas unstained and irregular pollen was classified as sterile. Meanwhile, the anthers of HengnongS-1 and Minghui63 were photographed using a LEICA Z16 APOA dissecting microscope.

Genetic analysis and mapping populations of HengnongS-1

For the inheritance of TGMS, the F_2 populations generated from the crosses of HengnongS-1 and the *indica* lines T92, T95, R96 and R98 were planted at the experimental station, Hangzhou, China, in the summer seasons. A large F_2 population of the HengnongS-1 and Minghui63 cross including 2,937 individuals was used for the mapping. All populations were evaluated for pollen fertility from August 1 to 15 in 2012 in Hangzhou, China. The daily average temperature was 27.9 °C, which is a typical high temperature, and these plants were grown under typical high temperature and long-day conditions (provided by Hangzhou Weather Bureau).

Marker development and fine mapping

Genomic DNA was extracted from the plants using CTAB buffer. A total of 506 SSR markers (http://www.Gramene.org) covering 12 rice chromosomes was used to detect polymorphisms between HengnongS-1 and Minghui63 using a previously described PCR-based approach (Deng et al. 2006). Based on primary mapping and genomic sequencing of the two parents, new dCAPS and CAPS markers were designed to further narrow down the candidate region. The reaction mixture (20 μL) for the PCR analysis consisted of 20 ng of template DNA, 10 μL of 2 × Kod Fx buffer, 2.0 μL of 2.5 mM dNTPs, 0.5 μL each of 10 mM primers and 0.5 U of Kod Fx (supplied by Solomon Biotechnology Co., Ltd). The thermal cycling program included an initial denaturation at 95 °C for 5 min, followed by 36 cycles of denaturation at 95 °C for 30 s, annealing at 50 °C for 30 s and extension at 68 °C for 1 min, and a final extension at 68 °C for 5 min. The PCR-amplified products were purified and sequenced by BioSune Biotech Co., Ltd. (Shanghai, China). The sequencing results of the HengnongS-1 and Minghui63 were analyzed using the DNA Star Software. CAPS and dCAPS marker primers were designed using the Primer Premier 5.0 software and the web-based tool dCAPS Finder 2.0 (http://helix.wustl.edu/dcaps/dcaps.html). Then, 10 μL of the PCR-amplified products was digested with a restriction enzyme (Fermentas) and analyzed using agarose gel (3.0 %) electrophoresis; the gels were photographed under UV light. The recombination frequency was converted into genetic distance (Kosambi 1944), and linkage analysis was conducted using MAPMARKER/EXP 3.0 (Lander et al. 1987).

Sequence analysis

Combined with the phenotype of HengnongS-1, the available gene annotation database (http://rgp.dna.affrc.go.jp/E/genomicdata/P0047B10/P0047B10.fr.html) and the fine mapping results, and the candidate gene for *tms9-1* was further analyzed. Based on the sequence of the AP005308 BAC clone, seven specific primers were designed to sequence the *OsMS1* gene and its promoter in the HengnongS-1 and Minghui63 varieties. Due to the SNP between HengnongS-1 and Minghui63, we also sequenced other rice accessions, including six P/TGM lines, seven *indica* varieties and nine *japonica* varieties with the specific primers. The PCR-amplified products were purified and sequenced by BioSune Biotech Co., Ltd. (Shanghai, China). The sequencing results were analyzed using the DNA Star Software. Multiple sequence alignment analysis was carried out with the deduced amino acid sequences of the OsMS1 protein from different rice varieties/germplasm, including *japonica* and *indica* and other eight plant species, using the ClustalW program (Chenna et al. 2003). Sequence homology searches in GenBank were carried out with the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The conserved domain prediction of the OsMS1 protein was performed with the web-based search (http://www.ncbi.nlm.nih.gov/cdd).

RT-PCR analysis

Total RNA was isolated from the different tissues of the HengnongS-1 and Minghui63 plants during the pre-heading stage using the RNeasy Plant Mini kit (QIAGEN). Reverse transcription was performed with the One-Step RT-PCR kit (Invitrogen) according to the manufacturer's protocol. The *OsMS1* gene transcript levels were detected by RT-PCR. RT-PCR was performed using the following PCR conditions: 95 °C for 3 min, followed by 35 cycles of 95 °C for 30 s, 58 °C for 30 s and extension at 72 °C for 30 s, and a final extension at 72 °C for 5 min. The PCR mixture (20 μL) contained 10 μL of 2 × GC I buffer (TaKaRa), 5 μg of cDNA template, 2.0 μL of 2.5 mM dNTPs, 0.5 μL each of 10 mM primers and 0.5 U of Taq polymerase (supplied by Beijing DingGuo Biotech co., Ltd.). The rice (*Oryza sativa* L.) *ubiquitin* (*Ubi-1*) gene was used as an internal control (Kachroo et al. 2003).

Results

HengnongS-1 is a thermo-sensitive genic male sterile line

HengnongS-1 is a TGMS line that was bred from a cross between wild rice and conventional *indica* cultivar, and this line is male sterile compared to the normal variety Minghui63, when grown during the summer seasons in Hangzhou, China (Fig. 1a). Under natural high temperatures, the anthers of HengnongS-1 were thin and pale yellow (Fig. 1b), whereas the anthers of Minghui63 were plump and dark yellow (Fig. 1c). The pollen from HengnongS-1 appeared different under the four temperature conditions. When grown under 23/21 °C (day/night), a large portion of the HengnongS-1 pollen was fertile (Fig. 1d). However, when the growth temperature increased to 27/25 °C (day/night), HengnongS-1 pollen was completely sterile, similar result was also observed with a growth temperature of 26/24 °C (Fig. 1e). When the growth temperature was reduced to 25/23 °C, a small portion of fertile pollen was produced in the anthers of HengnongS-1(~4.22 % of the total spikelet). We also observed that when the daily mean temperature was increased to 32 °C, no pollen was produced in the anthers of HengnongS-1(Fig. 1f). Therefore, HengnongS-1 is a strict thermo-sensitive genic male sterile line.

The TGMS locus of HengnongS-1 is non-allelic to other widely used TGMS genes

Three TGMS lines, C815S, Y58S and Zhu1S, are the most widely used lines in the two-line system of hybrid rice breeding in China, and each line was derived from a different TGMS gene source. The male sterility/seed settings

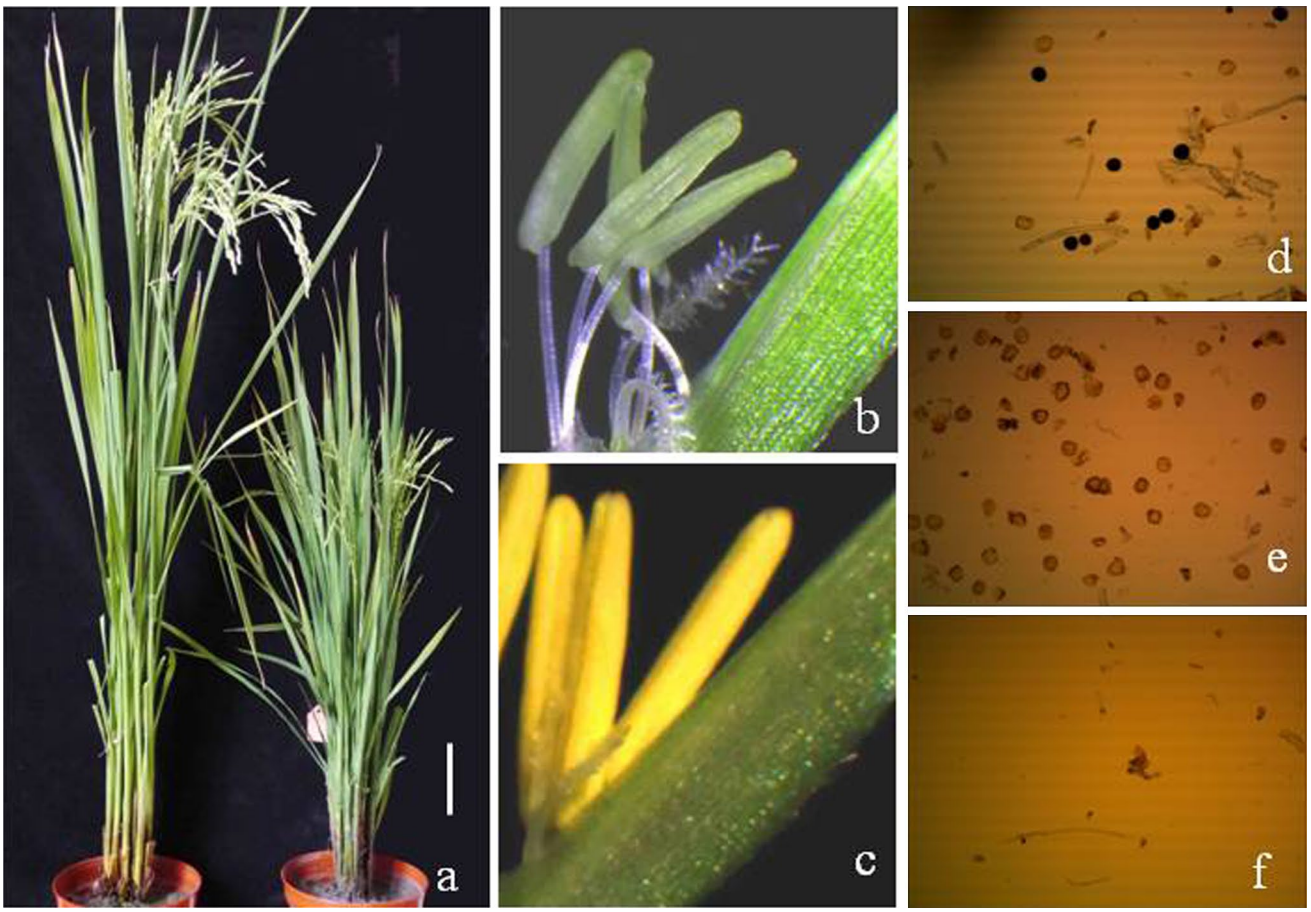

Fig. 1 Phenotype comparison of anthers of Minghui63 and HengnongS-1 and pollen fertility of HengnongS-1 under different conditions. **a** Plant morphology of Minghui63 (*left*) and HengnongS-1 (*right*). *Bar* = 10 cm. **b**, **c** Anthers of HengnongS-1 and Minghui63 during flowering, note that the HengnongS-1 anthers (**b**) are thin and *pale yellow*, in comparison with the *yellow* and plump anthers of Minghui63 (**c**). **d** Pollens of HengnongS-1 grown under 23/21 °C temperatures, showing that many of pollens were round and darkly stained with 1 % I_2–KI solution, which were fertile. **e** Pollens of HengnongS-1 grown under 27/25 °C temperatures, showing that all pollens were unstained and irregular, which were sterile. **f** No pollen was produced in the anther of HengnongS-1 grown under high temperature with a mean daily temperature of 32 °C

of the different F_1 lines from the crosses of HengnongS-1 and the three TGMS lines were examined (Table 1). These results showed that the setting rate of the three F_1 populations ranged from 57.4 to 68 %, restoring the fertile phenotype of each parent. Therefore, the TGMS gene of HengnongS-1 was non-allelic with the TGMS genes in the C815S, Y58S and Zhu1S lines. A similar result was also reported in an early genetic study of TGMS germplasm (Deng et al. 2001).

Genetic analysis of TGMS and primary mapping of *tms9-1*

The inheritance pattern of TGMS in HengnongS-1 was investigated using five F_2 populations. As shown in Table 2, the segregation ratios of the fertile plants to sterile plants in the five F_2 populations were at 3.51:1 ($\chi^2_{0.05} = 1.43 < 3.84$), 3.16:1 ($\chi^2_{0.05} = 0.09 < 3.84$), 3.33:1 ($\chi^2_{0.05} = 0.26 < 3.84$), 3.14:1 ($\chi^2_{0.05} = 0.11 < 3.84$) and 3.32:1 ($\chi^2_{0.05} = 1.55 < 3.84$) (Table 2), which fitted to 1:3, indicating that TGMS in HengnongS-1 was controlled by a single recessive gene. To map TGMS gene in HengnongS-1, a total of 506 SSR markers distributed on 12 chromosomes was used to detect polymorphisms between HengnongS-1 and Minghui63, using two DNA pools from 12 F_2 individuals with either sterile or fertile phenotype. Consequently, two SSR markers, RM5661 and RM5122, on chromosome 9 were found to be polymorphic between the two pools. To further confirm the location of the TGMS gene in HengnongS-1, four additional polymorphic markers, RM1026, RM24537, RM24413 and RM24349, were used to detect the linkage using 681 F_2 sterile plants. The results showed that the target gene was located between the RM24413 and RM24537 markers, with genetic distances of 1.39 and 1.61 cM, respectively (Fig. 2a), where no TGMS gene has been previously reported. Thus, the TGMS gene in HengnongS-1 was designated as *tms9-1*.

Fine mapping and candidate gene analysis of *tms9-1*

Five new markers (CAPS and dCAPS) were developed to fine map the *tms9-1*gene (Table 3), using the SNPs between HengnongS-1 and Minghui63. Finally, the *tms9-1* locus was further narrowed down to a 162-kb region between QY-9-27 and QY-9-19 on the BAC clones AP005575 and AP005308, with one recombination occurring between *tms9-1* and QY-9-27 and three recombinations between *tms9-1* and QY-9-19, respectively (Fig. 2b).

According to the gene annotation database (http://rgp.dna.affrc.go.jp/), the region between QY-9-27 and QY-9-19 contains a PHD finger protein (LOC_Os09g27620) that is a homolog of MALE STERILITY1 in *Arabidopsis thaliana*, which regulates pollen and tapetum development (Ito et al. 2007). Based on the BAC clone sequence, seven specific primers covering the *MALE STERILITY1* homolog (*OsMS1*) coding region and its promoter were designed to obtain PCR-amplified products from the HengnongS-1 and Minghui63, and the resulting PCR products were sequenced (Table S1). With the sequencing result, one

Table 1 TGMS lines used for allelic tests

TGMS lines	Origin	TGMS gene source	The seed setting rate of F_1 plants (%)
C815S	Selective breeding from the progeny of free cross-pollination (Anxiang S/xiandang//02428///peiai 64S)	*tms5* and *p/tms12-1*	57.4
Y58S	Selective breeding from the progeny of free cross-pollination (Annong S-1/changfei22B//Annong S-1/lemont///peiai64S)	*tms5* and *p/tms12-1*	61
Zhu1S	Natural mutation of F_2 population (Kangluozao//4342/02428)	*tms9*	68

Table 2 Pollen fertility of F_2 populations between HengnongS-1 and different male parents

Cross combinations	Number of sterile plants	Number of fertile plants	Segregation ratio (sterile:fertile)	$\chi^2_{0.05}$ (3:1) = 3.84
HengnongS-1 × T92	83	291	1:3.51	1.43
HengnongS-1 × T95	70	221	1:3.16	0.09
HengnongS-1 × R96	42	140	1:3.33	0.26
HengnongS-1 × R98	103	323	1:3.14	0.11
HengnongS-1 × Minghui63	204	678	1:3.32	1.55

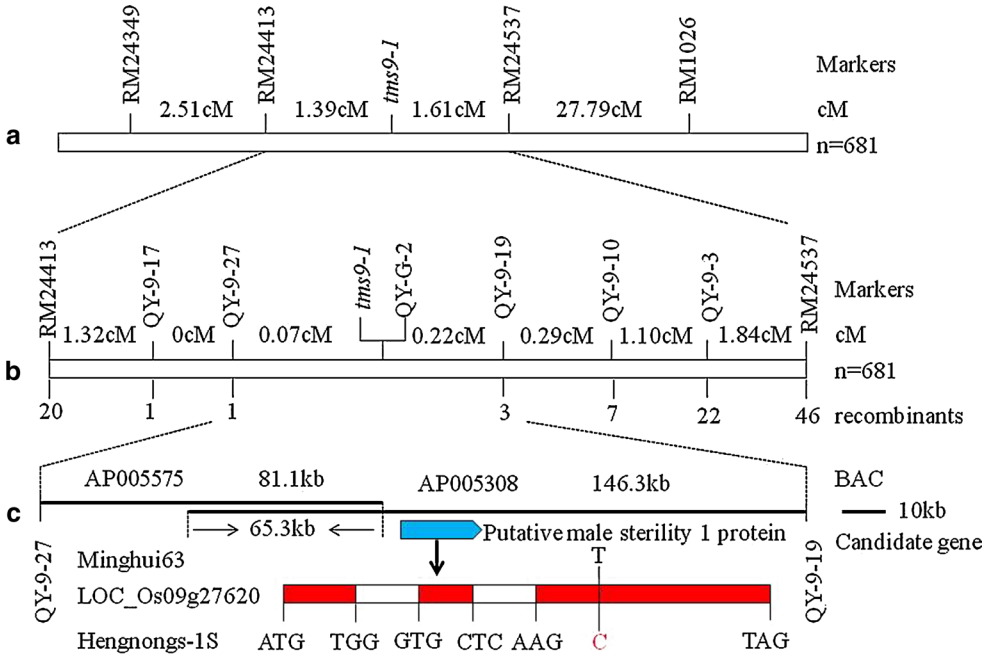

Fig. 2 Fine mapping and candidate gene of *tms9-1*. **a** Primary mapping of *tms9-1* gene. The *tms9-1* gene was mapped to the region between RM24537 and RM 24413, and the genetic distance was 1.61 and 1.39 cM, respectively. **b** Fine mapping of the *tms9-1* gene. The *tms9-1* gene was restricted to the region between QY-9-19 and QY-9-27, and the genetic distance was 0.22 and 0.07 cM, respectively. The marker QY-G-2 was co-segregated with the *tms9-1* gene. The number of recombinants among the markers and the *tms9-1* gene is shown on the linkage map. **c** The physical position of the *tms9-1* gene and the sequence analysis of the candidate gene. The *tms9-1* gene was finally mapped on a 162.1 kb region in the BAC clone AP005308 and AP005575. According to the annotation database of this region, there was a putative *MALE STERILITY1* (*OsMS1*) in this region. Sequence comparison between Minghui63 and HengnongS-1 showed that there was one SNP on the third exon of the *OsMS1* homolog gene

Table 3 Markers used for CAPS and dCAPS analysis

Markers	Forward primer (5′–3′)	Reverse primer (5′–3′)	Restriction enzyme	PCR products (bp)
QY-9-3	GAATAGTTTTGTTTGAAGTTTTGAACGAA	ACTGAATGCGTGACTGTTACCCCTTAG	TfiI	217
QY-9-10	AAGAGGACGGAACAATCAA	ACTAAACACGGCCAAGAGT	BstBI	591
QY-9-17	GATGAATTGAAGCAGTAGTAGGGTATAC	GTGTTGGGAGCACAAGGTTCCGAACAGAG	HinfI	286
QY-9-19	AGAAAAGAGATGGTTAGGGAGGCCCACTGA	GCGAGGGCGGTTTGCCGATT	DdeI	270
QY-9-27	ACGCGTTCACGGTGAGCCTACTACTCCCGA	CTCTGACCTGACCTCTGAGTGCTCGCAC	Hpy99I	538
QY-G-2	CTGGTGGACACGGCGAGGAAGGGCCAC	GCTCGAGCATGAAGCGGAGGAGGTCCCCG	SmaI	291

nucleotide substitution T to C was found, which resulted in an amino acid conversion from Leu in Minghui63 to Phe in HengnongS-1 (Fig. 2c). To determine that the SNP was exclusive to HengnongS-1, a new dCAPS marker (QY-G-2) was designed to detect the recombinants linked with the three dCAPS markers QY-9-10, QY-9-19 and QY-9-27. The result showed that no recombination occurred between *tms9-1* and QY-G-2, and the SNP co-segregated with the sterile phenotype (Fig. 2b). To further link the SNP with TGMS in HengnongS-1, we sequenced other rice accessions, including six P/TGMS lines, seven *indica* and nine *japonica* varieties (Table 4). We found that the SNP only occurred in HengnongS-1, and the 'T' allele was conserved in all of the analyzed lines and varieties. Therefore, *OsMS1* is most likely the candidate gene for *tms9-1* in HengnongS-1.

OsMS1 protein sequence and expression analysis

Multiple sequence alignment was performed with the deduced amino acid sequence of the OsMS1 protein and homologs from other eight plant species using the ClustalW program. As shown in Fig. 3, the amino acid sequence of the OsMS1 protein shares high levels of identity with

Table 4 Plant materials used for gene sequence analysis

Varieties	Subspecies	Origin	PTGMS gene	Chr. position	SNP	Phenotype
HengnongS-1	*O. indica*	China	*tms9-1*	Chr.9	C	TGMS
N5088S	*O. japonica*	China	*pms3*	Chr.12	T	PGMS
C815S	*O. indica*	China	*tms5* and *p/tms12-1*	Chr. 2 and Chr. 12	T	TGMS
Y58S	*O. indica*	China	*tms5* and *p/tms12-1*	Chr. 2 and Chr.12	T	TGMS
Zhu1S	*O. indica*	China	*tms9*	Chr. 2	T	TGMS
Xiangling628S	*O. indica*	China	*tms9*	Chr. 2	T	TGMS
Guangzhan63S	*O. indica*	China	*ptgms2-1*	Chr. 2	T	TGMS
9311	*O. indica*	China	/	/	T	Non-P/TGMS
Nanjing 11	*O. indica*	China	/	/	T	Non-P/TGMS
Zhehui7954	*O. indica*	China	/	/	T	Non-P/TGMS
Shuhui527	*O. indica*	China	/	/	T	Non-P/TGMS
Zhonghui8006	*O. indica*	China	/	/	T	Non-P/TGMS
Tianfeng B	*O. indica*	China	/	/	T	Non-P/TGMS
Yixiang B	*O. indica*	China	/	/	T	Non-P/TGMS
Lunhui422	*O. japonica*	China	/	/	T	Non-P/TGMS
Nipponbare	*O. japonica*	Japan	/	/	T	Non-P/TGMS
Koshihikari	*O. japonica*	Japan	/	/	T	Non-P/TGMS
Lemont	*O. japonica*	USA	/	/	T	Non-P/TGMS
Jefferson	*O. japonica*	USA	/	/	T	Non-P/TGMS
Balilla	*O. japonica*	Italy	/	/	T	Non-P/TGMS
C418	*O. japonica*	China	/	/	T	Non-P/TGMS
Xiushui09	*O. japonica*	China	/	/	T	Non-P/TGMS
Zhejing88	*O. japonica*	China	/	/	T	Non-P/TGMS

homologs from other species. The amino acid residue of Leu in Minghui63 is likely conserved in diverse species, which is located in the predicted central domain of Transcription factor S-II. We further examined the expression of the *OsMS1* gene in different tissues using RT-PCR with the specific primers (Table S1). As shown in Fig. 4, the *OsMS1* gene was highly expressed in the spikelet, but was hardly detected in the leaf and stem during the pre-heading stage. However, the expression pattern of the *OsMS1* gene was no different between Minghui63 and HengnongS-1. Therefore, the mutation in the *OsMS1* gene likely does not affect its expression.

Discussion

As important genetic resources, P/TGMS lines have been widely used in the two-line hybrid rice breeding system. The major P/TGMS lines were mainly derived from Nongken58S and AnnongS. HengnongS-1, one of the oldest *indica* TGMS lines, which was selected from the progeny of free cross-pollination (long wan wild rice/R0183//C64) (Zhou et al. 1988), has been used in two-line breeding programs in China, Hybrid rice varieties using HengnongS-1 have been bred and grown in South China since the 1990s (Xu and Fu 1993). In this paper, the *tms9-1* gene from HengnongS-1 was mapped to chromosome 9. We first show that the *tms9-1* gene is a novel TGMS gene.

In general, pollen phenotypes of male sterile rice are classified as non-pollen, typical abortion, spherical abortion and stainable abortion (Li 2000). In this study, we observed that the pollen fertility of HengnongS-1 differed from the other widely used TGMS lines. No pollen was produced in the anther of HengnongS-1 when grown at high temperatures, similar to the non-pollen TGMS lines including XianS (Peng et al. 2006), Zhu1S (Yang et al. 2007a, b) and HD9802S (Zhou et al. 2008). The sterility of HengnongS-1 was very stable under high temperature. However, when the growth temperature was reduced, pollen could be produced but was sterile. At environmental temperatures of 25/23 °C, fertile pollen was found in part of the anthers, suggesting that this temperature is the threshold for fertility emergence. In contrast, no any pollen was produced in other non-pollen TGMS lines including XianS (Peng et al. 2006), Zhu1S (Yang et al. 2007a, b) and HD9802S (Zhou et al. 2008) under high temperatures. This divergence likely results from the different mechanisms of pollen development mediated by *tms9-1* gene and other TGMS genes (Peng et al. 2010; Sheng et al. 2013; Zhou et al. 2008).

The emergence of the male sterility phenotype in TGMS lines depends on the environmental temperature during rice growth. However, little is known about the molecular

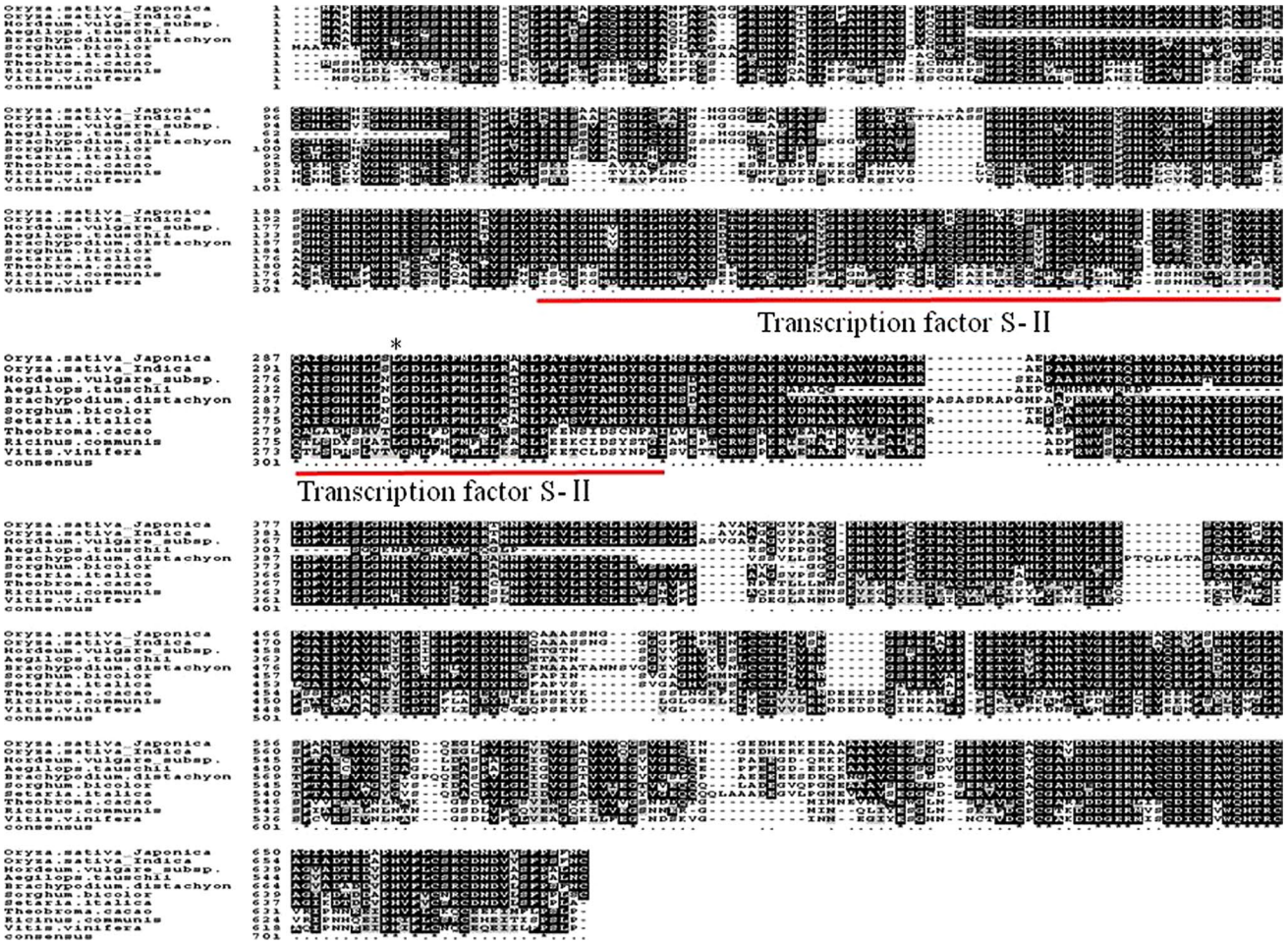

Fig. 3 Comparison of the predicted amino sequences of MALE STERILITY1 proteins in different plant species. The MALE STERILITY1 protein sequences were retrieved from database for rice (both *japonica* and *indica*) and other eight plant species. The *black boxes* indicate identical amino sequences, and the *gray boxes* represent amino sequences with a similarity of more than 50 %. The *asterisk* above the sequence indicates an amino acid conversion from Leu in Minghui63 to Phe in HengnongS-1. The sequences above the *red line* represent the predicted central domain of the transcription factor S-II, which regulates the transcription elongation by RNA polymerase II according to the protein prediction (http://www.ncbi.nlm.nih.gov/cdd)

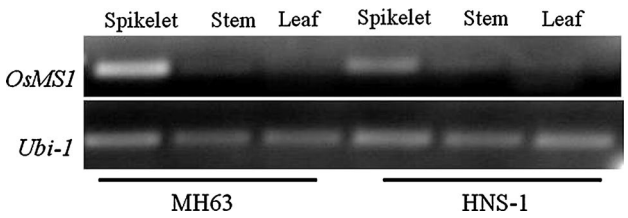

Fig. 4 RT-PCR analysis of the *OsMS1* gene in different tissues of the pre-heading rice plants of Minghui63 and HengnongS-1. The rice *Ubi-1* cDNA served as an internal control

mechanism of fertility conversion. Ku et al. (2003) reported that the male sterility of TGMS is associated with the premature programmed cell death of the tapetum in rice. UDP-glucose pyrophosphorylase1 is essential for callose deposition during pollen wall development, and its co-suppression in plants led to a TGMS phenotype (Chen et al. 2007). It has been recently recognized that *pms3* and *p/tms12-1* confers the PGMS phenotype in Nongken58S and Peiai64S, this gene encodes a long noncoding RNA (Ding et al. 2012; Zhou et al. 2012), suggesting epigenetic regulation of PGMS in rice. Moreover, it was proposed that temperature-sensitive splicing is an important regulation mechanism of TGMS in rice (Chen et al. 2009). All these studies suggest that TGMS is controlled by diverse molecular mechanisms.

In our study, *OsMS1* is most likely the candidate gene for *tms9-1*. The *MS1* gene encodes a PHD finger protein that functions as a transcription factor to regulate pollen and tapetum development in *Arabidopsis* (Ito et al. 2007). The rice *PTC1* gene also encodes a PHD finger protein

that is transiently expressed in the rice tapetal cells and microspores, and the loss of *PTC1* function will ultimately result in complete male sterility (Li et al. 2011). Multiple amino sequence alignment analysis showed that the MS1 protein family share high identity in different plant species, suggesting their conserved functions in regulation of male fertility. The conversion from Leu to Phe in the OsMS1 protein could be responsible for the TGMS phenotype in HengnongS-1. We propose that the mutation most likely loses the normal function of OsMS1 protein in HengnongS-1. The amino acid conversion is located in the predicted central domain of transcription factor S-II, which regulates transcription elongation by RNA polymerase II. We postulate that the amino acid conversion could reduce the stability of the OsMS1 protein under high temperature conditions, but not at low temperatures, resulting in transcription inhibition of target genes in HengnongS-1. Further experiments are necessary to functionally dissect the *OsMS1* gene. In addition, the markers linked with the *tms9-1* gene provide a practical tool for developing new TGMS lines by MAS in rice breeding program.

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Conflict of interest The authors declare that they have no conflict of interest.

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