Breeding Science 67: 535–539 (2017)
doi:10.1270/jsbbs.16138

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# Development and validation of a functional co-dominant SNP marker for the photoperiod thermo-sensitive genic male sterility *pms3* (*p/tms12-1*) gene in rice

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The fertility conversion of photoperiod thermo-sensitive genic male sterility (P/TGMS) lines in rice is mostly regulated by the P/TGMS genes in different environmental conditions. A point mutation with G-C on the *pms3* (*p/tms12-1*) gene regulates the pollen fertility of Nongken58S and a large amount of Nongken58S-derived lines. In this study, we developed and designed a functional co-dominant marker according to the SNP loci for the *pms3* (*p/tms12-1*) gene. We can differentiate the SNP loci in *pms3* (*p/tms12-1*) gene from another TGMS lines and inbred cultivars using the dpms3-54 marker. The results showed that 376 bp band was detected in the homozygous genotype for *pms3* (*p/tms12-1*), while 359 bp band was detected in the homozygous genotype for non-*pms3* (*p/tms12-1*), two bands with 376 and 359 bp were detected in the heterozygous genotype. The dpms3-54 marker can be used to test the purity of two-line hybrid rice seeds and to divide each of $F_1$ plant into homozygous and heterozygous genotypes at the seedling stage. Thus, this study provide a useful functional marker to detect *pms3* (*p/tms12-1*) gene in different genetic resources and populations, which can be applied to the development and breeding of P/TGMS lines in two-line hybrid rice system with marker-assisted selection.

**Key Words:** *Oryza sativa*, photoperiod-thermo-sensitive genic male sterility (P/TGMS), SNP, MAS, functional marker.

## Introduction

Rice (*Oryza sativa* L.) is one of the most important crops in the world. About more than half populations of the world depend on rice as their staple food (Khush 2001, Yuan 2004). Hybrid rice varieties have been planted in many countries due to the high yield over traditional inbred rice, especially for two-line hybrid rice (Cheng *et al.* 2007). Photoperiod thermo-sensitive genic male sterility (P/TGMS) line is an important genetic resource for the two-line hybrid rice system, which plays a very critical role in the increase in rice yield using the heterosis (Si *et al.* 2011). The P/TGMS lines can produce hybrid seeds under high temperature and/or long-day conditions as female parents, and self-propagated by itself under low temperature and short-day conditions. Thus two-line hybrid rice system has more advantages for seed production than conventional three-line hybrid rice system. Since the past few years, two-line hybrid rice varieties have been widely applied in the hybrid rice planting, popularization and application in China (Huang *et al.* 2015, Si *et al.* 2011).

The fertility conversion of P/TGMS lines is mostly regulated by the P/TGMS genes in different environmental conditions. To date, many P/TGMS genes were mapped and identified on different rice chromosomes. For example, *tms1*, *tms2*, *tms3*, *tms5*, *tms6*, *tms9-1* have been mapped on chromosomes 8, 7, 6, 2, 5 and 9, respectively (Lee *et al.* 2005, Pitnjam *et al.* 2008, Qi *et al.* 2014, Subudhi *et al.* 1997, Wang *et al.* 1995, 2003). *Pms3* (*p/tms12-1*) has been mapped on chromosome 12 and cloned (Ding *et al.* 2012, Zhou *et al.* 2012). In recent years, the underlying mechanisms of P/TGMS genes have been studied and recognized as P/TGMS genes have been cloned. It was reported that one base of G-C substitution on a long non-coding RNA (lncRNAs) of 1,236 bp in length was found in *pms3* gene. The point mutation on lncRNAs regulates the fertility conversion in Nongken58S controlled by *pms3* gene in different environmental condition. The lncRNAs may be involved in epigenetic modifications and cause unstable epigenetic state in Nongken58S (Ding *et al.* 2012). The same mutation was also found in the Nongken58S (PGMS) and Pei'ai64S (TGMS) and designated as *p/tms12-1*. The non-coding RNA encoded by *p/tms12-1* can cause a 21-nucletide small RNA. The mutation can lead to a loss-of-function of small RNA which regulates the fertility conversion for PGMS and

Communicated by Sang-Nag Ahn
Received August 24, 2016. Accepted June 1, 2017.
First Published Online in J-STAGE on November 15, 2017.
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TGMS in the *japonica* and *indica* genetic background, respectively (Zhou *et al.* 2012). A new point mutation from C to A creating a premature stop code has been found in AnnongS-1 and Zhu1S, which regulate the TGMS trait through the loss-of-function of RNase $Z^{s1}$ (Zhou *et al.* 2014). Many previous studies showed that two-line hybrid rice varieties widely planted are mainly bred with the P/TGMS lines containing *tms5* and *pms3*(*p/tms12-1*) in China (Huang *et al.* 2015, Zhang *et al.* 2014, Zhou *et al.* 2014).

Marker-assisted selection (MAS) is an effective and available breeding method to identify TGMS lines, selection for TGMS lines as breeding materials and purity test of two-line hybrid seeds due to the high efficiency and accuracy of selection for the targeted gene (Zhang *et al.* 2014). It is very important to develop the validate SNP markers in two-line hybrid rice system, as one base mutation has been found in different TGMS genes. Breeders use the marker to select breeding lines with the targeted traits in the laboratory to avoid time-consuming and heavy labor in the experimental field.

In this study, we designed a co-dominant functional dCAPS marker named as dpms3-54 based on the SNP in *pms3* (*p/tms12-1*) gene. The dpms3-54 marker can be used to differentiate the *pms3* (*p/tms12-1*) gene from another TGMS lines and inbred cultivars. We also can use the dpms3-54 marker to test the purity of different two-line hybrid varieties seeds bred by TGMS lines containing the *pms3* (*p/tms12-1*) gene as female parents. The high efficiency and validation of the dpms3-54 marker can provide a practical tool for the breeding in two-line hybrid rice.

## Materials and Methods

### Plant materials

N55S, a derived line from Nongken58S containing the *pms3* gene, was selected as the positive control to detect the validation and polymorphism of the dpms3-54 marker between N55S and other rice varieties and lines. Five TGMS lines including Zhu1S, Xiangling628S, 1892S, feng39S, Guangzhan63-4S in which TGMS traits were controlled by *tms5* gene, HengnongS-1 in which the TGMS trait was controlled by *tms9-1* gene and three conventional inbred varieties (Non-TGMS) including Minghui63, Xiushui134 and Nipponbare were selected to detect polymorphism between *pms3* (*p/tms12-1*) gene and others with the dpms3-54 marker. Liangyoupeijiu bred with pei'ai64S containing the *pms3* (*p/tms12-1*) gene as female parent and 9311 as male parent and Hualiangyou1206 bred with Hua201S containing the *pms3* (*p/tms12-1*) gene as female parent and T226 as male parent were selected to test the seed purity of two-line hybrid, respectively. All plants were planted in a paddy field at the experimental farm of the Zhejiang Academy of Agricultural Sciences, Hangzhou, China in the summer season.

### Development of the co-dominant SNP marker

Since the point mutation of G-C on *pms3* (*p/tms12-1*) gene can regulate the fertility conversion of the TGMS lines, the dCAPS marker was designed using the Primer Premier 6.0 software and the web-based tool dCAPS Finder 2.0 (http://helix.wustl.edu/dcaps/dcaps.html) according to the SNP. A mismatch base was introduced at the downstream of 3′ end of the dpms3-54R primer for the PCR amplified fragment can be digested by *Sal* I. The dpms3-54F primer was designed at the upstream of the SNP (**Table 1**).

### DNA extraction, PCR and digestion

Rice genomic DNA was extracted from the plants leaves with the length of 1–2 cm using CTAB buffer with minor modification. The reaction mixture (20 μL) for the PCR analysis consisted of 20 ng of template DNA, 2 μL of 10× PCR buffer, 2.0 μL of 2.5 mM dNTPs, 0.5 μL each of 10 mM primers and 0.5 U of Taq polymerase (supplied by Beijing Dingguo Biotechnology Co., Ltd.), adding up $H_2O$ to 20 μL. The PCR procedure included an initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 30 s and extension at 72°C for 30 s, and a final extension at 72°C for 5 min. After the PCR reaction finished, the amplified-products were digested with the *Sal* I (restriction enzyme (Fermentas)). The mixture for digestion reaction consisted of 10 μL PCR products, 2 μL of 10× digestion buffer, 0.5 U of *Sal* I, adding up $H_2O$ to 20 μL. The digestion reaction was placed in incubator at 37°C for 30 min. The digestion product was separated on a 3.5% agarose gel electrophoresis stained with ethidium bromide, and then the gels were took photo under UV light.

### Fertility examination

Anthers from the pre-flowering spikelets were collected, and the pollen was stained with 1 % $I_2$–KI solution. The anthers were removed from the spikelet, and then placed on glass slides, crushed into pollen powder and observed under a NIKON ECLIPSE E100 light microscope. Pollen fertility was assessed according to the staining results. Plants were considered as sterile if pollens were unstained and irregular, whereas the round and darkly stained pollens were classified as fertile.

## Results

### Development of the functional marker for pms3 (p/tms12-1) gene

According to the DNA sequence alignment between

**Table 1.** The dpms3-54 marker used in the detection of *pms3* (*p/tms12-1*) gene

| Marker name | Forward primer (5′-3′) | Reverse primer (5′-3′) | Restriction enzyme | PCR products (bp) |
|---|---|---|---|---|
| dpms3-54 | GAATGCCATCTAAACACT | ATTTTACTCTTGATGGATGGTC | *Sal* I | 376 |

*pms3* (*p/tms12-1*) gene and Nipponbare, one mismatch base was introduced at the downstream of 3′ end of the dpms3-54R primer. PCR amplification will generate one band of 376 bp with the primers of dpms3-54. While the genotype C exists in non-*pms3* (*p/tms12-1*) templates due to the introduction of one mismatch base, the PCR products containing the sequence of GTCGAC can be digested by *Sal* I enzyme, and then two bands of 359 and 19 bp will be produced. The band of 359 bp can be detected on the 3.5% agrose gel 140 U electrophoresis for 40 min. However, the genotype G exists in *pms3* (*p/tms12-1*) template, the PCR products cannot be digested by *Sal* I enzyme (**Fig. 1**).

### *Validation of the functional marker between pms3 (p/tms12-1) and other rice lines*

To validate the functional marker between *pms3* (*p/tms12-1*) and conventional inbred variety, we selected N55S, Nipponbare and their $F_1$ to detect the polymorphism using the dpms3-54 marker. The results showed that one band of 359 bp was detected since the amplified-product with Nipponbare template was digested by *Sal* I, whereas one band of 376 bp that was detected for the amplified-product with N55S template was not digested; and then the heterozygous bands with two bands were detected in the $F_1$ (**Fig. 2A**). To further confirm the validation of the marker, six TGMS lines controlled by different TGMS genes and two conventional inbred varieties were used to detect the polymorphism using the dpms3-54 marker. The results also showed that only amplified-product with N55S template was not digested by *Sal* I, and all of others were digested (**Fig. 2B**).

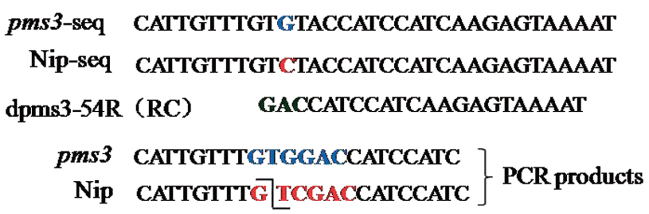


**Fig. 1.** Diagram of the dpms3-54 marker design. Nip indicate Nipponbare, dpms3-54R (RC) indicate the reverse complement sequence of the dpms3-54R primer.

### *Purity test of two-line hybrid seeds*

Two two-line hybrid rice varieties, Liangyoupeijiu and Hualiangyou1206, are the most wildly planted in China for many years. We randomly selected 200 $F_1$ individual plants of them to detect the seed purity using dpms3-54 marker, respectively. The detection results showed that 196 individual plants were heterozygous genotype with two band of 376 and 359 bp, and three individual plants were homozygous genotype with one band of 376 bp same with female parent (*pms3* (*p/tms12-1*)), but only one individual plant was homozygous genotype with one band of 359 bp same with the male parent in Liangyoupeijiu hybrid seeds (**Fig. 2C**). So, the seed purity of Liangyoupeijiu was 98% with the test of dpms3-54 marker. To further prove the validity of the seed purity with the pms3-54 marker, the fertility of three individual plants with the band of 376 bp and one individual plant with the band of 359 bp were examined with the staining results under the high-temperature and long-photoperiod environmental condition. The results showed that the pollen of three individual plants with the band of 376 bp were sterile; however, the pollen of one individual plant with the band of 359 bp was fertile, suggesting that the test of seed purity with the pms3-54 marker was valid. The test of seed purity of Hualiangyou1206 also showed that all $F_1$ individual plants were the heterozygous genotype at dpms3-54 marker locus suggesting their high seed purity.

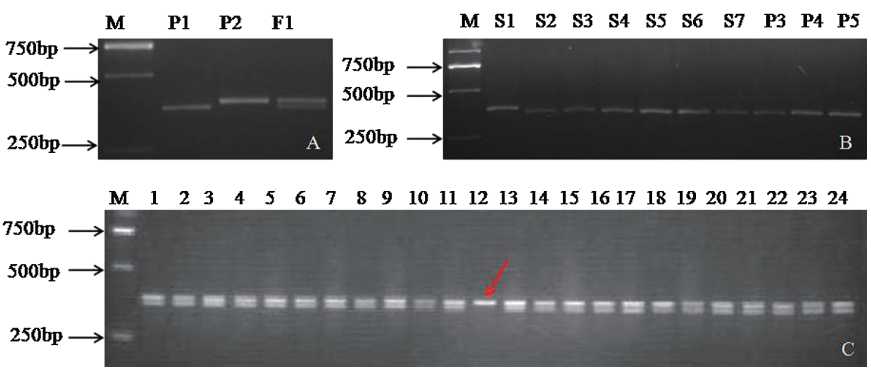


**Fig. 2.** Detection pattern of different rice accessions and the seed purity with the dpms3-54 marker. A. Detection pattern of N55S, Nipponbare and $F_1$. N55S with one band of 376 bp, Nipponbare with one band of 359 bp, $F_1$ share two bands of 376 and 359 bp. B. Detection pattern between N55S and other rice accessions including five TGMS lines and three inbred varieties (S1-7: N55S, Guangzhan63-4S, Feng39S, Xiangling628S, HengnongS-1, Zhu1S, 1892S P3-5: Minghui63, Xiushui134, Nipponbare). N55S with one band of 376 bp, other rice accession with one band of 359 bp. C. The seed purity test with the dpms3-54 marker in Liangyoupeijiu. Red arrow indicate the individual plant with one band of 376 bp same with female parent (Pei'ai64S) which is produced by self-propagation. Other individual plants share two bands of 376 and 359 bp which have the heterozygous genotype.

## Discussion

P/TGMS lines are very important genetic resources for the two-line hybrid rice system. Nongken58S and a large number of Nongken58S-derived P/TGMS lines were wildly used in hybrid rice production (Si *et al.* 2012). Although there are different explanations in the regulatory mechanism of pollen fertility in *pms3* and *p/tms12-1*genes, a point mutation with G-C on the lncRNAs controls the pollen fertility under different environmental conditions (Ding *et al.* 2012, Zhou *et al.* 2012, Zhu and Deng 2012). In this study, we designed and developed a functional co-dominant maker which can be used to analyze the genotype for the SNP loci on the *pms3* (*p/tms12-1*) gene in different rice accessions. In previous study, a molecular marker also has been developed according to the SNP in *pms3* (*p/tms12-1*) gene (Zhang *et al.* 2014). *Sal* I (Fermentas) used in this study is a regular restriction enzyme which is commonly used in all molecular biology laboratories due to low price and fast digestion reaction. Digestion reaction can be finished for 30 mins, so it is very fast and inexpensive. Compared to the previously published marker, dpms3-54 marker with low price and fast digestion reaction was used in this study. We can differentiate the SNP loci in *pms3* (*p/tms12-1*) gene from other TGMS lines and inbred cultivars using the dpms3-54 marker. It would be easy to operate and low cost and save time. It is also suitable to detect *pms3* (*p/tms12-1*) gene in a large amount of populations for two-line hybrid rice system.

The fertility of P/TGMS lines and the breeding materials is controlled by the temperature and photoperiod during the pre-flowering stages. However, a sudden drop in temperature can cause the fertility conversion of P/TGMS lines from sterility to fertility, P/TGMS lines can propagate to produce seed by itself, which can affect the two-line hybrid seed purity and result into the disastrous for seed production (Peng *et al.* 2010, Zhang *et al.* 2014). In general, two-line hybrid seeds are produced in the south of China in every summer and autumn under the high temperature and long-photoperiod environmental condition. The produced rice seeds were planted at the Hainan Island in winter and spring to detect the seed purity according to the phenotype of the hybrid rice varieties, which can be easily affected by the environmental factors including soil, temperature, humidity, photoperiod and so on resulting inaccuracy. In this study, we can use the dpms3-54 marker to test the seed purity of two-line hybrid rice and show the genotype for each of $F_1$ individual plant at the seedling stage. Three individual plants with the band of 376 bp in Liangyoupeijiu $F_1$ seeds which showed homozygous genotype and phenotype with the pollen sterility under high-temperature and long-photoperiod environmental condition were found. They are the seeds of pei'ai64S in which the pollen grains in a little part of spikelets was conversed to be fertile and sterile plants were self-propagated in the course of Liangyoupeijiu $F_1$ seeds production. One individual plant with the band of 359 bp in Liangyoupeijiu $F_1$ seeds which showed homozygous genotype and phenotype with the normal pollen fertility under the same environmental condition was also found, it is possible that other inbred rice variety or male parent seeds were mixed into the Liangyoupeijiu hybrid seeds in the course of seed production. It is evident that the functional co-dominant SNP marker developed in this study is highly efficient and precise to identify the allele of *pms3* (*p/tms12-1*) gene. This marker can be applied into a large number of seed purity tests which can be handled in a short period of time. Therefore, we can identify the seed purity with the functional co-dominant marker in advance to avoid the loss by the impurity of hybrid seeds.

## Acknowledgements

This study was supported by the Project of the Science and Technology Department of Zhejiang Province, China (grant number 2015C32051, 2016C02050), the Project of the Molecular Designed Japonica Breeding with High Quality and Yield in the Middle-Low Reaches of Yangzi River (Grant Number XDA08030104) and the Special Fund of Zhejiang Academy of Agricultural Science. The authors would like to thank the breeders for providing the rice varieties and TGMS lines.

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