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Genetics

Using bulked extremes and recessive class to map genes for photoperiod-sensitive genic male sterility in rice

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ABSTRACT **Photoperiod-sensitive genic male sterile (PSGMS) rice has a number of desirable characteristics for hybrid rice production. In this study we made use of a published rice genetic linkage map to determine the locations of PSGMS genes and we have characterized the effects of these genes on sterility by using molecular markers. A two-step approach was designed for mapping the genes: (*i*) identifying possible PSGMS gene-containing chromosome regions with bulked DNA from extreme fertile and extreme sterile plants of a very large F_2 population and (*ii*) determining the map locations of the genes in extreme sterile individuals. We show that this mapping method is much more cost effective and statistically efficient than using a random sample of an F_2 population. We identified two chromosomal regions each containing a PSGMS locus, one designated *pms1* on chromosome 7 and one designated *pms2* on chromosome 3. The existence of these two loci was confirmed by a large sample assay and with data on ratooning progenies of the F_2 plants. A marker-based analysis shows that the effect of *pms1* is 2–3 times larger than that of *pms2* and that dominance is almost complete at both loci. Implications in the breeding of PSGMS rice lines are discussed.**

A photoperiod-sensitive genic male sterile (PSGMS) rice was found in 1973 as a spontaneous mutant in a *japonica* (*Oryza sativa* ssp. *japonica*) rice cultivar (Nongken 58) grown in Hubei Province, China (1). Large numbers of studies conducted in the last decade have established that this novel mutant (referred to as Nongken 58S) possesses a number of desirable characteristics that might be useful in hybrid rice (2): pollen fertility of Nongken 58S is regulated by photoperiod length (3); it is completely sterile when grown under long-day conditions, whereas pollen sterility varies when it is grown under short-day conditions; and the critical stage comes between secondary branch differentiation and microsporogenesis during panicle development (4). Thus, PSGMS rice can be used to propagate itself under short-day conditions and also to produce hybrid seeds by interplanting it with normal fertile lines under long-day conditions. PSGMS rice may therefore provide opportunity to replace the widely used "three-line" (male sterile, maintainer, and restorer) system with a "two-line" system that promises to greatly reduce costs in labor, time, and resources in hybrid rice production. PSGMS rice has a broad spectrum of restoration; almost all normal rice strains restore the fertility of the F_1 hybrid. Deliberately bred restorer lines are consequently not required. Fertility is controlled by a relatively simple genetic system, usually one or two major Mendelian loci (1, 5). Thus it should be relatively easy to develop new PSGMS lines by transferring the PSGMS alleles from one genetic background to another, particularly if marker-aided systems of transfer can be developed. A further advantage is that the performance of PSGMS hybrids does not suffer from adverse effects of male sterile cytoplasm such as has commonly been the case with the three-line hybrids. Utilization of PSGMS rice for the development of two-line hybrids has thus become a major goal in many rice breeding programs in China (6). Many new PSGMS lines have been developed, and extensive field testing has shown several hybrid combinations to be promising. Rice breeders expect that two-line hybrids will occupy large acreage within a few years.

It has also been reported that photoperiodic induction of male fertility in PSGMS rice is independent of the photoperiodic reaction that regulates the shift from the vegetative phase to the reproductive phase in the life cycle (7). This has led to the notion of "two photoperiodic-reactions" in the PSGMS rice (2): the first reaction regulates transfer from the vegetative phase to the reproductive phase and the second regulates pollen fertility. In addition to photoperiod, temperature also plays an important role in regulating pollen fertility of PSGMS rice (8): higher temperatures usually shorten the length of photoperiod needed to induce male sterility, whereas low temperatures frequently cause variations in the fertility of different PSGMS lines. Photoperiod-sensitive male sterility clearly involves a complex set of phenomena that will require comprehensive studies designed to isolate individual degrees of freedom before its genetic and physiological basis is well-enough understood to permit fully efficient utilization of the PSGMS genes in hybrid rice breeding.

Mapping genes with molecular markers is usually laborious and costly. Bulked segregant analysis (9), which provides information simultaneously on polymorphism of the parents and possible linkage between a marker and a targeted gene using only two bulks and the parents, can reduce the cost and work load by severalfold. However, an individual-by-individual analysis of a segregating population is still necessary to determine the map distance between a marker and the targeted gene. Also, most mapping methods require complete classification of individuals in the population into discrete classes, which is sometimes difficult, especially when the expression of the trait is sensitive to environmental conditions, as in the case of the photoperiod-sensitive male sterility in rice.

In the study reported in this paper, we extended the bulked segregant analysis (9) to a two-step approach for mapping the PSGMS genes: (*i*) we used bulked DNA from extreme plants to identify the chromosomal segments likely to carry sterility vs. fertility alleles, which avoided classifying individuals into a fertile or a sterile class in a more or less continuously distributed population, and (*ii*) we determined the map locations of the genes by using only the extreme sterile individuals. It is our belief that this approach may have a broad range of applications in gene mapping. We anticipate that the markers identified in this study will be especially useful in

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Abbreviations: PSGMS, photoperiod-sensitive genic male sterile; RFLP, restriction fragment length polymorphism; cM, centimorgan(s).

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facilitating the transfer of PSGMS alleles in hybrid rice breeding, in characterizing the behavior of the PSGMS genes in various genetic backgrounds, and in isolating the PSGMS genes by a map-based cloning approach.

MATERIALS AND METHODS

Population Development. Numerous new PSGMS lines have been developed by transferring the PSGMS genes from Nongken 58S into both *indica* (*O. sativa* ssp. *indica*) and *japonica* strains of rice. It was known before the present study was started that PSGMS was more stably expressed in *japonica* than in *indica* genetic backgrounds. In 1990, we screened 21 newly developed PSGMS lines including both *indica* and *japonica* types and 5 normal rice cultivars for DNA restriction fragment length polymorphism (RFLP) with 50 probes from a published rice RFLP linkage map (10). This screening identified a pair of *indica* lines, 32001S (a PSGMS line developed by transferring the PSGMS genes from Nongken 58S) and Ming Hui 63 (a normal rice cultivar), that demonstrated a relatively high level of polymorphism (≈30%). Very low levels of polymorphism (5–8%) were detected among the *japonica* lines, making it difficult to construct a mapping population based on the *japonica* strains. A cross between 32001S and Ming Hui 63 was subsequently made, and large F_2 populations (>1500 individuals) were planted annually in the field during the 1991–1993 growing seasons under natural long-day conditions.

Each plant was examined for three attributes of fertility: (*i*) percent dark-staining pollen grains with iodine (I/KI), (*ii*) seed-setting rates on bagged panicles, and (*iii*) seed-setting rates on unbagged panicles. However, the correlation between pollen stainability and seed setting was low (≈0.3), and bagging often caused damage to the panicles under field conditions, resulting in excesses of plants that produced few or no seeds. Therefore, only seed-setting rates from unbagged panicles were subsequently used in assessing fertility.

Molecular Marker Assays. DNA was prepared from fresh leaf tissues of field-grown F_2 plants. DNA extraction and Southern blot hybridization followed published procedures (11, 12). Three hundred sixty-eight clones were surveyed, including 241 from the Cornell map [each clone covered about 5 centimorgans (cM) on the average (13)] and 127 other clones from mapping projects of barley, corn, and sorghum (M.A.S.M., unpublished data). All the 10 simple-sequence repeat markers of Wu and Tanksley (14) were also surveyed by published procedures (14, 15), thus bringing the number of markers surveyed to 378.

Use of Bulked Extremes to Identify Chromosomal Regions Containing PSGMS Genes. Two DNA bulks, F (fertile) and S (sterile), were made by selecting extreme individuals from an F_2 population of about 1500 plants in the 1992 rice growing season. The first bulk (bulk F) was made by mixing equal amounts of DNA from 30 highly fertile plants, and the second bulk (bulk S) was made by mixing equal amounts of DNA from 58 highly sterile plants. These two bulks and the two parents were digested with 6–21 restriction enzymes and probed for RFLP with the aforementioned 368 clones and also were assayed with the 10 simple-sequence repeat markers.

Based on prior information that male sterility is controlled by recessive alleles at one or more loci, we predicted that for a marker linked to a PSGMS locus, the paternal (the fertile parent) band(s) in the autoradiography would be less intense than the maternal (the sterile parent) band(s) in bulk S (Fig. 1) and that bands from the two parents would appear in similar intensity in bulk F. In contrast, for a marker unlinked to a targeted locus, we predicted that the intensity of the bands from the two parents would be about equal in both bulks. This proved to be the case, and we consequently refer to the markers that appeared to be linked to a PSGMS locus as positive markers.

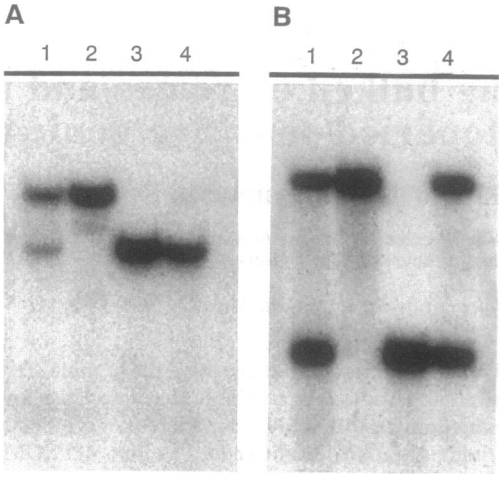

FIG. 1. Southern blots of DNA samples of the parents and bulked extremes demonstrating the identification of a chromosome region that is likely to contain a PSGMS locus. The blots were probed with RG30 (*A*), a marker locus linked to locus *pms1* or RG553 (*B*), which is not linked to a PSGMS locus. Lanes: 1, the bulk of fertile plants; 2, the fertile parent; 3, the sterile parent; 4, the bulk of sterile plants.

Determining the Chromosomal Locations of PSGMS Loci for the Extremely Sterile Individuals. Clones surrounding the positive markers in the RFLP linkage map were added to the survey until no more polymorphic clones were available. Each of the 58 sterile plants was assayed individually with all the positive markers identified above. By assuming that all the 58 sterile plants were homozygous for the recessive (sterile) allele at the targeted PSGMS locus, the recombination frequency (c) between a positive marker and the PSGMS locus was calculated by use of a maximum likelihood estimator (16), based on the RFLP data from the 58 sterile plants: $c = (N_1 + N_2/2)/N$, in which N is the total number of the sterile plants surveyed, N_1 is the number of individuals homozygous for the RFLP band from the fertile parent, and N_2 is the number of individuals heterozygous for bands from the two parents. The variance is given by $V_c = c(1 - c)/2N$.

To confirm the results of the bulked extremes and recessive class analysis, an additional sample of 224 individuals from the same F_2 population was assayed with two to four positive markers from each of the putative PSGMS gene-containing regions. This sample, together with the 58 sterile plants, brought the total number of individuals with data for each of these clones to about 270 (a few individuals were overlapping in the two samples). These 270 or so plants were maintained by ratooning and three to six vegetative progenies of each F_2 plant were transplanted in 1993 from which fertility was again examined. One-way and multiway analyses of variance (ANOVAs) were performed to assess further the existence of a locus for PSGMS in a given region and to characterize the genetic effects of the identified loci.

RESULTS

Fertility Segregation in F_2. The distribution of seed-setting rates on unbagged panicles of the F_2 individuals for fertility data collected in the 1992 rice growing season is given in Fig. 2; the 1992 season was exceptionally favorable for the expression of photoperiod-induced male sterility. The distribution for 1992 appeared to be bimodal, and χ^2 tests showed that breaking at any point in the apparent valley between 16% and 30% fit to a 15:1 ratio ($P > 0.10$). Similar results were also obtained in 1991 and 1993 (data not shown), suggesting a

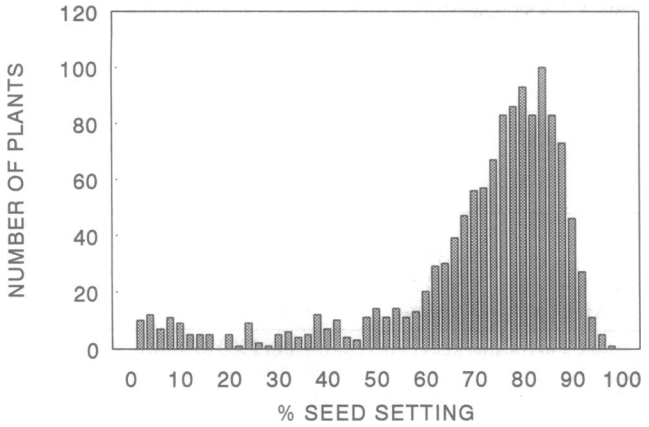

FIG. 2. Distribution of seed-setting rates on unbagged panicles of 1248 plants in the F_2 population in a cross between 32001S and Ming Hui 63 grown in the 1992 rice growing season. The seed-setting rates are 0.78 ± 0.80% (mean ± SD) for 32001S and 84.05 ± 6.16% for Ming Hui 63.

digenic segregation of male sterility in this population, in accord with previous studies (e.g., ref. 5).

Identifying Potential Chromosomal Regions Containing the PSGMS Loci. A total of 90 markers, covering about 92% of the Cornell RFLP linkage map, detected polymorphism between the parents for at least one of the 21 restriction enzymes. None of the SSR markers detected polymorphism between the parents.

The survey of bulked extremes identified markers (positive markers) from three regions, located on chromosomes 1, 3, and 7, that were likely to be linked to PSGMS loci. The bulks were subsequently probed with additional clones surrounding the positive markers, until 22 marker loci in total from these three regions had been surveyed.

To assess whether a PSGMS locus was present in each of these three chromosome regions, the 58 extremely sterile plants were assayed individually with each of the 22 clones. χ^2 tests showed that the segregation ratios for all the markers, except two from chromosome 1, deviated significantly from a ratio of 1:2:1, indicating that each of these three chromosomal regions was likely to contain a PSGMS locus.

The existence of the PSGMS loci on these chromosomal locations was assessed further with an additional sample of 224 individuals taken at random from our 1992 F_2 population, using eight positive clones: RG811 and RG655 from chromosome 1; RG348, RG191, RG450, and BCL033 from chromosome 3; and RG477 and RG511 from chromosome 7. One-way ANOVA of fertility, using marker genotypes as groups, showed that the locus on chromosome 7 had the largest effect followed by the locus on chromosome 3, whereas the effect of the putative locus on chromosome 1 was barely significant (Table 1). ANOVA of fertility data obtained in 1993 on the ratooning progenies of these 270 or so plants also indicated major effects of the loci on chromosomes 3 and 7 and possible minor effects of the putative locus on chromosome 1 (data not shown). When a three-way ANOVA was performed with the marker showing the largest effect from each of these three regions (RG811, RG191, and RG477), all of the effects involving the locus on chromosome 1 (marked by RG811) became insignificant (Table 2). These analyses established the existence of two loci, one on chromosome 3 and one on chromosome 7. We designate the locus on chromosome 7 as *pms1* and the locus on chromosome 3 as *pms2*.

Determining the Map Locations of the PSGMS Loci. The recombination frequency (c) between a positive marker and a targeted PSGMS locus was calculated (Table 3) by assuming that all the 58 sterile plants were recessive at the PSGMS locus. The c values for markers on chromosome 7 agreed reasonably well with the distances given in the Cornell map, which suggests that locus *pms1* is located between RG477 and RG511. However, the maximum likelihood estimate of the recombination frequency between RG477 and RG511, 16.3 ± 0.5%, based on our large random sample assay, was substantially smaller than the sum (20.7%) of c values between *pms1* and RG477 (4.3%) and that between *pms1* and RG511 (16.4%) as listed in Table 3. Using 16.3/20.7 as a correction factor, we estimate that locus *pms1* is located at 3.4 recombination units from RG477 and 13.0 recombination units from RG511, which are equal to 3.5 and 15.0 cM (17), respectively (Fig. 3).

The c values calculated for markers on chromosome 3 all indicated that locus *pms2* is between RG191 and RG348. However, the sum (56.3) of the c values between *pms2* and RG348 and between *pms2* and RG191 is about 3.8 times larger than that in the Cornell map and 2.4 times larger than the maximum likelihood estimate (23.3) obtained by using our own large sample from this same population (data not shown). The inconsistency in the estimated distance between our large sample and the Cornell map may be due to segregation distortion, a phenomenon frequently observed in rice crosses (18), which occurred in our population in all the markers within this region. Large differences between the c values calculated from the 58 extreme sterile plants and those calculated from our large sample suggest that the 58 extreme sterile plants were not necessarily all homozygous for the recessive allele at this locus. This in turn suggests that the effect of locus *pms2* is small.

The tentative map location of *pms2* was deduced as follows. The recombination values in Table 3 and the effects revealed by ANOVA (as evaluated by the square root of the mean square of the effect in Table 1) suggest that *pms2* is located at about two-fifths of the interval length between RG191 and RG348. The interval is ≈17.6 cM in the Cornell map, from which we infer that *pms2* is about 7.0 cM from RG191 and 10.6 cM from RG348 (Fig. 3).

Table 1. One-way ANOVA of the effect on male sterility of each of three chromosomal regions on the basis of genotypes of each RFLP locus

Locus	Chromosome	MS effect*	df error	MS error	F	P
RG811	1	3,472	265	842	4.1	0.02
RG655	1	142	262	864	0.2	0.85
RG348	3	9,802	234	783	12.5	0.00
RG191	3	20,147	265	716	28.1	0.00
BCL033	3	19,592	256	700	28.0	0.00
RG450	3	17,766	263	739	24.0	0.00
RG477	7	59,014	264	424	139.0	0.00
RG511	7	29,480	264	648	45.4	0.00

MS, mean square; df, degrees of freedom; F, F statistic.
*There are 2 degrees of freedom for the effect of each marker locus.

Table 2. Three-way ANOVA using one marker locus from each of the three putative PSGMS gene-containing chromosomal regions

Effect*	df	MS	F	P
1	2	5,263	15.0	0.00
2	2	21,616	61.7	0.00
3	2	406	1.2	0.32
1, 2	4	1,453	4.2	0.00
1, 3	4	485	1.4	0.24
2, 3	4	626	1.8	0.13
1–3	8	225	0.6	0.74

df, Degrees of freedom; MS, mean square; F, F statistic.
*Marker loci: 1, RG191; 2, RG477; 3, RG811.

Table 3. Recombination frequencies (*c*) between markers and a PSGMS locus, estimated by assuming that the 58 extreme sterile individuals are homozygous for the sterility allele at the targeted PSGMS locus

Locus	Chromosome	*c*, % (mean ± SE)
RG348	3	33.9 ± 4.5
RG191	3	22.4 ± 3.9
RG450	3	23.3 ± 3.9
RG266	3	25.4 ± 4.1
RG117	3	26.3 ± 4.1
RG335	3	26.3 ± 4.1
BCL033	3	20.2 ± 3.8
RG146B	7	31.0 ± 4.3
RG650	7	25.9 ± 4.1
RG678	7	19.0 ± 3.7
WG719	7	11.2 ± 2.9
RG30	7	11.2 ± 2.8
CDO533	7	9.6 ± 2.9
RG477	7	4.3 ± 2.0
RG511	7	16.4 ± 3.5
RZ272	7	16.4 ± 3.3
RG128	7	31.9 ± 4.3

Genetic Effects of the Two Loci. Genetic effects based on the marker genotypes were estimated for these two loci by using a two-locus model (Table 4). The marker-based estimates of fertility are expected to be biased upward for the recessive classes and downward for the dominant classes due to recombination between each marker locus and the PSGMS loci. Nonetheless, Table 4 shows that the effect of *pms1* is 2–3 times larger than that of *pms2* and that dominance is almost complete at both loci. The data of Table 4 suggest that alleles of these two loci interact more or less like alleles of duplicated loci: highly sterile individuals are apparently homozygous for recessive alleles at both loci, whereas several different genotypes appear to produce highly fertile individuals (Table 4).

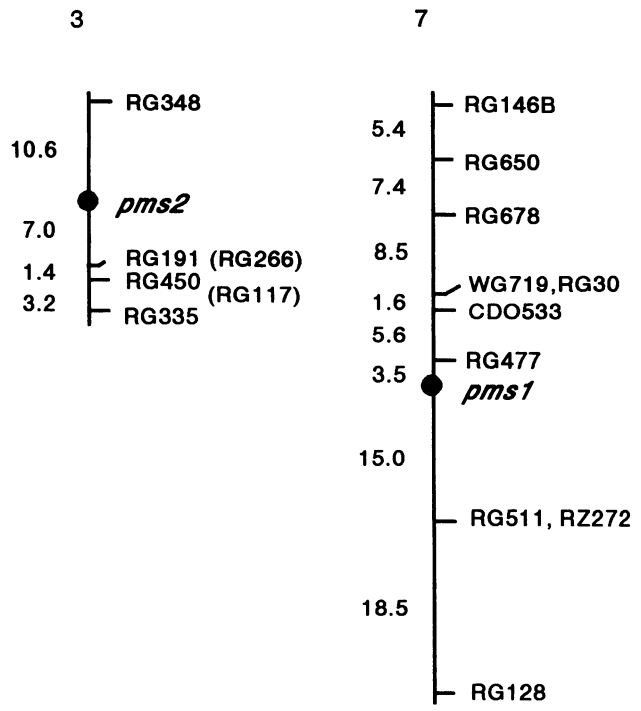

FIG. 3. The locations of loci *pms1* and *pms2* in the RFLP linkage map. Map distances (cM) between loci on chromosome 7 were deduced from the estimates made with the extreme sterile individuals, and the distances between *pms1* and RG477 and between *pms1* and RG511 were corrected on the basis of our large F_2 sample assay. The distances among markers on chromosome 3 are essentially those from the map of Tanksley *et al.* (13), except for that between RG191 and RG450, which was estimated by using data from our large F_2 sample assay.

Table 4. Average seed-setting rates on unbagged panicles for each of the two-locus genotypes (11, 12, and 22) marked by RFLPs of RG191 and RG477 on chromosomes 3 and 7, respectively

		% seed setting			
		RG477 11	RG477 12	RG477 22	Average
RG191	11	12.88	61.56	72.11	52.03
	12	35.94	73.12	75.28	64.36
	22	50.59	72.65	74.45	67.58
Average		33.84	70.11	74.28	

Allele 1 in the marker genotypes is from 32001S, and allele 2 is from Ming Hui 63. The average is weighted by a theoretical 1:2:1 ratio within each class of the F_2 population.

DISCUSSION

The bulked-extremes and recessive-class approaches allowed us to identify two loci (*pms1* and *pms2*) that govern photoperiod-sensitive male sterility and to determine the chromosomal locations of these two loci on a published RFLP linkage map. The large sample assay produced essentially the same results as the bulked-extremes and recessive-class analysis, confirming the existence of these two loci. It is clear that the bulked-extreme survey provided a fast and cost-effective means for identifying chromosomal regions that are likely to contain the genes controlling expression of a complex trait. The most distinct advantage of the recessive-class assay lies in its higher efficiency in estimating the recombination frequency than mapping with a random F_2 population. This is so because, on a per-assayed-individual basis, the variance associated with the maximum likelihood estimate of the recombination (*c*) using the recessive class [$V_c = c(1 - c)/2N$] is 2–3 times smaller than that using a random F_2 population (ref. 16, table 6, equation 5). Thus, for the same degree of accuracy, only one-third to one-half as many individuals need to be surveyed in the recessive-class assay as in mapping with a random F_2 population. This will result in considerable saving in effort, especially when very large numbers of individuals need to be assayed for molecular markers, as in the case of fine mapping for map-based cloning. In addition, selection of extremes avoided classifying individuals in a more or less continuously distributed population into a fertile or a sterile class, hence reducing the probability of misclassification. Thus, this method may be particularly useful for mapping genes whose phenotypes cannot be easily separated into discrete classes. Furthermore, this method may also allow one to determine the map locations of multiple loci that govern the same trait in the same group of individuals, provided that these individuals are homozygous for the recessive alleles at the targeted loci.

The original PSGMS rice Nongken 58S is a spontaneous mutant, and many studies have shown that fertility segregation in crosses between 58S and its wild-type progenitor is conditioned by a single Mendelian locus. It is thus interesting that a second locus has become involved in this system, and homozygosity of recessive alleles at both loci is required for expression of male sterility. This implies that the cultivar Nongken 58 was already homozygous for the recessive allele at the second locus before it mutated to become PSGMS rice. In this connection it should be noted that, in a previous study of a cross between two *japonica* lines in which the fertility

also displayed a typical two-locus segregation, Zhang *et al.* (19) reported a linkage between a PSGMS gene and a locus for dwarfism located on chromosome 5. Thus it seems likely that the "second locus" may not be the same in different lines and crosses. This is not surprising, because pollen fertility is the end result of many complex processes controlled by numerous loci, and mutation in any of the loci involved in these processes or pathways may have an impact in pollen fertility. However, it is almost certain that only one of the two loci, most likely the one that segregates between Nongken 58S and Nongken 58, triggers the photoperiod response for inducing male sterility; apparently the other is merely a locus for male sterility, like many of those identified previously in rice (20). Thus, which of the two loci identified in the present study governs the response to photoperiod induction, and also the molecular basis for the interaction between the two loci, still remains to be determined.

A major difficulty presently encountered in the utilization of PSGMS rice in two-line hybrid breeding is the temperature-mediated fertility variation observed in many newly developed PSGMS lines. The substantial amounts of self-pollinated seeds produced by these male sterile lines under some environmental conditions prevent their use in hybrid seed production: seed-setting rates of these male sterile lines under long-day conditions vary from zero under favorable growing conditions, to low percentages in average growing conditions, and to 30–40% in cooler-than-usual conditions. The extent of such fertility variations depends on the genetic background and, in general, it is more serious in *indica* than in *japonica* genetic backgrounds. There are several possible causes for such temperature-mediated fluctuation in male sterility, including: (*i*) the possibility that gene for the "second locus" differs from one PSGMS line to another, as discussed above; i.e., different genes for male sterility may be associated with the gene at the primary photoperiod locus in different PSGMS lines; (*ii*) the possibility that there may be multiple alleles at the second locus conditioning male sterility, as is the case in soybean and pea (21) and for a locus governing fertility restoration in corn (22), so that different lines may carry different alleles with varying degrees of temperature sensitivity; and (*iii*) the possibility that additional modifying genes with thermally sensitive expression may be involved in the system.

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