Development and utilization of the functional co-dominant KASP marker for thermo-sensitive genic male sterility in rice *Oryza sativa* L.

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**Abstract** Thermo-sensitive genic male sterility (TGMS) is an important genetic means of two-line hybrid rice breeding. Pollen fertility in TGMS lines is regulated by a single point mutation in TGMS genes. Based on single nucleotide polymorphisms (SNPs) and insertion-deletion mutations, Kompetitive Allele-Specific PCR (KASP) markers were developed and utilized in rice molecular breeding via high-throughput detection, which saves time and money. In this study, we converted the SNPs on TGMS genes (*p/tms12-1* and *tms9-1*) to functional co-dominant KASP markers and bred the resultant two-line hybrid rice. We differentiated the TGMS lines carrying *p/tms12-1* or *tms9-1* from other TGMS lines using the KASP assay. Pei’ai64S and Hua201S (containing *p/tms12-1*) had a homozygous GG genotype generating a blue signal. HengnongS-1 (containing *tms9-1*) had a homozygous CC genotype generating a red signal. The KASP assay for *tms9-1* was identified as a recessive Mendelian trait. Seed purity was tested using the KASP marker for the two-line hybrid varieties of Liangyoupeijiu and Hualiangyou1206, which was consistent with findings by the dCAPS marker. Moreover, new TGMS lines were generated by pyramiding *tms12-1* and *tms9-1* gene under the same genetic background. Therefore, the KASP marker to detect the TGMS genes developed in this study can be widely used in two-line hybrid rice breeding. It provides a visually convenient toolkit for breeders to select individual target plants using high-throughput screening in two-line rice breeding.

**Keywords** Two-line hybrid rice · Kompetitive allele-specific PCR · Thermo-sensitive genic male sterility · SNP · Marker-assisted selection

**Introduction**

Rice is the major food resource for half of the population worldwide (Khush 2001; Yuan 2004). Hybrid rice is cultivated in many counties owing to its potential higher production than traditional inbred lines. Presently, hybrid rice comprises two types as follows: three-line and two-line rice (Cheng et al. 2007). Two-line hybrid rice has many advantages over the conventional three-line one. The advantages include (but are not limited to) self-propagation of photoperiod-thermo-sensitive genic male sterility (P/
TGMS) lines under low-temperature and short-photoperiod conditions and male parents do not require restorer genes, eliminating the adverse cytoplasmic effects on agronomic traits in the hybrid variety (Yuan 1990; Virmani et al. 2003; Si et al. 2011). P/TGMS genes are key factors that regulate the fertility conversion of P/TGMS lines under different environmental conditions. These genes play a crucial role in two-line hybrid rice. Recently, many two-line hybrid rice varieties have been bred and widely applied in grain production in China after the initial identification and development of P/TGMS lines (Si et al. 2011; Mou 2016).

To date, many genes possessing a major P/TGMS trait locus have been mapped and identified into different chromosomes. These include TGMS genes tms1, tms2, tms3, tms4, tms5, tms8, tms6 (t), tms9, tms9-1, and tms in SA2, tmsX, ptgms2-1, as well as PGMS genes pms1, pms2, pms3, and p/pms12-1 (Fan and Zhang 2018). Nongken58S and its PGMS traits have been reported to be regulated mainly by pms3 and p/pms12-1 (Ding et al. 2012; Zhou et al. 2012; Zhu and Deng 2012). Pei’ai64S was also bred from Nongken58S but possessed a TGMS trait from the indica genetic background. In Pei’ai64S, a key single base substitution of C to G in p/pms12-1 results in a loss of function in the RNA osa-smR5864w (Zhou et al. 2012). Tms5 encodes an RNase ZS1 processing the Ubl40 mRNA, which was found in an early indica TGMS line, Annong S-1. A nonsense mutation (C to A mutation) on TMS5 creates a premature stop codon, which is the key factor that controls the TGMS trait in Annong5-1 and derived lines (Zhou et al. 2014). This gene is different from p/pms12-1, tms5 and tms9-1 mapped on the rice chromosome 9, which are other TGMS genes found in an early indica TGMS line, HenongS-1. A single nucleotide polymorphism (SNP) of C to T on the candidate gene OsMS1 results in an amino acid substitution, which regulates the TGMS trait of Hengnong S-1(Qi et al. 2014). Notably, SNPs on different TGMS genes (including p/pms12-1, tms5, and tms9-1) are the key factors controlling the TGMS trait in TGMS and its derived lines. Different TGMS traits were determined through SNPs. SNPs can be converted into functional markers (FMs) for use in marker-assisted breeding programs for the two-line hybrid rice.

SNPs and insertion-deletion mutations (indels) are common mutations in genes that determine many important agronomic traits in rice breeding. Indels are co-dominant markers owing to their detectability via PCR. Various methods have been developed to identify SNPs in rice, such as allele-specific PCR (Gaudet et al. 2009), cleaved amplified polymorphism sequence (CAPS), derived CAPS (dCAPS) (Chen et al. 2009), high-resolution melting (HRM) (Yang et al. 2019a), and temperature switch PCR (TS-PCR) (Thanh and Khoo 2014). With the identification and discovery of large amounts of SNPs on genes that contribute to agronomic traits in rice, the technology for SNP genotyping will provide useful information to breeders in the selection of parent combinations with desirable traits. Competitive allele-specific PCR (KASP) is a single-step genotyping technology based on terminal fluorescence readings similar to Taqman detection (Patil et al. 2017). Both methods require two fluorescent signals at an end-point through laser scanning to detect individual samples with different genotypes. KASP provides different fluorescent signals representing different SNPs on the same locus. KASP markers have been used in a wide range of applications, such as germplasm identification, SNP genotyping, bulked segregant analysis, and marker-assisted selection (MAS), as it has many advantages, including high throughput, high accuracy, good genetic stability, high flexibility, locus specificity, and low cost (Semagn et al. 2014; Mackay et al. 2014; Cabral et al. 2014; Leal-Bertioli et al. 2015; Wu et al. 2017; Yang et al. 2019a; Li et al. 2020; Makhoul et al. 2020). SNPs and indels can directly determine phenotypic traits and can be developed into FMs. Owing to the high efficiency and low cost, the conversion of FMs into KASP markers will provide a convenient toolkit for breeders to select the target individual plants from different combinations within a population.

In this study, SNPs in the two TGMS genes, p/tms12-1 and tms9-1, were converted into KASP markers and validated into MAS for two-line hybrid rice breeding. The newly developed KASP markers can be used to distinguish different TGMS lines, select individual plants from a mixed progeny, test the seed purity of two-line hybrid varieties, and enable the pyramiding of two TGMS genes into the same genetic background. The KASP assay provides a new reliable alternative method for high throughput detection in two-line hybrid rice breeding.
**Materials and methods**

Plant materials and fertility identification

Eighteen widely used TGMS lines for two-line hybrid rice production in southern China were selected to perform KASP assay for TGMS genes. The TGMS trait of Hua201S and Pei’ai64S regulated by p/tms12-1 and that of HengnongS-1 regulated by tms9-1, as well as others regulated by tms5 are presented in Table 1 (Zhang et al. 2014, 2015). To convert the SNPs in tms9-1 and p/tms12-1, two sets of KASP markers were designed for the SNP allele (Table 2). To validate the KASP assay for tms9-1, one cross combination of HengnongS-1 × Minghui63 was used to detect the SNP allele of tms-9 within the F2 population. KASP assay was performed on 164 individual plants within the F2 population. To validate the KASP assay for p/tms12-1, 200 seeds of each hybrid rice variety, including Liangyoupeijiu and Hualiangyou1206, were tested for purity. Liangyoupeijiu and Hualiangyou1206 were bred using Pei’ai64S or Hua201S as the female parent containing p/tms12-1 and tms9-1 into the same genetic background, a combination cross of HengnongS-1 × Hua201 was prepared under natural winter temperature conditions of Hainan Island, China. Individual plants with the TGMS trait were selected from the F2 population and used to generate F3 and F4 via self-propagation under low-temperature conditions. A total of 92 TGMS individual plants from the F4 population were used to detect p/tms12-1 and tms9-1 via KASP assay. All individual plants were grown at the end of May in the experimental farm of Zhejiang Academy of Agricultural Sciences, Hangzhou, China. Pollen from individual plants were collected from the anther at the stage of pre-flowering and stained with 1% I2-KI solution. The fertility of the pollens was identified using a NIKON ECLIPSE E100 light microscope. The plant pollens that remained unstained and irregular were classified as sterile. Conversely, round and dark stained pollens were classified as fertile (Fig. 1) (Qi et al. 2017).

DNA isolation and KASP assay

At the rice heading stage, genomic DNA was extracted from the plant, and the top 1–2 cm of the second leaf was immediately placed in CTAB buffer (Murray and Thompson 1980) according to pollen fertility to reduce potential sampling errors. DNA concentrations were measured using a Nanodrop onec-1000 spectrophotometer (Thermo Fisher Scientific, USA) and adjusted to a working concentration of 100 ng/L. To develop the marker to be used in the KASP assay, different SNP alleles at the same locus were analyzed following a previously described method (Zhou et al. 2012; Qi et al. 2014). To design the KASP marker for genotyping, approximately 200 bp of sequences upstream and downstream of the SNP locus were analyzed. KASP primers were designed following the standard KASP guidelines and obtained from LGC Limited, UK. The standard FAM (5′-GAAGGTGAC-CAAGTTCATGCT-3′) and HEX (5′-GAAGGTCG-GAGTCAACGGATT-3′) primers were used and linked with the allele-specific primer that targets the

| Table1 Photoperiod thermo-sensitive genic male sterile lines used in KASP assay |
|-------------------------------|-----------------|----------------|----------------|-------------------|-----------------|----------------|----------------|----------------|----------------|
| PTGMS lines                  | PTGMS gene     | SNP tms9-1 | SNP p/tms12-1 | PTGMS lines                  | PTGMS gene     | SNP tms9-1 | SNP p/tms12-1 |
| Hua201S                      | p/tms12-1      | T          | G              | Tai1S                       | tms5           | T          | C              |
| Pei’ai64S                    | p/tms12-1      | T          | G              | 1892S                       | tms5           | T          | C              |
| HengnongS-1                  | tms9-1         | C          | C              | Jing4155S                   | tms5           | T          | C              |
| Y58S                         | tms5           | T          | C              | Feng39S                     | tms5           | T          | C              |
| Zhu1S                        | tms5           | T          | C              | Longke638S                  | tms5           | T          | C              |
| LongS                        | tms5           | T          | C              | Chuang55S                   | tms5           | T          | C              |
| C815S                        | tms5           | T          | C              | Xiangling628S               | tms5           | T          | C              |
| FengS                        | tms5           | T          | C              | Guangzhian63S               | tms5           | T          | C              |
| Shen08S                      | tms5           | T          | C              | Guangxiang24S               | tms5           | T          | C              |
SNP at the 3′ end. A common reverse primer was designed using Primer 3 (Untergasser et al. 2012). The final product length was less than 100 bp. For the genotyping assay with KASP, genomic DNA was transferred into a 384-well plate and dried at 65 °C for 30 min. Then a 1 μl KASP assay mixture, which comprised 0.5 μl, 2 × KASP master mixture (Standard Rox, LGC), 0.014 μl of each primer mix, and 0.486 μl H2O, was added into each sample. The PCR reaction was performed using Hydrocycler-16 (LGC Limited, UK) with the following program: Hot start at 94 °C for 15 min, followed by 10 touchdown cycles (94 °C for 20 s; initial touchdown at 65 °C, decreasing by 1 °C per cycle for 25 s), followed by 26 cycles of amplification (94 °C for 20 s, 55 °C for 60 s). The PCR mixture was cooled to room temperature before the fluorescence signal was detected using a PheraStar scanning device (LGC Limited, UK). If no clear result was obtained, additional three cycles of amplification (94 °C for 20 s, 55 °C for 60 s) was performed. When SNPs of p/tms12-1 were genotyped using the KASP assay, the signal detected for the homozygous C/C, G/C, and C/G genotypes were red, blue, and green, respectively. When SNPs of tms9-1 were genotyped, the signal detected for the homozygous C/C, T/T, and C/T genotypes were red, blue, and green, respectively. All KASP assays were performed by Shanghai Baygene Biotechnologies Limited, Shanghai, China. Genotyping data were collected and viewed using SNP viewer2 (LGC Limited, UK).

Table 2 Primers for KASP assay used in this study

| Genes     | Primers   | Primer sequence (5′ → 3′) |
|-----------|-----------|--------------------------|
| P/tms12-1 | Kp/tms12-1-F1 | GAAGGTGACCAAGTTCCATGCTGATAAAATTTTACTCTTGATGGAT |
|           | Kp/tms12-1-F2 | GAAGGTCCGGAGTCAACGGATTTGATAAAAATTTTACTCTTGATGGAG |
|           | Kp/tms12-1-R  | GCATGGTGAAGCAAAGAAGTGCAT  |
| Tms9-1    | Ktms9-1-F1    | GAAGGTGACCAAGTTCCATGCTGCCAAAACGCTGCTAGCCT |
|           | Ktms9-1-F2    | GAAGGTCCGGAGTCAACGGATTTGCCAAAACGCTGCTAGCCT |
|           | Ktms9-1-R     | TCGAGCATGAAGCGGAGGAGGGT |

FAM-probe: GAAGGTGACCAAGTTCCATGCT, FAX-probe: GAAGGTCCGGAGTCAACGGATT

Fig. 1 Pollen fertility of rice under environmental conditions of high temperature and long photoperiod a Unstained and irregular pollen was classified as sterile plant; b: Round and dark stained pollen was classified as fertile plant
Results

Identification of TGMS lines and genetic analysis of \textit{tms9-1} using KASP assay

KASP assay showed that the two TGMS lines (Hua201S and Pei’ai64S) had the homozygous G/G genotype, and the other 16 TGMS lines had the homozygous C/C genotype of \textit{p/tms12-1}. HengnongS-1 had the homozygous C/C genotype, but the others had the homozygous T/T genotype of \textit{tms9-1} (Fig. 2). To validate the KASP marker for \textit{tms9-1}, a cross combination of HengnongS-1 × Minghui63 was prepared. We tested 164 individual plants from this F\textsubscript{2} population for the genotype analysis of \textit{tms9-1} using KASP assay. A total of 31 individual plants had the homozygous C/C genotype representing the TGMS trait; non-TGMS genotypes included 82 individual plants that had the heterozygous C/T genotype, and 51 individual plants that had the homozygous T/T genotype (Fig. 3). Chi square analyses of the F\textsubscript{2} genotypes showed that the segregation ratio of the three genotypes (C/C, C/T, T/T) was 1:2.65:1.65 ($\chi^2_{0.05} = 4.89 < 5.99$, $df = 2$), respectively, which fit the 1:2:1 pattern, indicating that the inheritance of \textit{tms9-1} followed a recessive Mendelian trait.

KASP assay for seed purity in the two-line hybrid rice varieties

To validate the KASP assay for the two-line hybrid rice varieties, two two-line hybrid rice varieties (Liangyoupeijiu and Hualiangyou1206) were selected.
to measure the seed purity of each variety. A total of 200 F₁ individual seeds of both Liangyoupeijiu and Hualiangyou1206 were randomly selected and used to detect the seed purity using KASP markers. The KASP assay showed that 196 individual Liangyoupeijiu plants were heterozygous with the C/G genotype (green signal), three plants were homozygous with the G/G genotype (blue signal, which was the same as the female parent), and only one plant was the homozygous with the C/C genotype (red signal, which was the same as the male parent or other inbred lines plants) (Fig. 4). Pollen fertility analysis of individual plants showed that the plants with a blue signal were sterile males, whereas plants with a green signal and red signal were fertile males. The KASP assay also showed that the genotype of all 200 F₁ plants of Hualiangyou1206 were heterozygous for the C/G genotype (green signal; Fig. 4).

Pyramiding of tms9-1 and p/tms12-1 via KASP assay

To generate new TGMS lines with different TGMS genes, including tms9-1 and p/tms12-1, and detect the TGMS genes using KASP markers, a total of 92 F₄ individual plants containing the TGMS trait from a single cross of HengnongS-1 x Hua201S were prepared. The KASP assay for tms9-1 showed that the genotype of 77 individual plants had the homozygous C/C genotype with a red signal, same as Hengnongs-1, and 13 individual plants had the heterozygous C/T genotype (green signal). However, the genotype of two individual plants could not be determined. The KASP assay for p/tms12-1 showed that 76 individual plants were homozygous with the G/G genotype (blue signal), similar to Hua201S; 15 individual plants were heterozygous with the C/G genotype (green signal), and one individual plant could not be genotyped. Among these, the genotypes of 74 individual plants were homozygous G/G for p/tms12-1 with blue signal and C/C for tms9-1 with red signal (Fig. 5). Therefore, p/tms12-1 and tms9-1 pyramided under the same genetic background via MAS combined with KASP assay.

Discussion

With the development of next-generation sequencing technology, a large number of SNPs have been detected in the diverse rice genome (Kumar et al. 2012). As functional SNPs can directly reflect natural genetic variations in rice accession, a high-throughput genotyping technology for SNPs is needed to allow
breeders to select parents for the desirable traits. Pariasca-Tanaka et al. (2015) identified a total of 2,015 KASP markers, of which, 1,890 can be used for indica rice. According to the sequences of the three principal genes (Wx, BADH2, and ALK) that determine rice quality, a functional KASP marker was developed to improve the quality using marker genotyping combined with a conventional breeding approach (Yang et al. 2019b). Rice breeding lines with low amylose were developed by applying molecular-assisted breeding to backcross populations using KASP markers (Kim et al. 2021). Nine SNPs in BADH2 were identified and developed into KASP markers to examine a total of 369 U.S. rice accessions, which represent the modern breeding germplasm. Of them, a single KASP SNP is unique to haplotype 6 and can be used to differentiate the aromatic traits from the non-aromatic rice varieties (Addison et al. 2020). Many KASP markers were developed and used to analyze the genetic diversity, germplasm classification, population evaluation, and polymorphism detection in Oryza glaberrima/O. sativa L. inter-specific crosses (Yang et al. 2019a; Pariasca-Tanaka et al. 2015). Moreover, a high-density KASP marker based on important agronomic traits was developed and used in genetic analyses and molecular breeding in wheat, cucumber, and cabbage (Makhoul et al. 2020; Fang et al. 2020; Zhang et al. 2020; Li et al. 2020).

The two-line hybrid rice is widely planted in southern China owing to its higher yield and various other advantages over three-line hybrid rice (Si et al. 2011; Qi et al. 2014). TGMS line is the most important genetic resource in two-line hybrid rice breeding. The conversion of its pollen fertility is regulated by TGMS genes in different environmental conditions. To date, although many TGMS genes have been identified and cloned in different rice chromosomes, only p/tms12-1, tms5, and tms9-1 have been used in two-line hybrid breeding in China (Zhou et al. 2012, 2014; Qi et al. 2014). Pollen fertility of different TGMS lines is regulated by the SNPs in TGMS genes. In the present study, we successfully converted the SNPs in p/tms12-1 and tms9-1 into KASP markers for application in two-line hybrid rice breeding. The functional co-dominant KASP assay was used to distinguish TGMS lines conferring the TGMS genes (such as p/tms12-1 or tms9-1) based on the different colored fluorescent signals.

Visual markers were used in genetic analysis to identify tms9-1 and detect the seed purity of the two-line hybrid rice varieties under investigation in our study. The genetic analysis results for the tms9-1 gene were consistent with our previous research (Qi et al. 2014), which validated the KASP assay for tms9-1 for use in two-line hybrid breeding through MAS. The seed purity in two-line hybrid rice varieties was also consistent with our previous study, in which we used a
co-dominant dCAPS marker (Qi et al. 2017). However, high-throughput genotyping and detection via the dCASP marker is difficult and expensive. The results of this study revealed that 384 samples could be detected at the same time using KASP markers. Therefore, KASP markers have distinct advantages over the previously published markers especially in large-scale detection (Zhang et al. 2014; Qi et al. 2017). KASP markers will facilitate breeders to precisely identify the genotype of rice breeds with different TGMS genes rapidly and at a low cost in two-line hybrid rice breeding when combined with conventional breeding. In this study, we used KASP markers to pyramid tms9-1 and p/tms12-1 into the same genetic background to generate new TGMS lines with TGMS traits regulated by tms9-1 and p/tms12-1. The utilization of KASP assay for p/tms12-1 and tms9-1 helps to overcome the difficulty in identifying the TGMS genes in p/tms12-1/tms9-1 TGMS lines for the regular phenotype analysis for pollen fertility.

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Authors’ contribution  YQ and JW conceived and designed the study. YQ, LW, JS, and GM performed the study test. YQ, LW, and JS contributed to the KASP assay. YQ and GM prepared the materials and populations. YQ and JW wrote the manuscript.

Declarations

Ethical Statement  The authors declare that they have no conflict of interest. This study complied with the ethical standards of China, where this research was carried out.

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