Genetics and Molecular Research

Development of SNP-based dCAPS markers for identifying male sterile gene *tms5* in two-line hybrid rice

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ABSTRACT. Molecular markers can increase both the efficiency and speed of breeding programs. Functional markers that detect the functional mutations causing phenotypic changes offer a precise method for genetic identification. In this study, we used newly derived cleaved amplified polymorphic sequence markers to detect the functional mutations of *tms5*, which is a male sterile gene that is widely used in rice production in China. In addition, restriction cutting sites were designed to specifically digest amplicons of *tms5* but not wild type (*TMS5*), in order to avoid the risk of false positive results. By optimizing the condition of the polymerase chain reaction amplifications and restriction enzyme digestions, the newly designed markers could accurately distinguish between *tms5* and *TMS5*. These markers can be applied in marker-assisted selection for breeding novel

thermo-sensitive genic male sterile (TGMS) lines, as well as to rapidly identify the TGMS hybrid seed purity.

Key words: Marker-assisted selection; *tms5* gene; dCAPS; Two-line rice; Purity detection

INTRODUCTION

Discovery and successful application of two-line male sterile rice germplasm resources play important roles in ensuring food safety in China (Dong et al., 2000; Fan and Zhang, 2014). Compared with three-line hybrid rice, two-line hybrid rice is not limited by restore gene(s) and maintainer gene(s), which widely broadens the range of germplasm resources (Nas et al., 2005; Peng et al., 2010; Qi et al., 2014). Therefore, two-line hybrid rice, in general, is superior to three-line hybrid rice, both in quality and in yield. Moreover, the self-reproduction characteristic of two-line male sterile lines simplifies the breeding procedure. Consequently, the growing area of two-line hybrid rice has expanded rapidly in China in recent years (Xu et al., 2011).

Based on the sources of male sterile genes, the two-line rice varieties widely used in production can be divided into two types; photo-sensitive genic male sterile (PGMS) derived from Nongken 58S (Shi, 1985), and thermo-sensitive genic male sterile (TGMS) derived from Annong S-1 (Deng et al., 1999). A single copy gene for PGMS has been cloned by Ding et al. (2012) and Zhou et al. (2012), in which a point mutation of a cytidine-5'-triphosphate in the wild type gene *PMS3* was replaced by a guanosine-5'-triphosphate in the mutated gene *pms3* (or *p/tms12-1*), which resulted in the sterility of Nongken 58S. As reported by Zhou et al. (2014), in TGMS, a cytidine-5'-triphosphate in the wild type gene (*TMS5*) was substituted with an adenosine-5'-triphosphate in the mutated *tms5* within a 71 bp coding region. This led to the sterility of Annong S-1 under high temperature. *TMS5* encodes RNase Z, while *tms5* is a loss-of-function mutant.

Obviously, *pms3* and *tms5* are key genes for breeding of two-line hybrid rice. Use of molecular markers on these two genes can improve both the efficiency and speed of the selection/breeding programs. Based on the functional mutation of *pms3*, a cleaved amplified polymorphic sequence (CAPS) marker was developed to detect the PGMS allele (Ding et al., 2012). For detection of the TGMS allele, Yang et al. (2011) developed an insertion/deletion (InDel) marker named SJ001. However, the InDel marker is located about 5 kbp away from the mutated site of *tms5*. Some inbred rice lines, such as Xieqingzao B, showed false positive results by this marker. Another InDel marker, RMZ-13, designed by Cao et al. (2011), is based on a six-base insertion that is located upstream of the start codon ATG in the *tms5* gene. This insertion was not the functional mutation of *TMS5*. According to sequencing results reported by Zhang et al. (2014a), many varieties of inbred rice, including 08EZ01, Hua201, Hua966, L718, R287, R288, Yi R88, and Zaoxian2430, showed false positive results after detection by the InDel marker RMZ-13. The authors also found three haplotypes, GC, TC, and TA at 70-71 bp of the *RNase Z* coding region. Among these rice lines studied by Zhang et al. (2014a), GC and TC were fertile, whereas TA serves as a *tms5* mutant that causes rice sterility. Zhang et al. (2014a) then developed two derived CAPS (dCAPS) markers (RZ2F1/R and RZ2F2/R), in which restriction enzymes *Hin*fI and *Sty*I were chosen to digest amplicons of wild type lines TC and GC, respectively, but not the TA mutation. In other words, if there were other haplotypes at this locus, *Hin*fI and *Sty*I would be unable to digest them, which still poses a potential risk of false positive detection. However, it is inconvenient to amplify and digest

twice for each sample to infer its genotype, and the scoring of the dCAPS markers is often complicated due to incomplete digestion of amplicons (Zhang et al., 2014b).

In this study, we developed dCAPS markers in which a restriction endonuclease specifically digests amplicons of *tms5*, rather than the wild type, to avoid any false positive detection. The new dCAPS markers can rapidly and accurately distinguish the *tms5* and *TMS5* alleles through a single one-time amplification and digestion. These dCAPS markers can be applied in marker-assisted selection and purity identification for hybrid rice seeds.

MATERIAL AND METHODS

Plant materials and growth conditions

In this study, we used 213 germplasm lines that included 15 TGMS lines and 198 non-TGMS lines. In addition, we used an F_2 population and two hybrid varieties. The 15 TGMS lines included 1892s, MengS, Xin 2S, Guangzhan 63S, Xuan 69S, Guangmo S, Y58S, P88S, Feng 39S, Annong S-1, N422s, H9802s, Xinan S, Shen 08s, and C815S. All these lines were sequenced for the *RNZ* gene (LOC_Os02g12290) and were shown to be male sterile when flowering in summer (July-August) in Hefei city, China; or partially male fertile in spring (mid-February-March) in the Hainan Province, China. Of the 198 non-TGMS lines, restorer lines Yangdao 6 and RH003 originated from Rice Research Institute, Anhui Academy of Agricultural Sciences, whereas the remaining lines came from the USDA Rice Mini-Core Collection (Agrama et al., 2009; Li et al., 2010).

The seeds of hybrid rice varieties Huiliangyou 6 (1892s x Yangdao 6) and Wandao 153 (1892s x RH003) were supplied by Huaan Seed Co., Ltd., China. Two hundred plants per variety were used for purity identification. An F_2 population from the cross of 1892s and Yangdao 6 were tested for co-segregation of the TGMS trait using designed dCAPS markers. All rice lines were planted in the paddy fields of Anhui Academy of Agricultural Sciences in Hefei city, China. The leaves of seedlings were collected for DNA extraction following the method of Li et al. (2013). The flowering stage appeared in August (average temperature >25°C) and panicles of each plant were collected for microscopic examination after staining with I_2-KI solution following Zhou et al. (2014).

Primer design and polymerase chain reaction (PCR) amplification

Primers were designed using the dCAPS Finder 2.0 software (Neff et al., 2002). Since no restriction site adjacent to the mutation site was available, mismatches were introduced into the primers. This was done to create restriction recognition sites on the amplicons of the *tms5* allele only, but not that of the *TMS5* allele.

Moreover, based on the availability and the cost of restriction enzymes, seven selective primers with cutting sites corresponding to endonucleases *Mae*I, *Mbo*II, *Xba*I, *Rsa*I, *Alu*I, *Sca*I, and *Sna*I were chosen (S1-S7 in Table 1). Among these, S1, S2, and S3 align to the sense strand of the gene, whereas S4, S5, S6, and S7 align to the anti-sense strand. These restriction enzymes targeted four primers (T1-T4; Table 1), resulting in 14 combinations of PCR amplifications (Table 2). Three additional target primers (T5, T6, and T7; Table 1) were designed by Primer Premier 5 software (http://www.premierbiosoft.com). All primers were commercially synthesized by Invitrogen Biotechnology Co., Ltd., Shanghai, China.

Table 1. Sequence, mismatch numbers, and mismatch positions of the designed dCAPS primers.

Type	Name	Primer sequence (5' to 3')[a]	Mismatch No.	Mismatch position[b]	Direction[c]
Selective primers	S1	CCGCGCCGCCACCGGGTCGGCCGAAC<u>T</u>	1	2	Sense
	S2	CCGCGCCGCCACCGGGTCGGCCGAAG<u>A</u>	1	1	Sense
	S3	CCGCGCCGCCACCGGGTCGGCCGA<u>TC</u>T	2	2 and 3	Sense
	S4	CTCGACGGTGAGGGGCGGCGCCTT<u>G</u>	1	1	Anti-sense
	S5	CTCGACGGTGAGGGGCGGCGCC<u>AG</u>C	2	2 and 3	Anti-sense
	S6	CTCGACGGTGAGGGGCGGCGCCT<u>AG</u>	2	2 and 3	Anti-sense
	S7	CTCGACGGTGAGGGGCGGCGCC<u>GT</u><u>A</u>	2	1 and 2	Anti-sense
Target primers	T1	CGGGAAGATGACGCAGGT	No	No	Anti-sense
	T2	CGCTCAGCGTCGGGAAGA	No	No	Anti-sense
	T3	GGCGAACAGCGGCAAGTC	No	No	Sense
	T4	GAACAGCGGCAAGTCATCG	No	No	Sense
	T5	GCGGCGGCGCCATGGCGAACA	No	No	Sense
	T6	GTGCCGGAACAACCCCCCACCACGG	No	No	Sense
	T7	CCGGCCCATCGTGCTTCGTGCCAAA	No	No	Sense

[a]Underlined letters indicate mismatched bases. [b]Numbers correspond to positions of mismatched bases relative to the 3' end of the selective primers. [c]"Sense" means that primer sequence aligns to the sense strand of the gene, while "anti-sense" means that primer sequence aligns to the anti-sense strand of the gene.

Table 2. Primer sets, expected product size, restriction enzymes, and recognition sites of the designed dCAPS primers.

Primer set No.	Primer pair (selective + target)	Expected product (bp)	Recognition site	Restriction enzyme
1	S1 + T1	110/83	CTAG	*Mae*I
2	S1 + T2	120/93	CTAG	*Mae*I
3	S2 + T1	110/72	GAAGA	*Mbo*II
4	S2 + T2	120/82	GAAGA	*Mbo*II
5	S3 + T1	110/85	TCTAGA	*Xba*I
6	S3 + T2	120/95	TCTAGA	*Xba*I
7	S4 + T3	94/68	GTAC	*Rsa*I
8	S4 + T4	91/65	GTAC	*Rsa*I
9	S5 + T3	94/70	AGCT	*Alu*I
10	S5 + T4	91/67	AGCT	*Alu*I
11	S6 + T3	94/68	AGTACT	*Sca*I
12	S6 + T4	91/65	AGTACT	*Sca*I
13	S7 + T3	94/69	GTATAC	*Sna*I
14	S7 + T4	91/66	GTATAC	*Sna*I

PCR amplifications and restriction enzyme digestions

Our results suggested several CpG islands in *TMS5* (see below). CpG islands often limit PCR amplifications. A CpG island is defined by >65% G+C content with a GC-rich region >200 bp. In order to obtain ideal amplification results of *tms5*, PCR conditions were optimized. Two DNA polymerases (*rTaq* and PrimeSTAR HS DNA polymerase) and five PCR buffers (10X PCR buffer, 2X GC buffer I, 2X GC buffer II, 5X PrimeSTAR buffer, and 2X PrimeSTAR GC buffer, all buffers contained Mg^{2+}) were purchased from Takara Biotechnology Co., Ltd., Dalian, China. The settings for the *tms5* PCR screenings followed the manufacturer recommendations.

For PCR condition 1 (regular PCR amplification), the amplification was conducted in a 25.0 μL volume, containing 2.5 μL 10X PCR buffer, 1.6 μL dNTPs (2.5 mM each), 1.0 μL each primer (10 μM), 0.2 μL *rTaq* (5 U/μL), 1.0 μL genomic DNA (50 ng/μL), and dH_2O to reach the target volume of 25 μL. For conditions 2 and 3, the 10X PCR buffer was replaced with 12.5 μL 2X GC buffer I, or 12.5 μL 2X GC buffer II, respectively. All other reagents were the same as in condition 1, with the exception of the dH_2O volume adjustment. In condition

4, 5.0 μL 5X PrimeSTAR buffer and 0.25 μL PrimeSTAR HS DNA polymerase (2.5 U/μL) were used to replace the 10X PCR buffer and 0.2 μL *rTaq* in condition 1, while all other components were the same as in condition 1 except the dH_2O volume. In condition 5, 12.5 μL 2X PrimeSTAR GC was used instead of 5.0 μL 5X PrimeSTAR buffer in condition 4. All other reagents were consistent with condition 4, except for the dH_2O volume.

The PCR program consisted of an initial denaturizing step at 95°C for 5 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, and a final incubation step at 72°C for 5 min. The DNA fragments were separated on 4% agarose gel and stained with ethidium bromide.

When digestion was needed, the PCR products were first purified by MiniBEST DNA fragment purification kit from TaKaRa, diluted with 20.0 μL 1X digestion buffer containing 1 U restriction enzyme, followed by incubation for 4 h at 37°C in a water or air bath. After the digestion, the samples were separated by gel electrophoresis on 4% agarose gel or 10% polyacrylamide gel (PAGE). All restriction enzymes were supplied from New England Biolabs.

RESULTS

Amplification of *tms5* fragments with GC-rich PCR buffers

Obtaining bright and specific PCR products are a prerequisite for the development of dCAPS. To amplify the mutation site of *tms5*, the 14 primer sets listed in Table 2 were tested under regular PCR conditions (condition 1, as described above). Five of 14 primer sets resulted in weak PCR bands (Figure 1A). Both primer sets 1 and 2 had a single band that was difficult to see after digestion. Primer sets 9 and 10 had bands with molecular weights inconsistent with the expected results. Primer set 11 had one clear band, but the fragment could not be digested using *Sca*I. Furthermore, the sequencing results for DNA extracted using primer set 11 showed that it had no homology with *tms5*, indicating that the S6/T3 primer set (set number 11) exhibited off-target effects. These results indicate that regular reaction conditions are not able to yield desirable results for *tms5*.

Then, we detected several CpG islands in *TMS5*. The expected PCR amplification region for *tms5* has a CpG island with a GC-ratio reaching 74%, which may result in difficult conditions for conventional PCR procedures. To optimize the PCR conditions at these GC-rich regions, we tried four different kinds of PCR buffers and two kinds of DNA polymerase to form four alternative reaction conditions (conditions 2-5), as described above. Figure 1B-E illustrates the electrophoresis results of the amplified PCR products from conditions 2-5.

Figure 1B showed that seven lanes contained relatively bright bands under condition 2 (*rTaq* in 2X GC buffer I). Five primer sets (1, 2, 3, 4, and 11) showed a single band of the expected size. For primer sets 7 and 8, apart from a target band, there were also some unexpected bands. The other primer combinations showed no bands. To confirm the amplified PCR results, each of the target bands was collected for sequence analysis. The results showed that the fragments taken from primer sets 1, 2, 3, and 4 were consistent with that of *tms5* gene, whereas primer set 11 still showed an off-target phenomenon.

Under the condition 3 reaction (*rTaq* in 2X GC buffer II), not only primer sets 1, 2, 3, 4 and 11 amplified a single band, but also primer sets 7 and 8 showed the same results (Figure 1C). The subsequent sequence analysis confirmed that all lanes were correct, except lane 11.

The results under conditions 4 and 5 (PrimeSTAR HS DNA polymerase in 5X

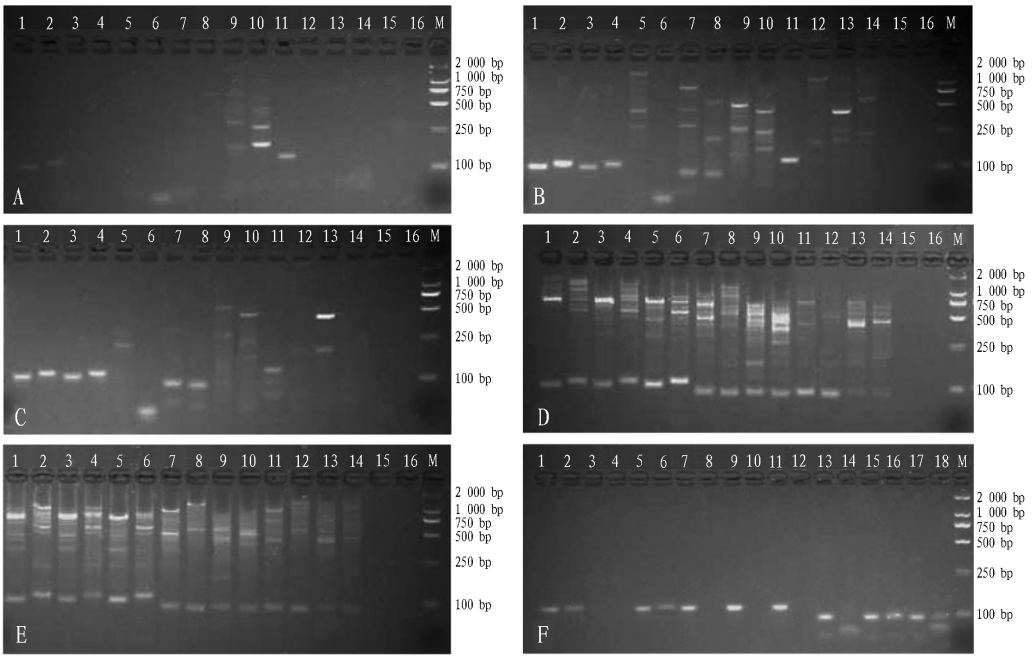

Figure 1. Results of PCR amplifications under different reaction conditions and digestions. **A.-E.** PCR results from conditions 1-5 are presented (as described in the material and methods-section). The number of each lane corresponds with the primer set numbers in Table 2. **F**. Digestion results of PCR amplicons using different restriction enzymes. *Lanes 1-6* are primer set number 2, *lanes 7-12* are primer set number 4, and *lanes 13-18* are primer set number 7. In each set, the DNA templates were derived from different rice varieties. The template in lanes 1, 2, 7, 8, 13, and 14 came from 1892s; the template in lanes 3, 4, 9, 10, 15, and 16 came from Yangdao 6; and the template in lanes 5, 6, 11, 12, 17, and 18 came from the F_1 hybrid of 1892s and Yangdao 6. In **F**, lanes with odd numbers (1, 3, 5, 7, 9, 11, 13, 15, and 17) are undigested PCR products, whereas lanes with even numbers (2, 4, 6, 8, 10, 12, 14, 16, and 18) are digested PCR products. *Lane M* indicates molecular weight ladder of DL2000 (TaKaRa).

PrimeSTAR buffer and 2X PrimeSTAR GC buffer, respectively) are presented in Figures 1D-E. Our results suggest that all lanes could amplify the target 100-bp band, indicating that the PrimeSTAR HS DNA polymerase has a strong ability for *tms5* PCR amplification. However, some unexpected bands with too high molecular weights also appeared in the gel. This suggests that the reaction conditions for this DNA polymerase need to be further adjusted.

The PCR amplification results of the mismatched primers could be found in Figure 1A-E. For selective primers with two mismatched bases (S3, S5, S6, and S7), no ideal band was obtained under any of the five amplification conditions. For primers with one mismatched base, three primers (S1, S2, and S4) could amplify a single target band either under condition 2 or 3 (Figure 1B and C). These results suggest that mismatches close to the 3' end of the primers have a relatively great impact on the PCR amplification results.

Digestion of amplified products of *tms5* with *Rsa*I

Since the selective primers with a single mismatch (S1, S2, and S4) could amplify ideal bands with all target primers with *rTaq* in GC buffer II, we selected two (T2 and T3) of

the four target primers to give three primer sets (sets 2, 4, and 7) to amplify the F_1 hybrid of Huiliangyou 6 and its parents 1892s and Yangdao 6. The amplified products were digested with the corresponding restriction enzymes as listed in Table 2.

When primer set 2 was used to amplify the genomic DNA of 1892s, Yangdao 6, and their F_1 hybrid, the PCR products from 1892s and the F_1 hybrid could not be digested with *Mae*I, whereas Yangdao 6 gave no PCR product (Figure 1F, lanes 1-6). For the PCR products amplified by primer set 4, target bands were bright but disappeared after *Mbo*II digestion (Figure 1F, lanes 8, 10, and 12), suggesting that *Mbo*II may have some effect of asterisk and was not suitable for the development of dCAPS. For PCR products amplified using primer set 7, three bright bands from each template were obtained with the expected molecular weights. After *Rsa*I digestion, the molecular weight of 1892s amplicon was reduced significantly (Figure 1F, lane 14), compared with the band without digestion (Figure 1F, lane 13). Amplicons of Yangdao 6 showed no change and the F_1 hybrid had two bands (Figure 1F, lane 18), which are the features of a co-dominant marker. This encouraging PCR amplification result from primer set 7 is consistent with the design purpose. The bands digested with *Rsa*I did not have a sufficiently high resolution on the agarose gel, due to their small molecular size. Therefore, a 10% PAGE was used to separate the low molecular weight DNA fragments (Figure 2). Among these, lane 4 is the completely digested *tms5* in 1892s, lane 5 is undigested in Yangdao 6, whereas lane 6 is heterozygous F_1 hybrid (*tms5*/*TMS5*) whose complementary bands came from its parents.

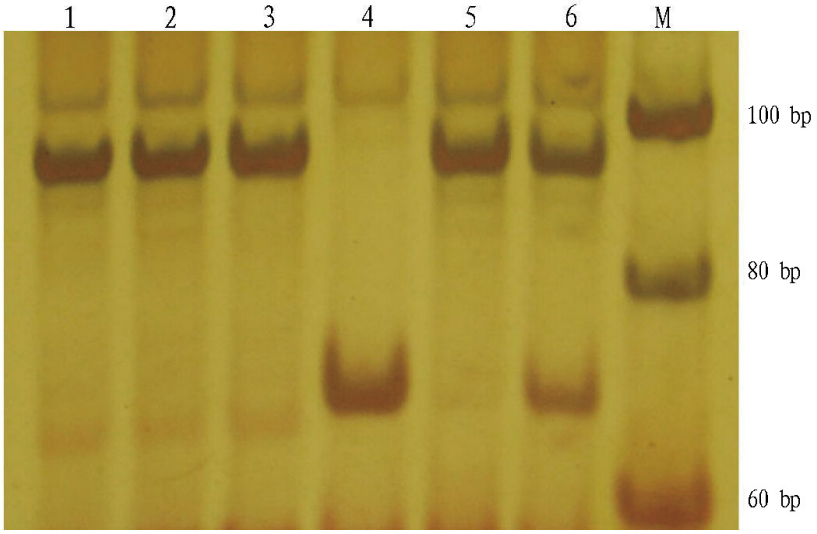

Figure 2. *Rsa*I digested PCR products using primer set 7 on 10% PAGE. *Lanes 1-3* are undigested PCR products and *lanes 4-6* are digested with *Rsa*I (*lanes 1* and *4* = 1892s, *lanes 2* and *5* = Yangdao 6, *lanes 3* and *6* = F_1 hybrid of 1892s and Yangdao 6, *lane M* = molecular weight marker). The molecular weights of the DNA fragments are 94 (*TMS5*, uncleaved type) and 68 bp (*tms5*, cleaved type), respectively.

The digested PCR products amplified with primer set 7 were unclear in agarose gel, due to their low molecular weight. Since agarose gel electrophoresis is popular in many labs, we improved the applicability by designing more target primers (T5, T6, and T7, see Table 1) to increase the molecular weights of the PCR products. These newly designed target primers

were well matched with selective primer S4 to amplify genomic DNAs of 1892s, Yangdao 6, and F_1 hybrid in buffer condition 3 (Figure 3). Only primer set S4/T7 resulted in a clear 172-bp target band, whereas the S4/T5 and S4/T6 primer sets resulted in multiple unexpected bands. When the 172-bp PCR products of S4/T7 were digested using *Rsa*I, our results indicated that the 1892s amplified PCR product could be digested, but not that of Yangdao 6. The digested F_1 hybrid PCR product resulted in heterozygous double bands (Figure 4). All digested bands were clear and polymorphic on the agarose gel. The sequencing results also suggested that the 172 bp S4/T7 fragment was consistent with *tms5*. We named the new dCAPS marker dCAPS-172.

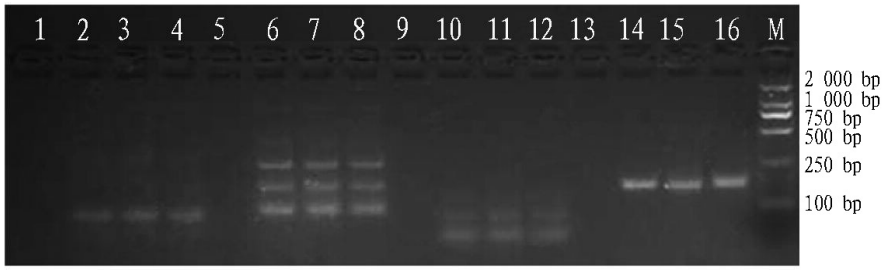

Figure 3. PCR amplification of F4 matching with newly designed target primers. *Lanes 1*, *5*, *9*, and *13* are negative controls; *lanes 2-4*, *6-7*, *10-12*, and *14-16* are PCR products amplified with primer sets S4/T3, S4/T5, S4/T6, and S4/T7, respectively. *Lanes 2*, *6*, *10*, and *14* = 1892s; *lanes 3*, *7*, *11*, and *15* = Yangdao 6; *lanes 4*, *8*, *12*, and *16* = F_1 hybrid of 1892s and Yangdao 6; *lane M* = molecular weight ladder.

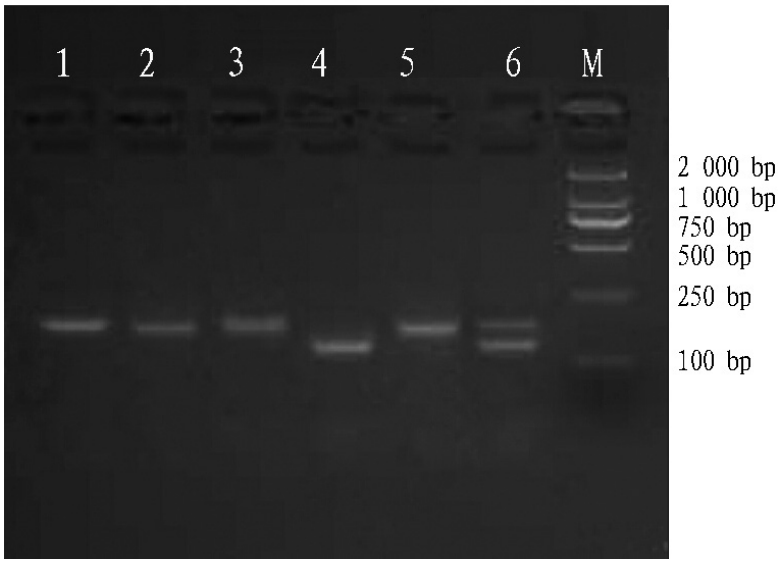

Figure 4. Digested PCR products of S4/T8 separated by 4% agarose gel electrophoresis. *Lanes 1-3* are undigested amplicons from 1892s, Yangdao 6, and F_1 hybrid of 1892s and Yangdao 6. *Lanes 4-6* are *Rsa*I digested amplicons from 1892s, Yangdao 6, and F_1 hybrid. *Lane M* = molecular weight ladder. The molecular weight for the restricted DNA fragments are 172 (*TMS5*, uncleaved type) and 146 bp (*tms5*, cleaved type).

PCR product purifications of restriction digestions are usually arduous, which can limit their usage in high-throughput detection. To simplify the process, 2 U *Rsa*I were directly added to the reaction tubes after the PCR amplification without digestion buffers. The reactions were incubated at 37°C, varying the incubation time from 1 to 16 h. Amplicons could be cleaved correctly in PCR 2X GC buffer II system with no electrophoresis profile changes among the extended digestion times, which indicates that *Rsa*I is suitable for large-scale screening.

Detecting *tms5* with the dCAPS-172 marker

The F_2 population derived from the cross between 1892s and Yangdao 6 was selected to analyze the reliability of the new dCAPS-172 marker. We investigated 192 F_2 population plants and found that 43 plants were sterile, showing the same bands as 1892s (cleaved type). The rest of the plants (149 plants) were fertile. Among these, 52 resulted in the same bands as Yangdao 6 when undigested by *Rsa*I. The other 97 fertile plants showed heterozygous bands, like their parents. The results of the molecular detection were completely consistent with the phenotypic identification.

We also tested the dCAPS-172 marker on the other TGMS lines. Among these, amplicons from 14 *tms5*-type TGMS lines sequenced for the *RNZ* gene could be cleaved correctly by *Rsa*I, but none of the USDA Rice Mini-Core Collections could be digested by *Rsa*I. We also retrieved haplotypes from the *Rsa*I restriction site (GTAG, Chr2:6397410-6397413, MSU 6.0) from sequencing data of 1479 rice accessions including landraces and improved varieties from 73 countries (http://ricevarmap.ncpgr.cn/). Only two haplotypes (GGCG and GTCG) were found that could not be digested by *Rsa*I. In conclusion, these results suggest that the dCAPS-172 marker can be used to test *tms5* allele accurately and that it is suitable for marker-assisted selection.

Purity detection of hybrid rice

Ensuring the purity of hybrid rice is important for hybrid rice production. During seed production of two-line hybrid rice, temperature fluctuations results in a potential problem when the female parent produces selfed seeds in addition to hybrid seeds. In theory, *tms5*-type hybrid rice is heterozygous on the locus, but inbred rice is homozygous. We believe that co-dominant dCAPS markers could be used to detect the purity of hybrid rice. To verify this hypothesis, 200 plants of two cultivars, Wandao 153 and Huiliangyou 6, were planted in a local paddy field. DNA samples from each cultivar were extracted and detected using the dCAPS-172 marker. In four plants of Wandao 153 and seven plants of Huiliangyou 6, a single 146 bp fragment was observed. This was the same band as that observed in cultivar 1892s. Pollen from these 11 plants could not be stained using the I_2-KI solution. In other plants that resulted in heterozygous double bands, the pollen was stained dark by the I_2-KI solution. These results indicated that the dCAPS-172 marker could be a useful tool for identifying the purity of *tms5*-type hybrid rice.

DISCUSSION

In China, both the *pms3* and the *tms5* genes are used as sterile male parents in two-line hybrid rice breeding. In practice, *tms5* appears more popular than *pms3* in rice seed production.

Zhou et al. (2014) found that *tms5*-containing TGMS lines accounted for at least 71% of all two-line hybrid rice cultivars and 83.8% of all the land used to grow two-line hybrid rice in China. Zhang et al. (2015) revealed that most of the two-line sterile lines in China harbored *tms5*. Accordingly, *tms5* serves as the major TGMS genetic resource for two-line hybrid rice breeding.

Genetic markers are DNA fragments that are associated with a certain location within the genome. The use of functional genetic markers is a precise way for genetic identification, because it detects genetic disorders that lead to phenotypic changes. Both CAPS and dCAPS markers are inexpensive and present an effective way to make point mutations or to transform SNPs to PCR-based markers. To make dCAPS markers, the design of selective primers is the first step. In this study, we designed seven selective primers, including three primers with one mismatched base and four primers with two mismatched bases. All primers with a single mismatch could successfully amplify the target band under specific conditions. However, of the four primers with two mismatched bases, three resulted in no amplified product, and one exhibited off-target results. Among the three primers with a single mismatch, two had a mismatched base located at the last position, by the 3' end, and the other had a mismatched base located at the second last position of the 3' end of the primer. The mismatches included G→C, T→A, and C→G. Regardless of the PCR settings, the PCR amplification results from this study suggest that the number of mismatched bases has a greater impact than the mismatch position or the base type.

For some genomic loci that are difficult to amplify, the expected results could be obtained by optimizing the PCR reagents. In this study, 2X GC buffer II was the most suitable buffer for amplification of *tms5*. PrimeSTAR HS DNA polymerase was found to be better at overcoming the mismatches than *rTaq* DNA polymerase. The seven selective primers with one or two mismatch bases were able to amplify target bands by PrimeSTAR HS DNA polymerase, although they often resulted in off-target PCR products. If the nonspecific amplification did not affect the detection results, PrimeSTAR HS polymerase was more suitable for dCAPS than *rTaq* DNA polymerase, although it is a bit more expensive.

Not all the restriction endonucleases investigated here were found to be suitable to digest dCAPS marker amplicons. In this study, we designed dCAPS markers with three different restriction enzymes that digest only the *tms5* mutation but not wild type amplicons, in order to avoid the possibilities of false positives. *Mae*I led to incomplete digestion, whereas *Mbo*II resulted in nonspecific digestion. Only *Rsa*I both cut well and resulted in the expected output.

In summary, we developed a new dCAPS marker that is able to rapidly and accurately distinguish between *tms5* and *TMS5*. We have shown that the dCAPS-172 marker can be applied in marker-assisted breeding selection as well as be used to identify the purity of *tms5*-type hybrid rice seeds. The development of this marker can also provide a useful reference for the design of other dCAPS markers.

Conflicts of interest

The authors declare no conflict of interest.

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