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Full Length Research Paper

Development of specific simple sequence repeat (SSR) markers for non-pollen type thermo-sensitive genic male sterile gene in rice (*Oryza sativa* L.)

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A key factor of two-line hybrid rice production is the development of thermo-sensitive genic male sterility (TGMS) lines. In this study, a TGMS line showing non-pollen type thermo-sensitive genic male sterility was used. Crossing between TGMS line (female parent) and normal pollen varieties; CNT1 and PTT1 (male parent) were performed and F_1 and F_2 populations were develop for each crosses. The phenotypic segregation ratio, 3:1 (fertile: sterile pollens) observed in F_2 populations of TGMS/CNT1 and TGMS/PTT1 crosses confirmed that the non-pollen type thermo-sensitive genic male sterility is controlled by a single recessive gene. The bulk segregant analysis (BSA) using simple sequence repeat (SSR) markers were deployed to identify the location and genetic effect of this gene. We have generated new set of SSR markers to identify progenies carrying *TGMS* gene in a cross TGMS/PTT1. The TGMS gene was located on chromosome 2 with 0.0 cM distance from T2 marker. This TGMS gene was located in the same region of previous identified *tmsX* gene. It might be the same allele of this gene of 2 different lines. This marker could be used for marker assisted backcrossing to transfer *TGMS* gene for developing female parent in two line hybrid rice system.

Key words: Hybrid rice, thermo-sensitive genic male-sterile, non-pollen type, molecular marker, marker assisted backcrossing.

INTRODUCTION

Rice (*Oryza sativa* L.) is one of the most important cereal crops. Hybrid rice technology exploits the phenomenon of hybrid vigor (heterosis) to increase the yield potential of rice varieties by 15 to 20 % over the commercial cultivars (Virmani et al., 1982; Hwa and Yang, 2008). China started hybrid rice research in 1964 and has been using it in commercial rice production since 1976 (Yuan, 1998). The fertility of thermo-sensitive genic male-sterile (TGMS) rice lines is regulated by environmental temperature changes. The pollens of TGMS line become fertile or sterile when plant has been exposed to lower or higher temperature than a critical sterility point temperature (CSPT). Consequently, its use is ideal as a male-sterile line and maintainer line. The use of a two line breeding system, instead of the three-line breeding system, as well as the discovery and successful application of TGMS lines in hybrid rice breeding has greatly contributed to hybrid rice seed production. The use of the TGMS in two-line breeding is

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Abbreviations: BSA, Bulk segregant analysis; **CMS,** cytoplasmic male sterility; **CSPT,** critical sterility point temperature; **PAGE,** polyacrylamide gel electrophoresis; **PCR,** polymerase chain reaction; **SSR,** simple sequence repeat; **TGMS,** thermo-sensitive genic male sterility.

labor- and time-saving, simple, inexpensive, effective and overcomes the limitations of the cytoplasmic male sterility (CMS) system (Rongbai et al., 2005). However, the introgression of this gene through conventional breeding is quite complicated. It involves the identification of TGMS plants in segregating generations and the subsequent induction of fertility by ratooning at the appropriate temperature. In order to accelerate the development of TGMS lines in different genetic background, marker-assisted selection (MAS) using DNA markers offers an attractive alternative. But to improve the selection efficiency of MAS, identification of tightly linked DNA markers is the pre-requisite (Collard et al., 2005).

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 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 completed than that of typical abortion (Peng et al., 2006). Previous cytological studies indicated that anther abnormal changes appeared earlier in non-pollen abortion than typical abortion during pollen development in male sterile lines. The low temperature triggers the development of non-pollen type uninucleate cell (Peng et al., 2009).

To date, nine TGMS genes have been identified in rice: *tms1* (Wang et al., 1995), *tms2* (Yamaguchi et al., 1997), *tms3* (Subudhi et al., 1997), *tms4* (Dong et al., 2000), *tms5* (Wang et al., 2003), *tmsX* (Peng et al., 2009), *tms6* (Lee et al., 2005), *rtms1* (Jia et al., 2001), and *Ms-h* (Koh et al., 1999), which have been genetically mapped on chromosome 8, 7, 6, 2, 2, 5, 5, 10, and 9, respectively. This information will be useful to identify new *tms* gene or the co-segregation of gene in the study to previous identified genes.

In this study, we aimed to map the TGMS gene and to analyze the inheritance of the TGMS genes in TGMS line and identifying SSR marker linked to this gene. This rice line was provided by Associate Professor Dr. Prapa Sripichitt, Department of Agronomy, Kasetsart University, Thailand. The findings of the present study 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 Thailand.

MATERIALS AND METHODS

Plant materials

The female parent (non-pollen type TGMS line) and male fertile parents (Chai Nat 1; CNT1 and Pathum Thani 1; PTT1) were used for genetic analysis in this study. Based on a relatively high level of polymorphisms between the parents, F_2 population of TGMS/PTT1 cross was selected for molecular mapping of the TGMS gene using bulk segregant analysis (BSA).

Phenotypic characterization of TGMS line

In order to determine the non-pollen critical sterility point temperature (CSPT) in TGMS line, rice plants at stem elongation stage were grown in a growth chamber in which daily mean temperatures were set for 12 h day-length at 20°C and 30°C. Six plants were sampled from each treatment to examine the type of pollen abortion. The plants of TGMS line, CNT1, PTT1, F_1 and F_2 population of each cross were photographed using a digital camera to determine the phenotypic changes of rice plants. Meanwhile, the mature spikelets of each plant were photographed under stereomicroscope. The intact anthers of each plant were squashed in 1 % I_2-KI solution and photographed with a light microscope.

Genetic analysis of TGMS line

TGMS line, CNT1, PTT1, F_1 and F_2 population of each cross were used to study the inheritance of TGMS gene. The parents, F_1, and F_2 populations were evaluated for pollen fertility in April 2010 at Kasetsart University, Thailand. 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, 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 plant. The average pollen fertility was expressed as percentage from three panicles. Plants with less than 5% stained pollens were considered as sterile, whereas all others were regarded as fertile. Chi square (X^2) test was applied to test the goodness of fit of F_2 segregation ratio for fertile and sterile pollen.

Molecular mapping of the TGMS gene

The TGMS gene was mapped with recessive class approach. The polymorphism between the parents was first detected by surveying SSR markers linked to the previous identified TGMS genes. Bulk segregant analysis (BSA) was applied to identify the linked marker to TGMS gene. This method was demonstrated by Collard et al. (2005). Then, the polymorphic markers were further used to detect the polymorphism between the sterile and fertile individuals from each F_2 mapping population. The amplified products were analyzed on the 6 % PAGE gel and detected by silver staining (Benbouza et al., 2006).

The map location of the TGMS gene was finally determined by using the male sterile plants of the mapping population and the polymorphic markers. The recombination frequency (*c*) was calculated with the formula described by Zhang et al., (1994). Genetic map was constructed by using MapChart software (Voorrips, 2002).

SSR analysis

Form the identifying region of TGMS gene, new SSR primers were designed by using sequence information of the TGMS gene region in rice genome (http://www.ncbi.nlm.nih.gov) and simple sequence repeat identification tool (SSRIT; http://wsmartins.net/wabsat). The new SSR primers were used to narrow down the region of TGMS gene.

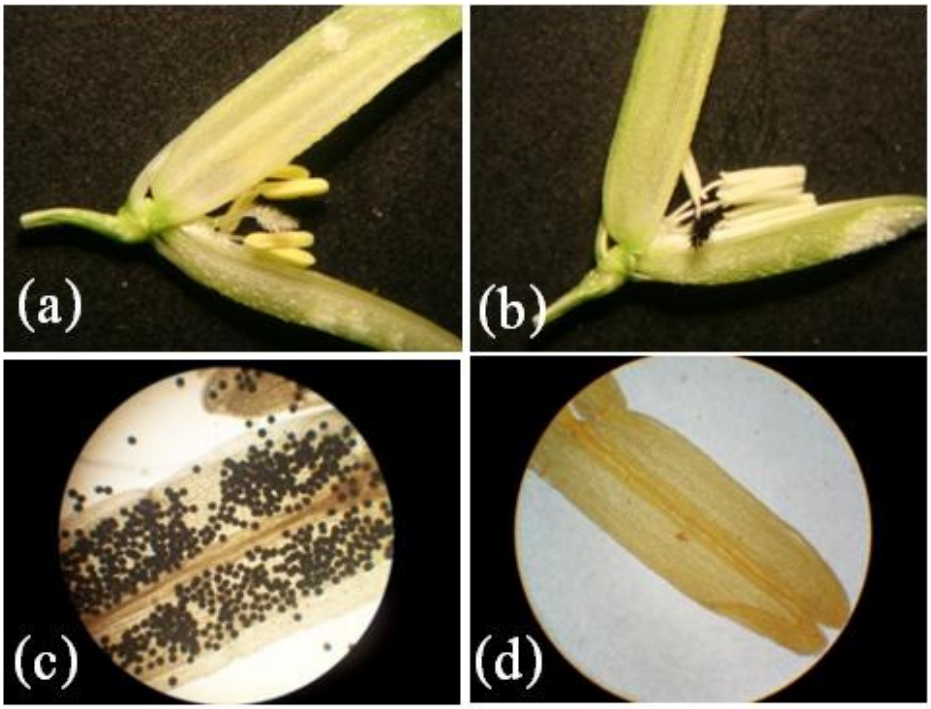

Figure 1. Comparison, normal-type PTT1 and non-pollen type TGMS line. (a) a spikelet of PTT1 with normal anthers;(b) a spikelet of TGMS line with shriveled anthers; (c) a mature anther of PTT1 was squashed with 1% I2-KI solution, in which pollens were round and darkly stained; (d) a mature anther of TGMS line was squashed with 1% I2-KI, in which no pollen was found.

RESULTS

TGMS line is a non-pollen type TGMS line

TGMS line showed male sterility under high temperature but male fertility under low temperature, of critical sensitive point temperature. Pollen abortion type observations at nursery condition showed that this TGMS line belonged to non-pollen type TGMS line. When grown in nursery condition, the average temperature was 30°C, which is suitable for normal growth of rice, anthers in variety PTT1 were normal with yellow color (Figure 1a), while those in the TGMS line were shriveled with white color (Figure 1b). In addition, mature pollens were normal in PTT1 anther (Figure 1c), while no pollen was found in anther of TGMS line (Figure 1d).

Location of the TGMS gene

To analyze the inheritance behavior of the thermo-sensitive male sterile gene of TGMS line, two different F_2 populations were used for segregation analysis. The segregation pattern of fertile to sterile plants in these F_2 populations followed 3:1 ratio (Table 1). The results indicate that a single recessive nuclear gene controls the thermo-sensitive male sterility of this line.

Polymorphisms between female and male parents were identified using already linked SSR markers covering nine previously reported TGMS genes. The RM154 and RM300 markers on chromosome 2 linked to *tmsX* gene showed polymorphism between parental lines and pooled-DNA of sterile and fertile F_2 plants. Subsequently, the polymorphic markers were further used to screen polymorphism between the sterile and fertile F_2 plants individually. The linkages of two polymorphic markers to this gene were further confirmed by using sterile and fertile F_2 plants from the cross TGMS/PTT1 (Data not shown). These markers co-segregated with the *tmsX* gene, reported by Peng et al., (2009). As a result, both markers showed polymorphism and linkage with *tmsX.* Thus, this TGMS line carrying *tmsX* gene which it located between RM154 and RM300 markers on chromosome 2.

For further fine mapping the *TGMS* gene, we designed 5 SSR markers (Table 3) from the region surrounding *TGMS* that reported by Peng et al., (2009). The F_2 population was composed of random selections of sterile and fertile plants from the cross TGMS/PTT1. Unfortunately,

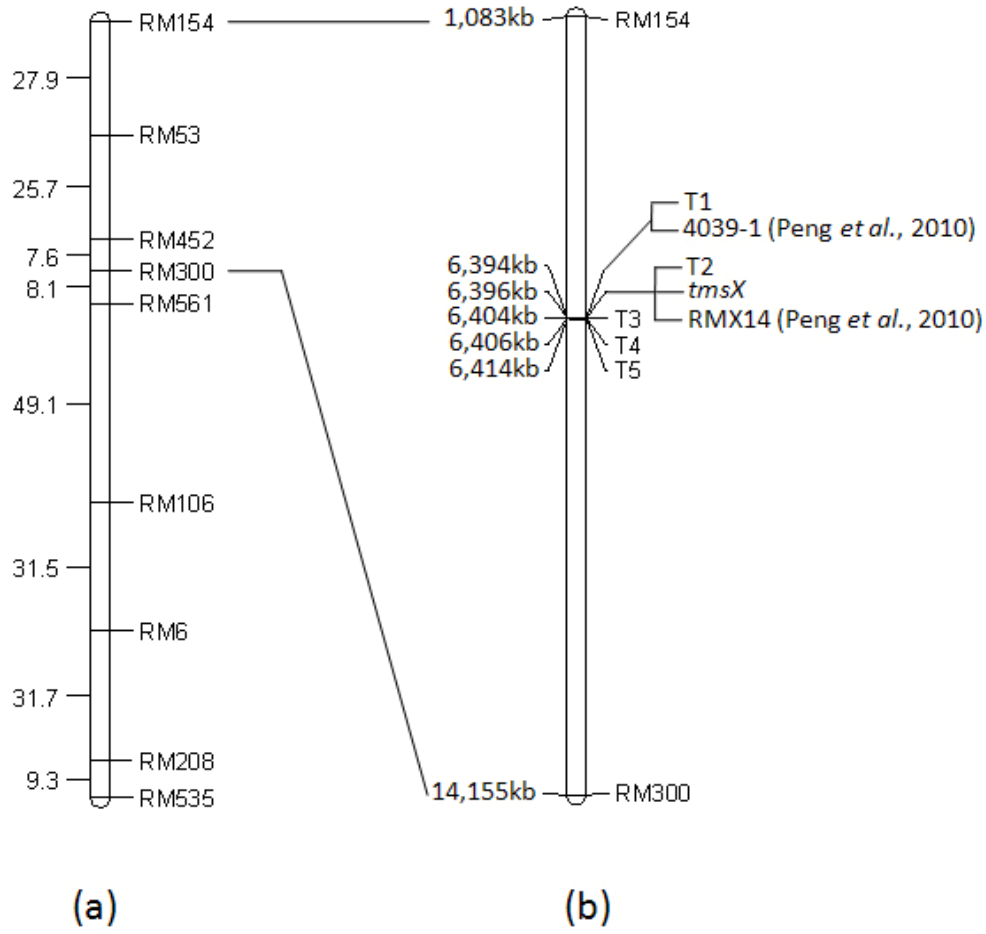

Figure 2. Molecular mapping of the *tmsX* gene, using F_2 population of TGMS/PTT1. (a) genetic map information from http://www.gramene.org and (b) physical map of the *tmsX* gene on chromosome 2 base on http://www.ncbi.nlm. (modified from Peng et al., 2006).

Table 1. The segregation pattern of fertile to sterile plants in these F_2 populations.

Crosse of F_2 population	Number of observed plant			X^2 (3:1)
	Sterile pollen	Fertile pollen	Total	
TGMS/CNT1	43	143	186	0.351^{ns}
TGMS/PTT1	63	168	231	0.636^{ns}

^{ns}: No significant at tested ratio.

only one marker, T2, showed polymorphism between both parents of mapping population. As a result, marker T2 showed that heterozygous and homozygous dominant bands followed 3:1 ratio (Table 2). This type of band was detected in fertile plants. The T2 marker was detected as homozygous allele in all sterile plants. According to the physical mapping information from publicly available resources (http://www.ncbi.nlm.nih.gov), T2 marker is located in the position of 6,396 kb on chromosome 2 of rice.

DISCUSSION

The male sterility of TGMS used in two-line system is mainly regulated by environmental temperature changes.

Table 2. The genotypic score with T2 marker in F_2 population of TGMS/PTT1 cross.

Phenotype	Number of fertile plant		Number of sterile plant	χ^2 (3:1)
	63		30	2.61 ^{ns}
Genotype	Homozygous dominance	Heterozygous	Homozygous recessive	χ^2 (1:2:1)
	22	46	30	2.05 ^{ns}

^{ns}: No significant at tested ratio.

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

Molecular marker	Forward primer (5'-3')	Reverse primer (5'-3')
T1	CTAGGCCCAATTTATTCTCA	AAAACGAGAGAGAGAGACCA
T2	TTCACCCTTCCAACCTCTTA	GATGACTTGCCGCTGTTC
T3	AGGAGGTTCGTCTTCTACCT	ATCCTACACAGCACCATCTC
T4	CCTCCTTCCTGTTCTTCTCT	TTCTGTTAATTTCCCTGCTG
T5	CCTCCTTCCTGTTCTTTTCT	TTCTGTTAATTTCCCTGCTG

Sudden drop in temperature could be disastrous during hybrid seed production because the sterile plants start producing fertile pollens which may lead to self-fertilization of the female parent. Therefore, the stable male sterility is critical for the application of TGMS line (Peng et al., 2009). The cytological studies indicated that abnormal changes in anthers appeared earlier in non-pollen abortion type than the typical abortion type during pollen development in male sterile lines. Thus, the non-pollen abortion is more difficult to reverse from sterile to fertile phase than typical abortion. Under the same critical sterility point temperature, the male sterility of non-pollen abortion is more stable than that of typical abortion, thus, the TGMS lines of non-pollen abortion could be safer than that of typical abortion in two-line hybrid seed production (Feng et al., 2000; Ku et al., 2003; Peng et al., 2006). Moreover, using TGMS lines in hybrid rice production can be simplified and diverse germplasm available for parents.

The *tmsX* gene, a single recessive gene, with stable male sterility was mapped on chromosome 2. The male sterility of this variety belongs to non-pollen type of TGMS (Peng et al., 2010). The TGMS line used in this study showed similar type of male sterility, and also found the same *tmsX* gene reported earlier. It might be the same allele of *tmsX* gene. Identification of markers that co-segregate with the gene of interest can be useful in marker assisted selection. It can be used for selecting the target genotype in new genetic recombination (Sreewongchai et al., 2010). The T2 marker showed polymorphic between parents and it completely co-segregated with *tmsX* gene in TGMS line. Thus, it will be used as molecular marker to select plant carrying this gene in breeding programs.

The genetic background of the two different male parents (CNT1 and PTT1) did not show effect on the expression of *tmsX* gene. The F_2 populations showed segregation between non-pollen and normal pollen phenotype 1:3 when grown at temperature above critical sensitive temperature. This gene could be transferred to varieties with good adaptability and combining ability using backcross breeding program. The linked marker could be helpful for selecting of the favorite allele of the gene.

The DNA markers tightly linked to favorite gene can be cost effectively used for marker-assisted selection of target trait (Collard et al., 2005; Sreewongchai et al., 2010; Wongsaprom et al., 2010). Developing TGMS lines is the basic steps in two-line rice hybrids research. However, some problems are being encountered by breeders in selecting the breeding lines because of the inherent of the TGMS trait was controlled by recessive gene. Plant breeders have been working on the development of an efficient breeding program to generate new TGMS lines. With the availability of tightly linked DNA markers, DNA marker assisted backcrossing was considered a promising approach to solve these problems (Lopez et al., 2003). This germplasm and DNA marker will accelerate the two line hybrid rice research in Thailand.

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