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DEVELOPMENT AND VALIDATION OF GENE-SPECIFIC DCAPS MARKERS FOR A TEMPERATURE-SENSITIVE MALE STERILE GENE *TMS5* IN RICE

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Abstract

With the popularization of two-line hybrid rice, the corresponding two-line rice lines have been studied extensively. Two pairs of derived cleaved amplified polymorphic sequence (dCAPS) markers were designed for the single-base mutation (C/A) of thermo-sensitive genic male sterility (TGMS) rice gene *tms5* in this study to quickly and effectively breed thermosensitive genic male sterile lines, and 48 indica and japonica rice accessions and one F_2 population were used to validate the accuracy of two markers. The results showed that two dCAPS markers could accurately detect the *tms5* gene. The polymerase chain reaction products that could be digested by restriction enzymes corresponded to normal fertile materials, whereas those that could not be digested were related to high-temperature sterile lines. The two dCAPS markers developed in this study can be used to determine whether the rice material is thermo-sensitive sterile and to breed TGMS lines and identify the purity of two-line hybrid rice seeds in marker-assisted selection.

Key words: DCAPS Marker· rice, Temperature-sensitive male sterile, *TMS5*.

Introduction

Rice is one of the most important grain crops in China and a staple food crop for more 50% of the population in China (Kusano *et al*., 2015; Zhou *et al*., 2016). With the improvement in people's living standard, the taste quality of rice has been emphasized increasingly (Lu *et al*., 2010). Therefore, high yield and good quality are important standards for breeding new rice varieties. The heterosis of hybrid rice is especially important to enhance rice yield potential and quality. At present, two hybrid rice systems three-line hybrid rice and two-line hybrid rice exist; the main differences between the two methods are in the selection and breeding of sterile lines. In the two-line method, the male sterile line has the advantages of one-line dual use, free combination, and simple seed production, and thus a rapid development in breeding (Nas *et al*., 2005; Peng *et al*., 2010; Qi *et al*., 2014; Song *et al*., 2016).

In the past, two-line sterile lines were selected based on the field performance during heading and pollen microscopic examinations. The disadvantages for the selection of two-line sterile lines include cumbersome steps, low accuracy, long time, and slow process (Hai, 2012; Liu *et al*., 2001; Xu *et al*., 2011; Yang *et al*., 2007; Zhang *et al*., 2016). However, with the development of molecular biology, molecular markers have been applied to the breeding of two-line sterile lines, greatly improving the efficiency of selection. At present, a large number of rice TGMS lines have been identified and several rice TGMS genes have been cloned, which is a great impetus to the application of two-line hybrid rice (Zhang J *et al*., 2016). The invention is based on the cloned temperature-sensitive male sterile gene *tms5*; a series of gene-specific markers have been developed based on this gene and used to breed TGMS lines in rice. The Institute of Genetics, Chinese Academy of Sciences, used the F8 recombinant inbred line of AnnongS-1 × Nanjing 11, with the male sterile gene mapped in AnnongS-1. The thermo-sensitive male sterile gene was mapped to Chr-II, located between the markers C365 and G227 and named *tms5* (Jia *et al*., 2003). Further, the expressed sequence tag (EST) marker HN57 was found by Southern China Agricultural University, which completely co-segregated with *tms5*. The marker was located at 31.2 cM of chromosome 2 in the 181-kb region between simple sequence repeat (SSR) markers RMAN7 and RMAN54 (Jiang *et al*., 2006; Wang *et al*., 2003). A C-A mutation was found at the position of the 71st base of the Os02g0214300 gene by sequencing of AnnongS-1 and Zhu 1S in South China Agricultural University. This mutation resulted in the loss of RNase ZS1 function. The RNase ZS1 was capable of processing mRNAs of three ubiquitin ribosomal L40 fusion protein genes (UbL40) into multiple fragments *in vitro* and *in vivo*. High temperature elevated the mRNA levels of UbL40 in the *tms5* mutant, while RNase ZS1 function was missing. The excessive accumulation of UbL40 mRNA resulted in reduced pollen yield and male sterility (Jin *et al*., 2020; Pan *et al*., 2014). Wild-type fertility was normal because excess of UbL40 mRNA could be cleaved by RNase ZS1 (Zhou *et al*., 2014).

Tms5 is a gene that lacks function caused by a C-A mutation at the 71st base in the open reading frame. If the 71st base is C, the rice cultivars are fertile under both high and low temperatures. If the 71st base is A, the rice cultivars are sterile under high temperatures and fertile under low temperatures (Ding *et al*., 2012; Fan & Zhang, 2014).

Based on the single-nucleotide mutation (C/A) at the *tms5* locus, which induced thermo-sensitive genic male sterility in rice, we designed two cleaved amplified polymorphic sequence (CAPS) markers that specifically amplified wild-type C and mutant A. It could effectively detect the thermo-sensitive genic male sterile gene *tms5*.

Table 1. Rice accessions used in this study.

No.	Name	Type	Source
1.	Dongjin	*Japonica*	Korea
2.	Liaoxing1	*Japonica*	Liaoning
3.	Nipponbare	*Japonica*	Japan
4.	Xiushui09	*Japonica*	Zhejiang
5.	Huanghuazhan	*Indica*	Guangdong
6.	Kasalath	*Indica*	India
7.	KDML105	*Indica*	Thailand
8.	9311	*Indica*	Jiangsu
9.	ChaotaiB	Maintainer	China
10.	Chuanxiang 29B	Maintainer	China
11.	YixiangB	Maintainer	Sichuan
12.	ZhongzheB	Maintainer	Zhejiang
13.	Chenghui 727	Restorer	Sichuan
14.	Neixianghui 2156	Restorer	Sichuan
15.	Yuehui 9113	Restorer	Hunan
16.	Zhonghui 161	Restorer	Zhejiang
17.	1892S	TGMS	Anhui
18.	2301S	TGMS	Anhui
19.	ZhongguangS	TGMS	Beijing
20.	FulongS	TGMS	Fujian
21.	Shen 08S	TGMS	Guangdong
22.	BPH68S	TGMS	Hubei
23.	C815S	TGMS	Hunan
24.	Guangxiang 24S	TGMS	Hunan
25.	H638S	TGMS	Hunan
26.	LongS	TGMS	Hunan
27.	TannongS	TGMS	Hunan
28.	Xu 98S	TGMS	Hunan
29.	Zhu 1S	TGMS	Hunan
30.	33S	TGMS	Hunan
31.	628S	TGMS	Hunan
32.	Guangzhan63S-4	TGMS	Liaoning
33.	Jingda1S	TGMS	Zhejiang
34.	Ke 08S	TGMS	Zhejiang
35.	MS	TGMS	Zhejiang
36.	Rui 97S	TGMS	Zhejiang
37.	WangS	TGMS	Zhejiang
38.	Yu 03S	TGMS	Zhejiang
39.	Yu 06S	TGMS	Zhejiang
40.	Y04S	TGMS	Zhejiang
41.	ZhanS	TGMS	Zhejiang
42.	089S	TGMS	Zhejiang
43.	128S	TGMS	Zhejiang
44.	134S	TGMS	Zhejiang
45.	152S	TGMS	Zhejiang
46.	163S	TGMS	Zhejiang
47.	764S	TGMS	Zhejiang
48.	PA64S	TGMS	Tianjin

Materials and Methods

Plant materials: Overall, 48 rice accessions, including 4 indica cultivars, 4 japonica cultivars, 4 maintainers, 4 restorers, and 32 TGMS lines were employed in this study (Table 1). A total of 94 F_2 individuals from cross Shen 08S/YixiangB were also used to validate dCAPS markers.

Development of dCAPS markers: According to *tms5* SNP mutation, two dCAPS markers for *tms5* gene were designed using the software Lasergene 7.1 and Primer Premier. It is designed for the design and evaluation of PCR, sequencing primers and hybridization probes. Its main functions are sequence editing, primer design, enzyme digestion analysis and motif analysis. The two dCAPS were dtms51 and dtms52, and their restriction enzyme were *Pvu*I for dtms51 and *Bsi*WI for dtms52 (Table 2).

Polymerase chain reaction (PCR) and restriction enzyme digestion: We performed PCR with a 20-μL reaction system: Tris-HCl (pH 8.8), 33.5mM; $(NH_4)2SO_4$, 8.0mM; $MgCl_2$, 1.5mM; Tween-20, 0.05%; Deoxy-ribonucleoside triphosphates (dNTPs), 0.2mM; both upstream and downstream primers were 3.3 ng/μL; Taq DNA polymerase, 2.0 U; and template DNA, 50 ng. PCR reaction conditions were consistent except for annealing temperature. Specific parameters were as follows: 2 min at 94°C, followed by 30 cycles of 45 s at 94°C, 45 s at 63°C, 1 min at 72°C, and a final extension at 72°C for 8 min. The annealing temperature of dtms51 was 63°C. The annealing temperature of dtms52 was 66°C.

Restriction endonuclease reaction of two labeled PCR products comprised the following: reaction volume 10 μL, including 3.0 U endonuclease, 1.5 μL of 10× buffer, and ddH_2O added to 15 μL. Reaction conditions were as follows: Dtms51-PvuI RE for tms51 at 37℃ for 3 h; Dtms52-BsiWI RE for tms52 at 55°C for 3 h.

Enzymatic cleavage product electrophoresis and color development: The sample buffer was added to the digested product, and 8 μL of each sample was loaded into 3% agarose gel; the electrodes were turned on followed by electrophoresis at 100 V constant voltage for 4 h; subsequently, the electrodes were turned off, and the gel was removed and stained with GelRed nucleic acid staining solution.

Field trials: The F_2 population of Shen 08S/YixiangB was planted at the experimental base of China National Rice Research Institute in Hangzhou, 28 Shuidaosuo Road, Fuyang, Hangzhou. These materials were sown on May 20 and transplanted on June 15 with routine field management. The pollen test was taken for each plant to verify the sterility characteristics under high-temperature conditions in the heading stage.

Results and Discussion

Development of the markers to detect *tms5*: Two dCAPS markers dtms51/*Pvu*I and dtms52/*Bsi*WI were developed for rice *tms5*. The primers for PCR are listed in Table 2, and their position and mutation points are shown in (Fig. 1).

The two dCAPS markers were developed to detect the C/A SNP. The forward primer of tms51 introduced two point mutations that produced a cleavage site of restriction endonuclease *Pvu*I-HF (CGAT/CG). When the PCR product of tms51 was digested with *Pvu*I-HF, the undigested 165-bp band indicated genotype A, whereas the digested 142-bp band indicated genotype C.

Table 2. Primer sequences for PCR and restriction enzymes used in the present study.

Marker	Forward (5–3')	Reverse (5'–3')	Restriction enzyme	Restriction site	PCR product (bp)	RE product (bp)
dtms51	CATCGTGCTTCGTGCCAAAACGG	TCGACGGTGAGGGGCGGCGCGATC	*Pvu*I	CGAT/CG	165	23+142
dtms52	CCCATCGTGCTTCGTGCCAAAAC	TCGACGGTGAGGGGCGGCGCGTAC	*Bsi*WI	C/GTACG	167	20+147

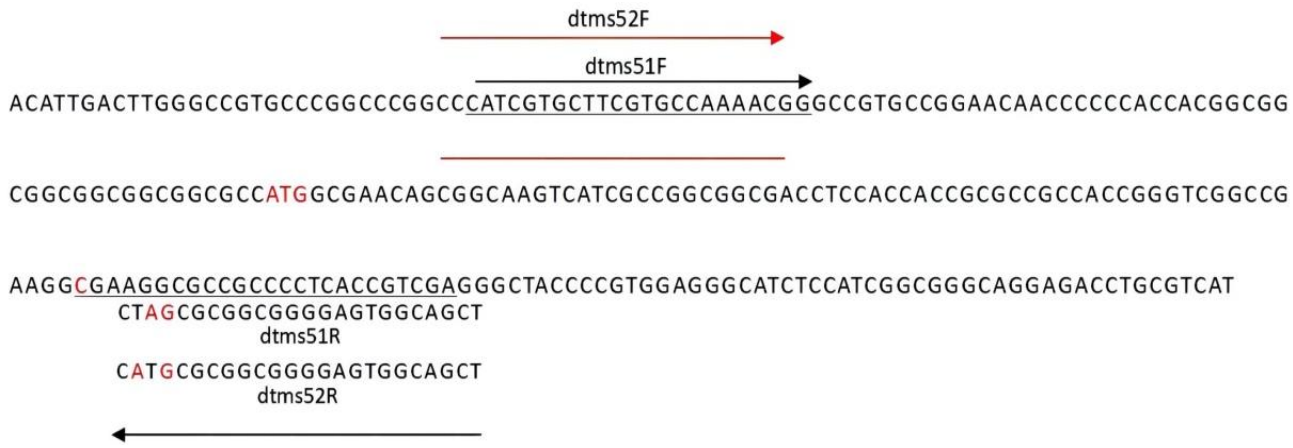

Fig. 1. Os02g0214300 gene and C/A SNP at the 71-bp position.

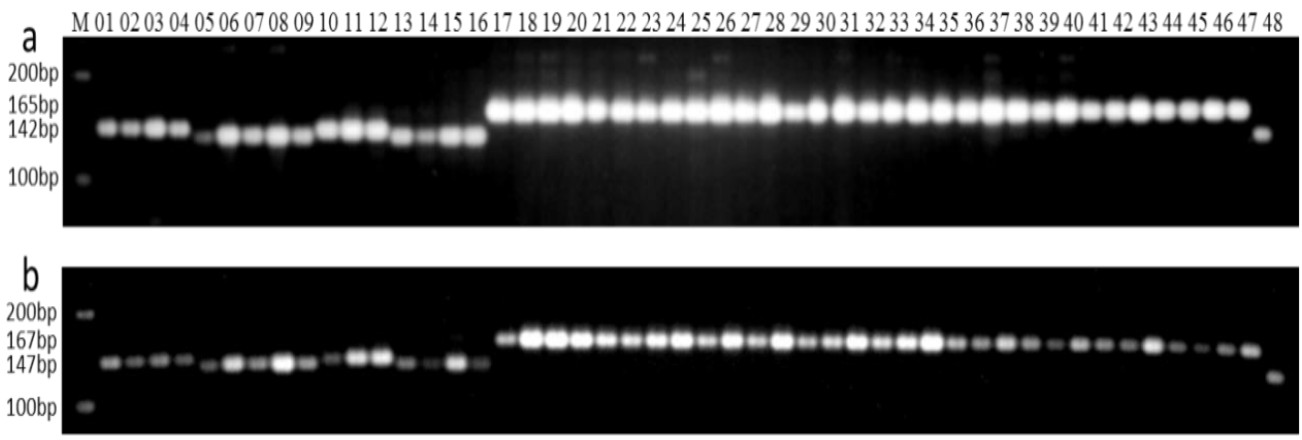

Fig. 2. Gel image showing different dCAPS to detect the C/A SNP among different rice accessions; 1–16 were fertile rice varieties and 17–48 were TGMS. (a) dtms51 amplification and digestion products; 165 bp was *TMS5*, and 142 bp was *tms5*. (b) dtms52 amplification and digestion products; 167 bp was *TMS5*, and 147 bp was *tms5*.

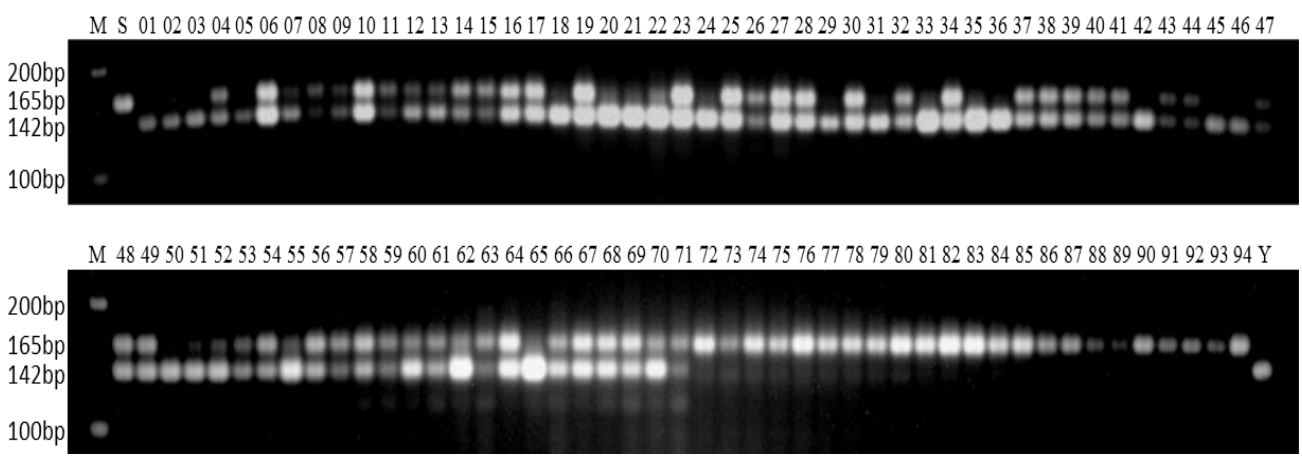

Fig. 3. Application of dtms51 in the detection of thermo-sensitive male sterile plants in the F_2 population of Shen 08S/Yixiang B. 01–71: Carrying either homozygous *TMS5* allele or heterozygous *TMS5*/*tms5* alleles. 72–94: Carrying homozygous *tms5* allele. S: Female parent Shen08S. Y: Male parent Yixiang B.

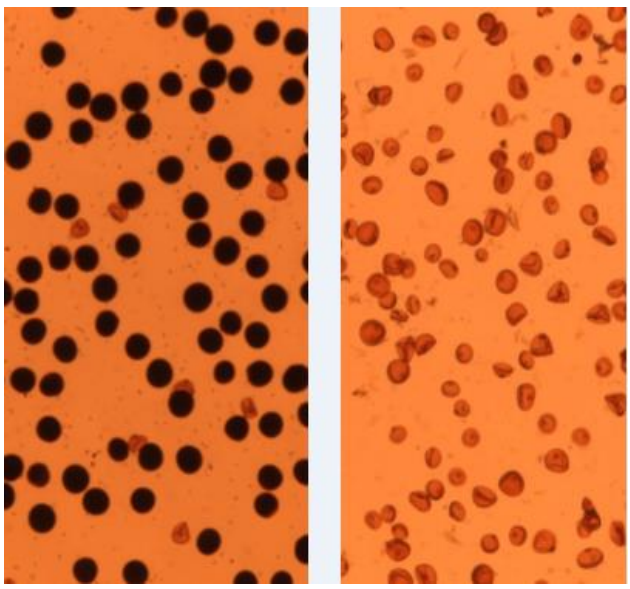

Fig. 4. (A) Pollen grains of fertile plants stained with 1% I_2-KI solution. (B) Pollen grains of sterile plants stained with 1% I_2-KI solution.

The forward primer of tms52 introduced two point mutations that produced a cleavage site of restriction endonuclease *Bsi*WI (C/GTACG). When the PCR product of tms52 was digested with *Bsi*WI, the undigested 167-bp band indicated genotype A, whereas the digested 147-bp band indicated genotype C.

dCAPS analysis of rice accessions: We tested 48 rice varieties to confirm the effectiveness of these dCAPS markers (Fig. 2A). The results showed that PCR products of 16 fertile rice accessions, including Nipponbare, Dongjin, Liaoxing 1, Xiushui09, Kasalath, KDML105, Huanghuazhan, 9311, TaichaoB, ZhongzheB, YixiangB, Chuanxiang 29B, Neixianghui 2156, Chenhui 727, Yuehui 9113, and Zhonghui 161, were specifically digested with *Pvul*-HF, exhibiting that these accessions contained *TMS5*. The PCR products of other accessions except PA64S were undigested, indicating that they had *tms5* related to infertility. PA64S had a noncoding RNA at other loci that could produce small RNAs, showing a P/TGMS trait. It had a different sterility mechanism from other CMS materials (Zhou *et al*., 2012; Ding *et al*., 2012). These two dCAPS markers discriminated the same C/A SNP for the same rice resource (Fig. 2B and 2C). Our results indicated that the two markers dtms51 and dtms52 could specifically recognize the dominant allele *TMS5* and the recessive allele *tms5*.

dCAPS analysis of the F_2 population: The segregation of C/A SNP detected by the dtms51 marker was conducted in the F_2 individuals from the cross between Shen 08S and YixiangB. Shen 08S is a TGMS line, and YixiangB is fertile. Among the 94 F_2 individuals, 22 plants with the same band pattern as YixiangB were fertile, 23 plants showing the same band pattern as Shen 08S were sterile, whereas the other 49 plants having 2 bands were fertile (Fig. 3). The ratio of 22:49:23 conformed to the expected 1:2:1 segregation (Fig. 3). The dtms52 markers displayed the same results. These findings indicated that two dCAPS markers could be used for the selection of temperature-sensitive male sterile lines.

As shown in Figure 4, the pollen of 71 fertile plants No. 01–71 carrying homologous *TMS5* allele or heterozygous *TMS5*/*tms5* were fertile (Fig. 4A), whereas the pollen of plants No. 72–94 carrying homozygous *tms5* allele was abortive (Fig. 4B).

Conclusions

Traditional breeding is mainly dependent on the field phenotype of plants for selection. However, this is influenced by many factors such as environmental conditions, gene interaction, and genotype and environment interaction, so that the selection efficiency is reduced. Besides, the sensitive period of TGMS-induced fertility transformation was from the pollen mother cell to the pollen monocytic stage, the most sensitive period was pollen mother cell meiosis. If the temperature in the fertility conversion sensitive period is low, the fertility fluctuates obviously, affecting the accuracy of selection (Yufang *et al*., 2014). The blindness and uncertainty of traditional selection can be avoided using molecular marker–assisted selection. MAS is not restricted by weather conditions, and the selection can begin in an earlier stage; in the seedling stage, TGMS with the *tms5* gene can be selected.

The new dCAPS markers were successfully developed to detect *tms5* SNP in this study. The development of the two molecular markers could help select the rice temperature-sensitive male sterile lines with the *tms5* gene accurately and efficiently by molecular marker–assisted selection. It can save breeding time, speed up the breeding process, and reduce the cost of breeding. The whole testing process is simple and accurate.

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