# *pms*3 is the locus causing the original photoperiod-sensitive male sterility mutation of 'Nongken 58S'*

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**Abstract** Photoperiod-sensitive genic male sterile (PSGMS) rice is a very useful germplasm for hybrid rice development. It was first found as a spontaneous mutant in a japonica cultivar 'Nongken 58'. *pms*3 on chromosome 12 was determined to be the locus where the original PSGMS mutation occurred, changing the normal cultivar Nongken 58 to PSGMS Nongken 58S. Large amounts of RAPD and AFLP analyses were also conducted for the fine mapping of the *pms*3 genomic region, which resulted in 4 molecular markers linked to *pms*3. Although these markers somewhat increased the marker density of this region, the *pms*3 locus is still located in a marker-sparse region.

**Keywords: photoperiod-sensitive genic male sterile (PSGMS) rice, gene mapping, restriction fragment length polymorphism (RFLP), randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP).**

Photoperiod-sensitive genic male sterile (PSGMS) rice 'Nongken 58' (abbreviated as 58S) was discovered as a spontaneous mutant from a japonica (*Oryza sativa* ssp. *japonica*) rice cultivar 'Nongken 58' (abbreviated as 58N) in 1973[1]. Studies have established that this mutant is completely male sterile under long-day conditions, and shows various degrees of male fertility under short-day conditions. Thus it is able to propagate itself under short-day conditions, and also can be used as a male sterile line to produce hybrid seeds under long-day conditions by interplanting it with normal varieties. Now great efforts have been made to develop 'two-line' hybrid rice making use of this PSGMS rice to replace the widely used 'three-line' system.

A large number of studies have been conducted on the inheritance of the photoperiod-sensitive male sterility. Results from several dozens of crosses have consistently shown that the fertility segregation is controlled by two Mendelian loci in crosses between 58S and many japonica varieties used in the studies[2], and single locus segregation was observed in crosses between 58S and 58N or a few other late japonica varieties[3]. It has now been generally accepted among rice breeders that the genetic change associated with the photoperiod sensitive male sterility between 58N and 58S was a mutation of a single gene.

Studies have also been conducted to determine the chromosomal locations of the PSGMS genes. Results from a morphological marker-based genetic analysis of crosses using 58S as the PSGMS parent

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suggested a linkage between one of the PSGMS loci and a locus for dwarfism on chromosome 5[4]. However, the RFLP-based analysis of Zhang et al.[5] showed that the two loci segregating for male sterility were located on chromosomes 3 and 7, using a cross between Minghui 63, an indica variety, and 32001S, an indica PSGMS line selected from a cross between IR8 and 58S. They also determined that the effect of the locus on chromosome 7 (*pms*1) was much larger than the one on chromosome 3 (*pms*2), whereas the results of Wang et al. (1997)[6], who tested linkage between molecular markers linked to the *pms*1 locus and male sterility in an $F_2$ population of a cross between 58S and 58N, showed that *pms*1 is not the locus for the PSGMS mutation that changed 58N to 58S.

Recently, Mei et al.[7], using RFLP-based analyses of $F_2$ populations from the crosses of (58S × 1514) and (58S × Lunhui 422), showed that the two loci segregating for male sterility in both populations are located on chromosomes 7 and 12, respectively. They also determined that the locus on chromosome 7 was the same as the *pms*1 locus identified previously by Zhang et al. (1994)[5], but the locus on chromosome 12 was new, designated as *pms*3.

The study reported in this paper was undertaken to resolve the location of the mutant locus associated with fertility difference between 58S and 58N. We also tried to fine map the genomic region of the *pms*3 locus where the PSGMS locus segregating between 58S and 58N was located, according to the results of this study.

## 1 Materials and methods

### 1.1 Experimental populations and fertility examination

Experimental materials were the $F_2$ populations of two crosses (58S × 58N) and (58S × 1514). The seeds of 58S were from the original photoperiod-sensitive genic male sterile rice. '1514' is a japonica variety selected from 'Pei C115'. The parents and $F_2$ populations of the two crosses were planted in the experimental fields in the 1995—1997 rice growing seasons under natural long-day conditions in Wuhan. Seed-setting rates and pollen fertility were examined and used as the fertility scores. In addition, the fertility reversal of the sterile plants in the $F_2$ populations under short-day conditions (late in the growing season) was also examined to provide additional evidence for photoperiod sensitive male sterility.

### 1.2 DNA extraction and preparation of the bulks

Fresh leaf tissues were harvested from the plants growing in the fields. DNA extraction followed essentially the cTAB method[8]. Two bulks, a sterile bulk and a fertile bulk, were prepared for each of the $F_2$ populations. The sterile bulk was made by mixing equal amounts of DNA from 10 highly sterile plants of the population and the fertile bulk was made by mixing equal amounts of DNA from 10 highly fertile plants. The bulks were used to screen for molecular markers, such as RFLP (restriction fragment length polymorphism), RAPD (randomly amplified polymorphic DNA) and AFLP (amplified fragment length polymorphism), that are likely to be linked to the PSGMS loci.

### 1.3 Molecular marker assay

1.3.1 RAPD analysis. A total of 1 047 random decamers were used as primers for RAPD analysis. Of the 1 047 primers, 1 010 were purchased from Operon Technologies (USA) including 490 from OPA to OPZ series and 520 from OPAA to OPAZ series, and 37 (RA series) were synthesized in our

laboratory. Single primer assay was performed using the two bulks from the (58S × 1514) cross with all 1047 primers according to the method of Williams et al.[9]. Double-primer RAPD analysis was performed with 710 primer pairs, by combining the 30 RA primers with 9 Operon primers. DNA fragments that were polymorphic between the sterile and fertile bulks were cut off the agarose gel under long wave UV lamp, cleaned using the Sephaglas™ Bandprep kit (Pharmacia Biotech) and cloned using the Invitrogen Original TA cloning kit (Invitrogen Corporation).

1.3.2 AFLP analysis. Together, 266 pairs of *Eco*RI + 3 and *Mse*I + 3 primers were used in this study. AFLP analysis followed essentially the methods by Vos et al.[10]. Briefly, 1.25 μg of total cellular DNA per sample was doubly digested using 5 units each of *Eco*RI and *Mse*I. The digests were ligated to the adapters specifically designed for the enzymes. All fragments containing *Eco*RI-ends were collected using magnetic beads and pre-amplified with 'plus one' primers (i.e. *Eco*RI + 1 and *Mse*I + 1), and the pre-amplification product was collected and used as the template for subsequent AFLP survey. AFLP reactions were conducted using an MseI + 3 primer and a [$\gamma$-$^{32}$p] ATP labeled *Eco*RI + 3 primer. The reaction products were separated on a denaturing polyacrylamide gel. After electrophoresis, the gel was dried and exposed to an X-ray film.

1.3.3 RFLP analysis. Approximately 2.5 μg of DNA for each sample was digested with 6—21 restriction endonucleases. DNA digestion, electrophoresis and Southern blot hybridization followed the methods described previously[11].

## 2 Results

### 2.1 The PSGMS mutant locus of 58S and its genetic effect

2.1.1 Fertility segregation of the $F_2$ population in the cross between 58S and 58N. The fertility showed a discontinuous and bimodal distribution in the $F_2$ population (173 plants) grown under the natural long-day conditions of Wuhan in 1995 (fig. 1), which fit the 3∶1 ratio for a single Mendelian locus inheritance ($\chi^2 = 0.002$, $P > 0.90$). A repeated examination of this population (120 plants) in the summer of 1997 consistently showed a single locus segregation ($\chi^2 = 0.011$, $0.70 < P < 0.90$). Such typical single Mendelian locus segregation was in accord with the results of many previous studies[2,3].

2.1.2 Determination of the mutant locus of 58S. Mei et al. (1998)[7] obtained 33 markers (positive markers) in a survey of bulked extremes from the $F_2$ populations of (58S × 1514) and (58S × Lunhui 422), using 306 RFLP probes selected from the Connell and Japanese Rice Genome Research Program (RGP) maps[12,13]. Nine of the positive markers were linked to *pms*1 on chromosome 7, and 24 were linked to *pms*3 on chromosome 12. Wang et al.[6] determined, using markers linked to the *pms*1 locus, that the *pms*1 locus did not segregate in the $F_2$ population of (58S × 58N).

In order to determine the location of the mutant gene of 58S, all the positive markers from chromosome 12 were surveyed for RFLP between 58S and 58N with up to 21 restriction enzymes. Two probes, R2708 and R643, detected polymorphism between 58S and 58N with *Dra*I, *Bgl*II and *Sca*I; the variant bands resolved by R2708 were co-dominant and that by R643 were dominant (band present in 58N and absent in 58S). Further survey of the sterile and fertile bulks from the $F_2$ population using

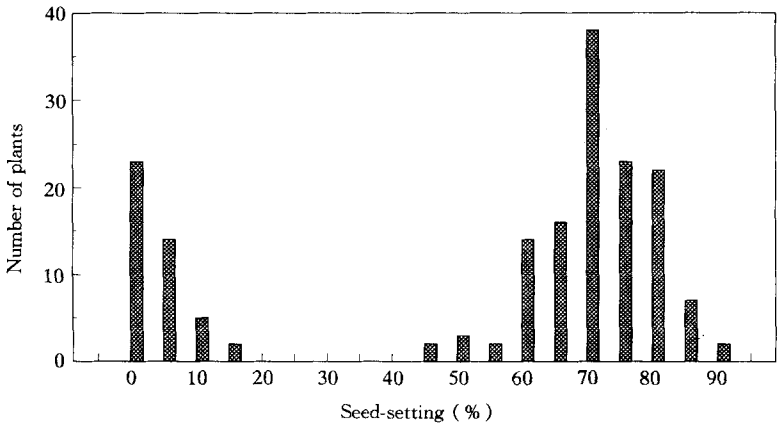


Fig. 1. Distribution of fertility in the $F_2$ population of (Nongken 58S × 58N) under natural long-day conditions in Wuhan observed in the 1995 rice growing season.

both probes revealed polymorphism between the two, thus indicating that both R2708 and R643 were linked to the locus controlling fertility segregation in the $F_2$ population of this cross.

To determine the distance between the two markers and the PSGMS locus, 37 highly sterile plants from the $F_2$ population of (58S × 58N) cross was assayed individually with R2708 and R643. R2708 recovered 7 plants heterozygous for bands from the two parents, the same 7 plants contained the dominant band of 58N resolved by R643. Because R2708 and R643B were located in the same position in the RGP map[13], we assume that these 7 plants were also heterozygous, i.e., the two markers co-segregated in the 37 sterile plants. Using a maximum likelihood estimator[14], we calculated the genetic distance (9.56 cM) between the two markers and the PSGMS locus. This value was very consistent with the results obtained from study of the (58S × 1514) cross (9.54 cM using the data of 1995 and 8.31 using the data of 1996)[7]. Thus, clearly, the locus causing fertility segregation between 58S and 58N is the same as the *pms*3 locus identified in our previous study. The chromosomal location of this locus is illustrated in figure 2.

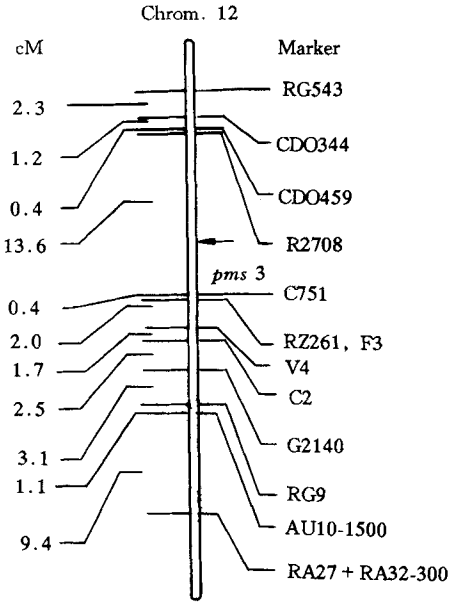


Fig. 2. The location of *pms*3 in the molecular marker linkage map. F3 and V4 are markers derived using AFLP technique and AU10-1500 and RA27 + RA32-300 are obtained using RAPD technique. The remaining are RFLP markers whose locations are adapted from Mei et al.[7].

2.1.3 Analysis of genetic effect of *pms*3. To assess quantitatively the effect of the *pms*3 locus on fertility, an one-way analysis of variance was performed with the three genotypes of R2708 locus as the groups using the fertility scores of 120 plants from the $F_2$ of the (58S × 58N) cross (table 1). The effect of the locus as

marked by R2708 was highly significant using both seed-setting and pollen fertility as the criteria. Further comparison of the fertility scores of the three genotypic classes revealed very large differences among the three groups. The fertility was very low when the R2708 locus was homozygous for the band from 58S, and high when R2708 was homozygous for the band from 58N. However, individuals heterozygous at the R2708 locus was only semi-fertile, as opposed to the expectation of being highly fertile based on a complete dominance effect at the PSGMS locus. It should also be noted that the genetic effect of the *pms*3 locus was obviously underestimated due to recombination between the molecular marker and the PSGMS locus.

Table 1 One-way analysis of variance of the effect on male sterility of *pms*3 based on the marker genotypes at the R2708 locus

| Fertility | MS effect | MS error | *F* | *P* |
|---|---|---|---|---|
| Pollen | 38816.52 | 754.16 | 51.47 | 0.000 1 |
| Seed-setting | 42656.5 | 401.70 | 106.12 | 0.000 1 |

There are 2 degrees of freedom for the effect and 117 degrees of freedom for the error term.

Table 2 Fertility for each of the genotypes of the *pms*3 locus as marked by R2708 based on 120 plants from an $F_2$ population of (58S × 58N)

| R2708 genotype | Number of plants | Pollen fertility (%) (mean ± SD) | Seed-setting rate (%) (mean ± SD) |
|---|---|---|---|
| 11 | 28 | 11.40 ± 27.57 | 7.73 ± 17.51 |
| 12 | 57 | 61.06 ± 29.13 | 59.48 ± 23.85 |
| 22 | 35 | 80.24 ± 24.38 | 80.03 ± 14.20 |

11, Homozygote for the allele from 58S; 22, homozygote for the allele from 58N; 22, heterozygote.

## 2.2 Further mapping of *pms*3 region

Although we used up all RFLP markers in the *pms*3 region that were available from the two published maps, the molecular markers in this region were still sparse[7]. Therefore, we conducted large amounts of RAPD and AFLP screening with the objective of fine mapping this region.

2.2.1 RAPD screening. Eight of 1 047 primers used in single primer RAPD analysis amplified polymorphic fragments between the sterile and fertile bulks made of the individuals from the $F_2$ population of (58S × 1 514). Polymorphic bands were also detected in 6 of the 710 double-primer reactions. When the polymorphic RAPD fragments were cloned and used as RFLP probes, two clones (OPAU10-1500 and RA27 + RA32-300) detected polymorphism in the bulked extreme analysis, indicating that the fragment in each of the two clones was from a region linked a PSGMS locus. The remaining 4 clones did not detect polymorphism in the survey.

2.2.2 AFLP screening. Altogether, 253 of the 266 AFLP primer pairs could amplify clear and readable bands, and each of the 253 primer pairs produced about 40 bands. Polymorphic bands between 58S and 1514 were observed in amplified products by 230 primer pairs. However, only 20 primer pairs detected differences between the two bulks. Thus, 20 fragments were cloned and used as RFLP probes for Southern analysis. Twelve of the 20 clones were of single- or low copy, 4 were of multiple-copy, and the remaining 4 were repetitive sequences. Using the 12 single- or low copy clones as probes, it was shown that 6 clones detected polymorphism between the two parents. However, only

2 (F3 and V4) of the 6 probes detected differences between the two bulks, thus were likely linked to a PSGMS locus.

2.2.3 The genetic distance between the positive fragments and the PSGMS locus. Genetic distances between the positive markers identified above and the PSGMS locus were calculated using the method of Zhang et al.[5], based on 52 highly sterile plants of the $F_2$ population from the (58S × 1514) cross planted in 1995 (table 3). By comparing the recombinant events that occurred in the 52 highly sterile plants, we deduced that all four positive markers derived from the RAPD and AFLP analyses were linked to and located on the same side of the *pms*3 locus (figure 2).

Table 3 Distances between RAPD and AFLP derived markers and the *pms*3 locus calculated using highly sterile individuals from an $F_2$ population of (58S × 1514) planted in 1995

| Marker | Recombination (%) ± SD | cM |
|---|---|---|
| AU10-1500 | 9.61 ± 2.89 | 9.73 |
| RA27 + RA32-300 | 18.27 ± 3.79 | 19.15 |
| F3 | 5.77 ± 2.28 | 5.80 |
| V4 | 7.69 ± 2.61 | 7.75 |

## 3 Discussion

The main finding of this study was that the locus where the original photoperiod sensitive male sterile mutation occurred is the *pms*3 locus on chromosome 12. The change that made the normal cultivar 'Nongken 58' become photoperiod-sensitive male sterile rice Nongken 58S is due to the mutation of the allele at the *pms*3 locus. In theory it should not be possible to use a cross between 58S and 58N for mapping the PSGMS locus, because the genetic backgrounds of 58S and 58N were supposed to be identical. In fact, however, there has been quite a few different versions of the 'Nongken 58' cultivar as a result of wide spread cultivation for several decades. Thus 'Nongken 58' maintained by different institutions showed conspicuous differences in morphological traits, and even DNA polymorphism could be detected on several chromosomes[6] which provided markers for the present study to determine the locus for the PSGMS mutation.

The PSGMS loci reported thus far involve 4 chromosomal locations including chromosomes 3, 5, 7 and 12[4, 5, 7]. However, previous studies have established that fertility segregation in the progeny of many crosses is controlled by one or two major loci[2]. Thus, apparently, photoperiod-sensitive male sterility in different crosses and lines has different genetic bases that involve different PSGMS loci. For example, although '32001S' was developed using 58S as the donor for PSGMS genes, the PSGMS loci are clearly different between '32001S' and 58S. The *pms*1 locus was detected in the crosses involving both 58S and '32001S' as the PSGMS parents. However, the *pms*3 locus on chromosome 12 was not segregating in the (32001S × Minghui 63) cross, instead, a different locus on chromosome 3 (*pms*2) was involved in the fertility segregation[5]. There has been evidence (Mei Minghua et al., unpublished data) suggesting that the RFLP patterns of 32001S in the *pms*3 genomic region is the same as its indica parent (IR8) and different from that of 58S. Thus the DNA segment of the *pms*3 genomic region was not even transferred from 58S to 32001S. Furthermore, the results of Zhang et al.[5] showed that there was significant segregation distortion in the *pms*2 region in the $F_2$

population of (32001S × Minghui 63), suggesting that the sterility may somehow be related to indica-japonica hybridization. The differences between 58S and '32001S' in the genomic regions of PSGMS loci imply that the transfer of the complete set of the PSGMS genes from the donor parent is not necessary for the development of new PSGMS lines.

Another objective of this study was to increase the maker density in the *pms*3 region, or fine mapping of this locus. It is unfortunate that this region is marker-sparse both in the Cornell and the RGP maps. Although our effort of the large amounts of RAPD and AFLP analyses detected many polymorphic bands in the gels, only a few positive markers were recovered after converting them to RFLP probes which slightly increased the marker density of the *pms*3 region. Nonetheless, we did not find any markers that were more tightly linked to the *pms*3 locus than the ones we previously identified. Consequently the *pms*3 region is still marker-sparse. This seems to suggest that this region may be rich in repetitive sequences. Future studies have to address this problem in order to isolate and clone this PSGMS gene.

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