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# The rice thermo-sensitive genic male sterility gene *tms9*: pollen abortion and gene isolation

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**Abstract** The development of two-line hybrid rice has done great contribution to the food security. It is imperative to study the male sterility mechanism of rice photo-thermo sensitive genic male sterile (PTGMS) line which is the core component of two-line hybrid rice. Zhu1S is a rice thermo-sensitive genic male sterile line used frequently as female parent in two-line hybrid seed production. A cytological examination of the development of the Zhu1S anther wall and the microspores showed that *tms9* encoded male sterility is caused by the failure of the tapetum to degenerate normally, thereby starving the microspores and finally leading to pollen abortion. A fine-scale genetic map based on a large F2 population allowed *tms9* to be located within a 30.2 Kb segment of chromosome 2 harboring seven open reading frames. A comparison between Zhu1S under sterile temperature condition (high temperature, male sterile) and fertile temperature condition (low temperature, fertile) showed that only one of the seven genes, *LOC_Os02g12290*, was differentially transcribed, with its transcript abundance being much lower under the sterile temperature growing condition. *LOC_Os02g12290* encodes a nuclear ribonuclease Z. Re-sequencing demonstrated that the Zhu1S *LOC_Os02g12290* allele differed from that present in non *tms9* carrier alleles by two contiguous nucleotides in the first exon, inducing a truncated mRNA.

**Keywords** Cytological examination · Pollen abortion · Thermo-sensitive genic male sterile (TGMS) · Fine-scale mapping · *tms9* · Rice

## Introduction

The development of F1 hybrid rice cultivars over recent years has raised the yield potential of the crop by about 30 % (Chen et al. 2007). Current hybrid seed production systems are based on either a three-line or a two-line method. The core component of the latter method which enjoys a number of advantages (Sheng et al. 2013), is a photo-thermo sensitive genic male sterile line (PTGMS), which becomes male sterile under an inductive daylength or temperature (Yuan 1992). Rice PTGMS line whose fertility could reversion along with the changes of environmental conditions have attracted much attention of rice breeders and geneticists all the time (Peng et al. 2010), and tremendous progress on the mechanism of rice PTGMS have been achieved in the past few decades.

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Previous researches of rice PTGMS mechanism were mainly focused on cytological characterization of pollen development, such as the characteristics changes of small spore morphology and morphological structures of four layers anther wall cells (epidermis, endothecium, middle layer and tapetum layer) during pollen abortion period (Shi 1985). A critical structure is the tapetum, the function of which is to channel nutrients to the developing microspores (Li et al. 2004). When the timing of the tapetum's degeneration is inappropriate, microspore development is compromised and the pollen aborts (Li et al. 2006). More interestingly, PCD mechanism involves in the tapetum cells degeneration during the pollen abortion period of rice PTGMS lines revealed by TUNEL staining (Ku et al. 2003). By the reason of the earlier initialed tapetum PCD of rice PTGMS lines, together with the slower rate of PCD compared with that of normal rice varieties that caused tapetum could not provide sufficient nutrients for the developing small spores timely, then finally result in the pollen abortion (Li et al. 2011).

Currently, a number of distinct genes responsible for PTGMS have been identified and genetically mapped. However, cases of PTGMS gene cloning are rarely reported, an exception is *pms3*, which was previously mapped on Chr.12 by using Nongken 58S has been cloned (Ding et al. 2012; Zhou et al. 2012). Although numbers of rice PTGMS genes have been mapped, the really cloned of which is rare. In addition, the understanding of rice PTGMS molecular mechanism is very superficial (Peng et al. 2008). Therefore it is of great importance to further identification and excavation new rice PTGMS genes.

The rice thermo-sensitive genic male sterile line (TGMS) Zhu1S is a widely used source of male sterility (Yang et al. 2000). The characteristic change of pollen development cytology between Zhu1S under sterile temperature condition and fertile temperature condition were studied. And a F2 population derived from Zhu1S/D50 (tropical japonica variety) was constructed to fine mapping the TGMS gene *tms9* on the basis of previously mapping. The candidate genes in the fine mapping interval were identified, then expression examination of the candidate genes between Zhu1S sterile and fertile temperature conditions were performed. Furthermore, re-sequencing analysis of the candidate genes was conducted. Finally, one of the candidate genes which encoding nuclear ribonuclease Z was predicted most likely the target gene of Zhu1S. The research results above would lay the foundation for the further function complementary and gene function analysis of rice TGMS gene *tms9* of Zhu1S. Furthermore, the research results would facilitate the breeding process for excellent rice TGMS lines by molecular markers assistant selection.

## Materials and methods

### Plant materials and pretreatments

Zhu1S is an early season rice TGMS line bred from geographically distant hybridization of Kangluozao × ((Kefuhong2 × Xiangzaoxian3) × 02428), which is male fertile when the temperature is below 23 °C, but is sterile above this temperature. Zhu1S plants were grown under natural high temperature condition (sterile temperature condition) in normal rice production season during 2012–2013 in the experimental field of China National Rice Research Institute in Hangzhou, Zhejiang province (Fig. 1). During young panicle differentiation stage, young panicles which within microspores meiosis stage were harvested to extract total RNA for gene expression analysis. At the meaning time, during young panicle initial differentiation stage, ten Zhu1S plants were placed in the fertile temperature condition of 22.5 °C for 10 days, then the young panicle total RNA was also isolated for further genes expression detection. D50 is a tropical *javanica* rice variety with long-grain which was used as male parent of Zhu1S/D50 F2 mapping population.

### Ultrathin ultrastructural examinations

Spikelets and anthers of Zhu1S under both sterile and fertile temperature conditions with different development stages were sampled and fixed for 4 h in cacodylate buffer (pH 7.2) containing 2 % v/v paraformaldehyde (Sigma-Aldrich) and 2 % glutaraldehyde (Sigma-Aldrich). The material was rinsed in the same buffer and post fixed for 1 h in cacodylate buffer containing 1 % w/v osmium tetroxide. After dehydration and embedding in London White Resin (London Resin co., Reading, UK), the material was sliced into ultrathin (40–60 nm) sections, which were collected

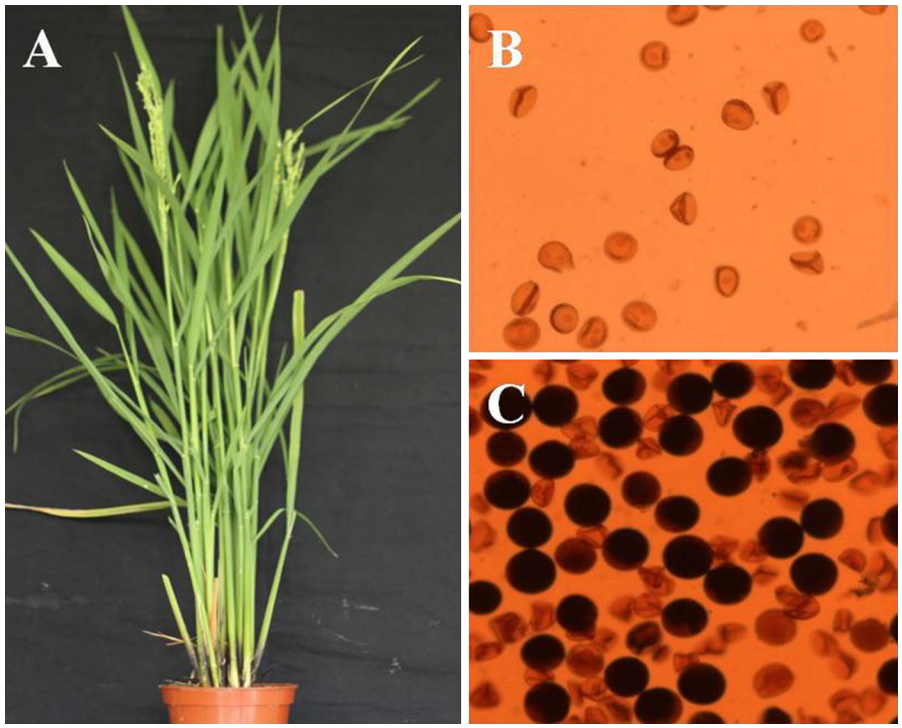



**Fig. 1** Plant morphological and pollen fertility of Zhu1S. **a** The plant morphological of heading period of Zhu1S. **b** Sterile pollens produced under high temperature condition. **c** Fertile pollens produced under low temperature condition

on uncoated nickel grids (300 mesh) and stained in 4 % w/v uranyl acetate. The stained sections were subjected to transmission electron microscopy at 80 kV (JEOL 1200, JEOL, Tokyo, Japan).

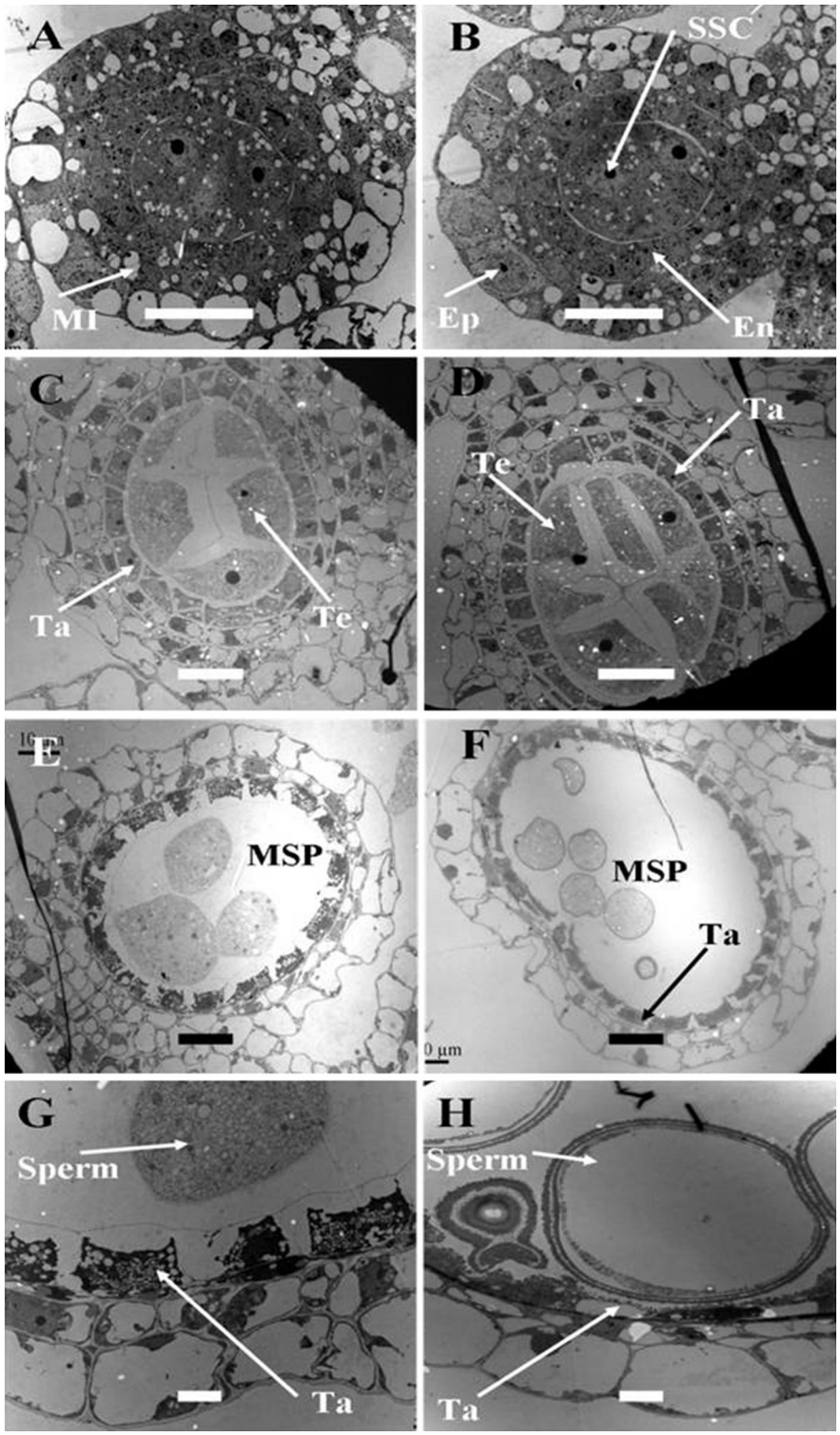
## Genomic DNA exaction and genotyping

Genomic DNA was extracted from fresh-frozen leaves of each sterile plant from Zhu1S/D50 F2 population by using the SDS method (Zhu et al. 2009). Based on a previously obtained map location for *tms9*, 103 indel assays within the critical genomic region were designed from a comparison between the full genome sequences of Nipponbare and 9311. The 103 pairs of In-Del markers were first used to analysis polymorphism between the two parents (Zhu1S and D50), then selected ones were further applied to genotype the male sterile Zhu1S/D50 F2 individuals. Each 10 μL PCR reaction volume contained 25 ng template DNA, 1.0 μL 10 × PCR buffer, 0.1 mM dNTP, 0.1 μM primer pairs and 0.1 μL Taq DNA polymerase. The amplification protocol included an initial denaturation at 95 °C for 3 min, followed by 35 cycles of denaturation of 30 s at 95 °C, annealing of 30 s at 55 °C, and extension of 40 s at 72 °C. The amplicons were electrophoretically separated through a 6 % native polyacrylamide gel, and silver-stained for visualization.

## Transcription analysis

Total RNA was prepared from immature panicles using Trizol reagent (Invitrogen). The subsequent RT-PCRs were based on TaKaRa Ex Taq DNA polymerase, amplifying over 25 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 58 °C, and extension for 90 s at 72 °C, followed by a final extension for 10 min.

qRT-PCR was performed on a Rotor-Gene RG3000A detection system (Corbett Research) using SYBR Green I master mix (Generay Biotech, USA). All PCR experiments were conducted using 40 cycles of 94 °C for 20 s, 58 °C for 20 s, and 72 °C for 20 s, in a reaction mixture containing 10 pmol of each primer, 3 mM magnesium chloride and a 1:10 dilution of each cDNA pool (per biological replicate) as a template. All reactions were performed in triplicate,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◀**Fig. 2** Inappropriate degeneration of the taptum leaded to pollen abortion of Zhu1S. **a**, **b** The submicrostructure of early pollen mother cell stage of Zhu1S under sterile and fertile temperature conditions. **c**, **d** The submicrostructure of tetrads of Zhu1S under sterile and fertile temperature conditions. **e**, **f** The submicrostructure of mature small spores of Zhu1S under sterile and fertile temperature conditions. **g**, **h** The submicrostructure of mature pollen of Zhu1S under sterile and fertile temperature conditions. *SSC* second sporogenous cell, *Ep* epidermis, *MI* middle layer, *En* endothecium, *Ta* tapetum, *Te* tetrads, *MSP* mature small spores, *Sperm* mature pollen grain. *Bars* 5 μm

with the *actin1* gene used for normalization. The transcript abundance of Zhu1S under sterile temperature condition was used as control.

## Results

### Inappropriate degeneration of the taptum leaded to pollen abortion of Zhu1S

Transmission electron microscopy monitoring of microspore development detected no difference between Zhu1S under sterile and fertile temperature conditions until mature microspores stage (Fig. 2a–d). During mature microspores stage, the tapetal layer cells of Zhu1S under sterile temperature condition appeared to have dense cytoplasm, while the tapetum of Zhu1S under fertile temperature condition appeared indistinct and degraded (Fig. 2e, f). By mature pollen period, there were still four layer cells in the anther walls of Zhu1S under sterile temperature condition, the cytoplasm of tapetum further condensed. Conversely, the anther wall of Zhu1S under fertile temperature condition was represented by just the epidermis and endothecium, tapetum was degraded completely, and the anther locules were filled with mature pollen grains (Fig. 2g, h). The implication is that due to the tapetum of Zhu1S under sterile condition unable to undergo normal degradation that lead to the abortion of microspores, and finally caused male sterility of Zhu1S.

### Fine mapping of Zhu1S TGMS gene *tms9*

Based on the primary mapping of Zhu1S TGMS gene *tms9*, a much bigger Zhu1S/D50 F2 population with 7,000 plants was constructed to further fine mapping of *tms9*. Among the 7,000 plants of Zhu1S/D50 F2 population, 1,623 plants performed male sterile. At the meaning time, among the 103 pairs of In-Del markers, 31 pairs of which performed polymorphism between Zhu1S and D50. Then the 31 pairs of In-Del markers with polymorphism were used to further analysis the recombinant individuals of the 1,563 male sterile plants of the Zhu1S/D50 F2 population. Finally Zhu1S TGMS gene *tms9* was fine mapped between markers Indel91 and Indel101, the physic distance between the two markers was 30.2 Kb (Fig. 3; Table 1).

### Identity of the candidate genes of *tms9*

The gene contents within this segment, as obtained from the rice genome annotation project (http://rice.plant.biology.msu.edu), consisted of seven open reading frames (*LOC_Os02g12240, 12,250, 12,260, 12,270, 12,280, 12,290* and *12,300*), five of which (*LOC_Os02g12240, 12,250, 12,260, 12,270, 12,280*) encoded an expressed protein, *LOC_Os02g12290* expressed a nuclear ribonuclease Z and *LOC_Os02g 12300* encoded pectate lyase precursor.

A comparison of transcript abundance between Zhu1S under sterile temperature condition and fertile temperature condition for each of the seven genes showed that there are more or less differences, but the only one which was greatly differentially transcribed was *LOC_Os02g12290* (which encoded nuclear ribonuclease Z) (Fig. 4); for this gene, the level of transcription in the Zhu1S under sterile temperature condition was considerably lower than that in the fertile ones (Fig. 4).

Re-sequencing each of the genes in Zhu1S and other rice cultivars (93-11, Nipponbare, D50) failed to demonstrate any variants at any of *LOC_Os02g12240, 12,250, 12,260, 12,270, 12,280* or *12,300* (re-sequencing primer: Seq-1, Seq-2, Seq-3). However, a sequence polymorphism at 2 contiguous nucleotides in the 24th codon of the first exon of *LOC_Os02g12290* introduced a premature stop codon (Fig. 3; Table 1). Thus, based on both transcription and genome sequence evidences, *LOC_Os02g12290* appears to be the most likely candidate gene for *tms9*.

## Discussion

Rice TGMS has proved to be a very useful source of male sterility in two-line hybrid rice seed production (Shi 1985). While a substantial research effort has

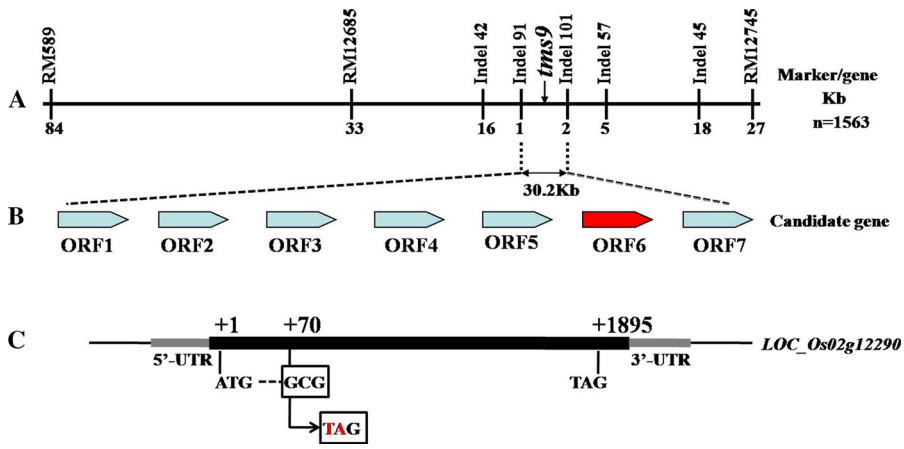


**Fig. 3** Fine mapping and candidate genes prediction of TGMS gene *tms9* of Zhu1S. **a** Fine mapping of *tms9*. **b** Candidate genes prediction of *tms9*. **c** The target gene *LOC-Os02g12290* of *tms9* which has two contiguous nucleotides mutation

**Table 1** Partial information of molecular markers for *tms9* fine mapping and qRT-PCR

| Marker | Forward primer (5′–3′) | Reverse primer (5′–3′) |
|---|---|---|
| Indel 91 | TAAACAAGCAGGAGCTCTGTC | CGACATAAAATGTTTGAGGAAAT |
| Indel 101 | GATGATGATCCGGGTGAC | ATATTTAACTCAAATCTCCAGCA |
| DL-1 | GTCGAGTCACTGGAGCTTGTCGG | ATAGGTGGCATTGGCGTCTG |
| DL-2 | ATGTCGTCGCTCTTGCTGTC | GCCATCATCGTGCTTCATCTC |
| DL-3 | AACCATCCTCGCCGTGTCCC | CGCCACCGTTCAATATCGTAG |
| DL-4 | GTGGACGGCGGCCATACTCC | AGGGCGGTGACCAAAAGCAAC |
| DL-5 | CTCAAACTCGGAGAAGGAAGGAG | GCGTGCTGGTCATCGTCATC |
| DL-6 | GCTCAGCCACAACCTCGTC | GGGAATGGCGTGGTAGGT |
| DL-7 | GAGCCCTACCATCCTCAGCC | CCACGCCCAGTTCTTCCAC |
| Seq-1 | ATCGTGCTTCGTGCCAAA | TTCAGGACTCAAGAGTTAAGGATTC |
| Seq-2 | TCACCCTTCCAACCTCTTAC | GTTTCAGGACTCAAGAGTTAAGG |
| Seq-3 | CAGAGCAGAGGTGCGAGTG | TCAGCAATGAGCAGCAAAGT |

been devoted to mapping and isolating rice male sterility genes, there has been rather less directed at uncovering its mechanistic basis. It has been suggested that the TGMS expressed by Peiai64S is an epigenetic effect (Chen et al. 2013); meanwhile, cloning of the PGMS gene *pms3* from the cultivar Nongken 58S pointed to the possibility that the phenotype was due to a single nucleotide polymorphism which resulted in a reduced abundance of a small non-coding RNA, possibly processed from a long precursor (Zhu and Deng 2012).

It has been proposed that temperature-sensitive RNA splicing may in some cases underlie the temperature induced change from sterility to fertility (Chen et al. 2007). Chen et al. found that there are two homologous *UGPase* genes (*Ugp1* and *Ugp2*) in rice genome. *Ugp1* silencing by RNA interference or co-suppression resulted in male sterility. More interestingly, *Ugp1*-cosuppressing plants contained unprocessed introns containing primary transcripts derived from transcription of the over expression construct, and these aberrant transcripts undergo temperature-sensitive splicing in florets, leaded to a novel thermosensitive male sterility. In addition, Mi-Ok Woo et al. (2008) reported that rice genic male sterility mutant gene *ms-h* is recessive and has a pleiotropic effect on the chalky endosperm. The suppression of target gene *UGPase* by the introduction of a *UGPase1*-RNAi construct in wild type plants nearly eliminated seed set because of the male defect, with developmental retardation similar to the *ms-h* mutant phenotype, whereas over expression of *UGPase1* in *ms-h* mutant

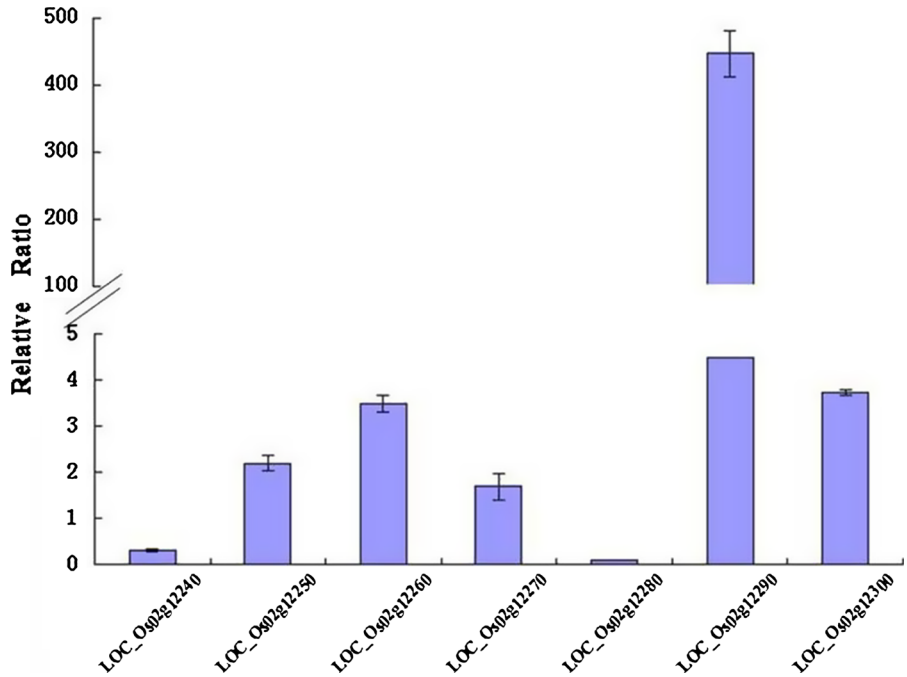


**Fig. 4** qRT-PCR based assessment of the seven candidate genes transcript abundance in Zhu1S between sterile and fertile temperature conditions. The transcript abundance of Zhu1S under sterile temperature condition was used as control

plants restored male fertility. In spite of rice PTGMS mechanism could be elucidated by some researched results, but it could not guide the rapid development of applied research of two-line hybrid rice well. Therefore the enhancing research of rice PTGMS molecular mechanism would be imperative.

Here, we have demonstrated genetically that *tms9* lies within a 30.2 Kb segment of chromosome 2, and that this segment harbors seven open reading frames. A transcription and a genomic DNA sequence analysis both identified the same candidate gene, which is predicted to encode a nuclear ribonuclease Z. Curiously, the same candidate gene has been identified for the PTGMS gene *ptgms2-1* present in the cultivar Guanzhan63S (Xu et al. 2011). The relationship between *tms9* and *ptgms2-1* needs further researches, while the results researched both indicated that the ribonuclease Z gene is most likely the target gene of rice PTGMS. The further *tms*9 interference vector and over-expression vector which used to *tms*9 transgenosis were constructed for analysis the function of *tms9*. The research results above could lay foundation for the further gene function complementary and gene function analysis of *tms9*.

Rice PTGMS line is an important part of two-line hybrid rice system (Chen et al. 2007). An excellent rice PTGMS line can generate many excellent two-line hybrid rice combinations by crossing with many different male parents, which could improve rice yield greatly and ensure food security of our nation (Chen et al. 2010). Zhu1S is an elite early season TGMS line, which has been the female parent of more than 50 two-line hybrid rice combinations that have passed the approval of new rice varieties (Yang et al. 2000). So transduction the *tms9* into other rice genetic foundations could select many excellent rice TGMS lines efficiently by using molecular marker assistant selection. Therefore our recently research results on *tms9* molecular marker development is of great importance in application research of two-line hybrid rice.

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