Mol Gen Genomics (2005) 273: 507–511
DOI 10.1007/s00438-005-1155-4

ORIGINAL PAPER

Q. Lu · X. H. Li · D. Guo · C. G. Xu · Q. Zhang

Localization of *pms3*, a gene for photoperiod-sensitive genic male sterility, to a 28.4-kb DNA fragment

Received: 25 December 2004 / Accepted: 20 April 2005 / Published online: 24 May 2005

Abstract Photoperiod-sensitive genic male-sterile (PSGMS) rice, in which pollen fertility is regulated by day-length, originally arose as a natural mutant in the rice cultivar Nongken 58 (*Oryza sativa* ssp. *japonica*). Previous studies identified *pms3* on chromosome 12 as the locus of the original PSGMS mutation. In this study we have assigned the *pms3* locus to a 28.4-kb DNA fragment by genetic and physical mapping. A cross between Nongken 58S (PSGMS line) and DH80 was used to produce an F_2 population of about 7000 plants, from which 892 highly sterile individuals were obtained for recombination analysis. By analyzing recombination events in the sterile individuals using a total of 157 RFLP probes from a BAC contig covering the *pms3* region, the *pms3* locus was localized to a sub-region of less than 1.7 cM. Further analysis of recombination events using 49 additional probes isolated from this sub-region identified markers flanking the *pms3* region on each side; these markers are only 28.4-kb apart. Sequence analysis of this fragment predicted the presence of five ORFs, found high homology with two ESTs in public databases, and detected three SNPs between the mutant and the wild-type parents, which may be helpful for identifying a candidate gene for *pms3*.

Keywords *Oryza sativa* L. · *pms3* · Genetic mapping · Physical mapping · BAC contig

Introduction

Photoperiod-sensitive genic male sterile (PSGMS) rice was first found as a spontaneous mutant in the *japonica* variety Nongken 58 in Hubei Province, China (Shi 1985); hereafter the mutant is referred to as Nongken 58S. A major characteristic of the PSGMS rice is that pollen fertility is regulated by day-length: the pollen is completely sterile under long-day conditions, while the fertility varies from partial to full under short-day conditions (Zhang and Yuan 1987). This characteristic makes it a desirable germplasm for developing "two-line" hybrids in rice breeding programs, promising to save costs in labor and resources compared with the traditional "three-line" hybrids. Two-line hybrids developed using the PSGMS system now account for a large proportion of rice production in China.

Many studies have established that the sterility of the PSGMS rice is controlled by one or two Mendelian loci, depending on the genetic backgrounds of the materials used in the crosses (Jin 1995). Typical single locus segregation of male fertility was consistently observed in crosses between Nongken 58S and Nongken 58 (Mei et al. 1999a, b). On the other hand, in the progeny of a cross between the *indica* cultivar Minghui 63 and 32001S, a PSGMS line of the *indica* type that derived its PSGMS genes from Nongken 58S, the fertility trait displayed two-locus segregation (Zhang et al. 1994). These two loci were designated *pms1* and *pms2*, and have been localized on chromosomes 7 and 3, respectively. Zhang et al. (1994) further showed that the effect of *pms1* on pollen fertility was much larger than that of *pms2*. Mei et al. (1999b) studied the segregation of male fertility in the F_2 progenies from a cross between Nongken 58S and the *japonica* breeding line 1514, and a cross between Nongken 58S and the widely compatible line Lunhui 422. They observed two-locus segregation in both populations, and localized the two loci to chromosomes 7 and 12, respectively. Their study also determined that the locus on chromosome 7 was the same as the *pms1* identified by Zhang et al. (1994), while the one on chromosome 12 was a new locus for PSGMS which they designated *pms3*. Further work revealed that *pms3* was the locus at which the

Communicated by R. Hagemann

Q. Lu · X. H. Li · D. Guo · C. G. Xu · Q. Zhang (✉)
National Key Laboratory of Crop Genetic Improvement and National Center of Plant Gene Research (Wuhan), Huazhong Agricultural University, Wuhan, 430070, China
E-mail: qifazh@mail.hzau.edu.cn
Tel.: +86-27-87282429
Fax: +86-27-87287092

original mutation occurred in the cultivar Nongken 58 to give rise to the PSGMS rice Nongken 58S (Mei 1999a), while fertility did not segregate with the *pms1* locus in progeny of the cross between Nongken 58S and Nongken 58 (Wang et al. 1997). Liu et al. (2001) constructed a physical map of the *pms1* region and localized the *pms1* locus to a DNA fragment of 85 kb in length.

To map the *pms3* locus with greater precision, Li et al. (2001) developed doubled haploid (DH) lines by anther culture of F_1 plants from a cross between Nongken 58S and 1514. One line, DH80, was found to have the 1514 genotype at the *pms3* locus and the Nongken 58S genotype at the *pms1* locus, and was used in a cross with Nongken 58S. The fertility trait showed typical single-locus segregation in the progeny of this cross, which allowed Li et al. (2002) to map the *pms3* locus to a region of about 3.2 cM.

In the study reported in this paper, we further localized the *pms3* locus to a genomic DNA fragment of 28.4 kb in length, thus achieving a major step toward our ultimate goal of isolating this gene.

Materials and methods

The mapping population

The DH80, a line developed by anther culture of F_1 plants from a cross between Nongken 58S and 1514 which has the 1514 genotype at the *pms3* locus and the Nongken 58S genotype at the *pms1* locus, was crossed with Nongken 58S (Li et al. 2001). An F_2 population of 7000 plants was planted in the field under long-day conditions in 2000 on the Experimental Farm of Huazhong Agricultural University in Wuhan, China. Spikelet fertility was examined by scoring three panicles per plant that headed before September 3rd to ensure that all the examined panicles developed under long-day conditions. Plants with spikelet fertility below 10% were considered to be sterile, and the seeds of these plants were harvested after transplanting and growing the stubs under short-day conditions in the winter season of 2000–2001 on Hainan Island, China. The resulting F_3 families were planted in the summer of 2001 in Wuhan under long-day conditions, and examined again for spikelet fertility. In this way, 892 highly sterile F_2 individuals showing no segregation of fertility in the F_3 progenies were obtained.

DNA extraction and southern hybridization

Fresh leaves were harvested from field-grown plants and genomic DNA was extracted by using the CTAB method (Murray and Thompson 1980). DNA digestion, Southern blotting and hybridization were conducted following the protocol described by Liu et al. (1997).

Physical map construction and molecular marker identification

A *Hin*dIII bacterial artificial chromosome (BAC) library of the *japonica* cultivar Nipponbare from Clemson University (Clemson, SC, USA) was used to construct a contig map encompassing the *pms3* region. Both BAC clones and pUC plasmids were extracted as previously described (Liu et al. 2001). Subclones from BAC clones were obtained by digestion with *Hin*dIII and ligation with the pUC19 vector, then electroporated into the *E. coli* strain DH10B. The resulting subclones (0.5–2.0 kb in length) were used as probes to identify additional RFLP markers with which to survey polymorphisms in the *pms3* region. Some shotgun-sequenced subclones were also used as RFLP probes to detect polymorphisms in this region. Plasmid inserts were obtained from the subclones by PCR amplification.

Results

Mapping of the *pms3* locus to a 1.7-cM region

Previous work had restricted the *pms3* locus to a genetic interval of 3.2 cM defined by the closest linked markers on either side: C751 and RZ261 on one side, 1.6 cM from *pms3*, and M36 on the other, also 1.6 cM from *pms3* (Li et al. 2001). Two RFLP markers, M36 and C751, were used to genotype the 892 F_2 plants that were identified as male sterile in the field experiment and whose phenotype was confirmed by examining F_3 progeny. Analysis using M36 identified 17 recombinant plants and C751 detected 43 recombinants.

To obtain more RFLP markers in this region, M36, a subclone derived from BAC clone 48M9 in the Minghui 63 BAC library (Peng et al. 1998), was employed to screen the *Hin*dIII BAC library of Nipponbare. Nine positive clones were recovered, all of which were anchored to the same contig on chromosome 12 (Contig No. 96), based on the contig information released by Clemson University Genomics Institute (this information is now available at http://www.genome.arizona.edu). We selected a total of 13 BAC clones from this contig (Fig. 1a), including two (35P12 and 71J16) of the nine mentioned above, for fingerprinting. The clones were digested with *Hin*dIII, fractionated on a 0.8% agarose gel, transferred to a nylon filter and hybridized using each of the BAC clones as the probe. The overlap relationships between the clones were determined on the basis of banding patterns on the X-ray films, which confirmed that they were indeed members of the same contig (data not shown).

A total of 157 subclones were prepared from these 13 BAC clones and used as probes to assay for polymorphism between Nongken 58S and DH80, with genomic DNAs digested with up to 21 restriction enzymes (*Acc*I, *Apa*I, *Bcl*I, *Bgl*I, *Bgl*II, *Cfo*I, *Cla*I, *Dra*I, *Eco*RI, *Eco*RV, *Hpa*II, *Hin*cII, *Pst*I, *Pvu*II, *Sac*I, *Sal*I, *Sca*I, *Sac*II, *Sst*I,

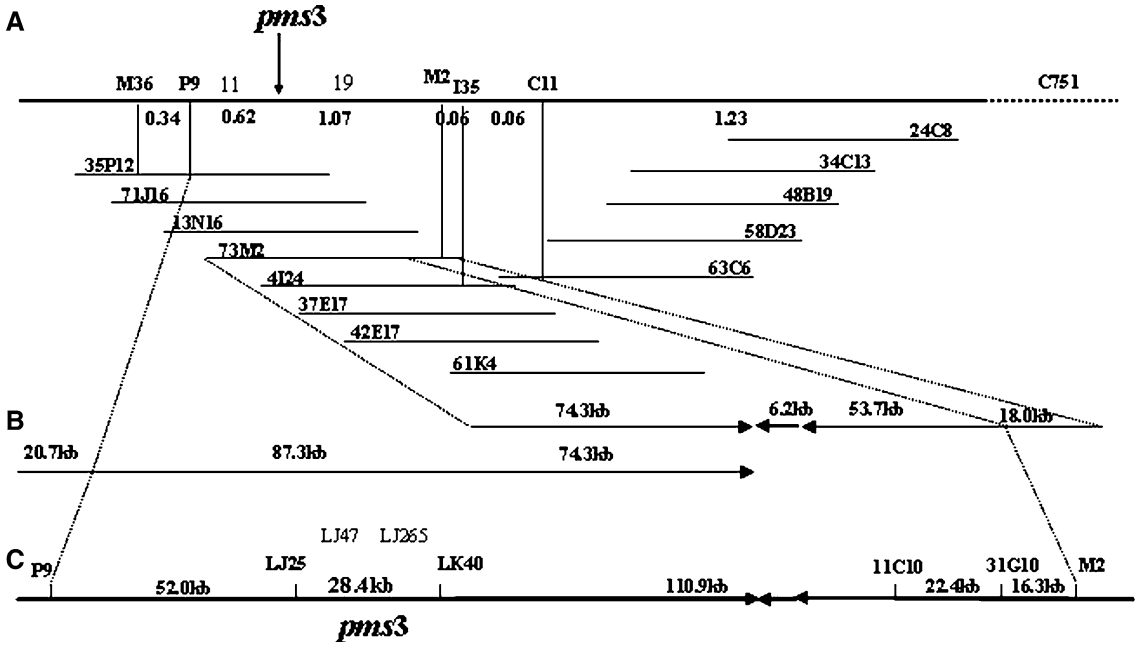


Fig. 1 **a–c** Maps of the region encompassing the *pms3* locus. **a** The contig covering the *pms3* region. The M36 and C751 are from a published linkage map (Li et al. 2001). The P9, M2, I35 and C11 are subclone markers obtained during the course of this work. The *thick line* at the *top* represents the genomic region covered by BAC clones, and the *broken segment* at the *right end* indicates the region not covered by BAC clones. The distance from C11 to C751 is now known to be 558.4 kb. The numbers *between* markers *below* the *top line* give the genetic distances calculated on the basis of the population of highly sterile individuals. The *bars below* the *thick line* indicate the BAC clones. **b** Sequence contigs in the *pms3* genomic region. The three *arrowed lines* represent the three contigs assembled from the OSJNBa0073M02 shotgun sequences by Sequencher 3.0, the single *arrowed line below* them represents the sequence contig extended by searching the Monsanto database. **c** Positions of the subclone markers developed in this study relative to the *pms3* locus. The numbers shown *above* the *arrowed line* represent the physical distances between adjacent markers. The *pms3* locus lies between LJ25 and LK40, which define an interval of 28.4 kb. The LJ47 and LJ265 co-segregate with *pms3*

*Xba*I and *Xho*I). This approach, combined with an analysis of the recombination events between the *pms3* locus and the polymorphic markers in the 60 recombinant individuals identified by C751 and M36, led to the identification of four subclones that were located within the region containing polymorphisms detectable with M36 and C751. Two of the subclones (P9 and M2) from two different BAC clones, OSJNBa0035P12 (35P12) and OSJNBa0073M02 (73M2) that overlapped substantially with each other, detected the fewest recombinants between the markers and the *pms3* locus, with 11 recombinant plants on the P9 side and 19 recombinants on the M2 side (Fig. 1a). Using the formula given by Zhang et al. (1994), the recombination frequencies were calculated as 0.62 between P9 and *pms3*, and 1.07 between *pms3* and M2 (Fig. 1a). Thus, the *pms3* locus was mapped within a region of less than 1.7 cM.

Localization of the *pms3* locus to a 28.4-kb DNA fragment

To determine the physical distance between the gene and the markers, and also to facilitate the identification of candidate genes for future studies, 73M2 was completely and 71J16 partially sequenced using the shotgun sequencing method (Birren et al. 1997). Using Sequencher 3.0 (Gene Code), the 162-kb sequence of 73M2 was assembled into three contigs with only two gaps. By searching the sequence data provided by Monsanto Company (http://www.rice-research.com/registration.html), another 108-kb sequence was obtained which extended the contiguous sequence in the direction of 71J16, increasing its total length to about 270 kb (Fig. 1b). Sequence analysis of the two RFLP markers P9 and M2 showed that they were located within the 270-kb sequence, some 230 kb apart. Thus, the *pms3* locus had to lie within this 230-kb region.

To narrow down the *pms3*-containing region further, 49 probes were prepared from subclones of the 230-kb fragment, spaced at distances of one clone per ~5 kb. DNA samples from the two parental lines were digested using the 21 restriction enzymes mentioned above and assayed with each of the probes. Polymorphisms were detected with 11 of the probes, which were subsequently used to assay the recombinant plants identified by P9 and M2. Two subclones (LJ25 and LK40) located 28.4 kb apart, which detected four and two recombinants with the *pms3* locus, respectively, were identified as the nearest markers on either side. In addition, two subclones, LJ47 and LJ265, were shown to co-segregate with the *pms3* locus (Fig. 1c). Thus, the *pms3* locus was delimited to a 28.4-kb DNA fragment and co-segregates with the markers LJ47 and LJ265 (Fig. 1c).

Putative genes in the 28.4-kb region

Analysis of the 28.4-kb region using GenScan1.0 (http://genes.mit.edu) identified five putative ORFs (Fig. 2), none of which showed any homology with known genes. A BLAST search of the 28.4-kb sequence against the EST database in GenBank found homology with two expressed sequences. The first was a full-length cDNA (Gene 1) from panicle and mature leaf that was identical to the region between positions 8.1 kb and 14.7 kb in this fragment, interrupted by three introns (Fig. 2). The second was an EST from callus tissue that was highly homologous to one of the intronic regions of Gene 1 (referred to as Gene 2). Neither the cDNA nor the EST displayed any homology to genes of known function. In addition, an incomplete copy of Gene 2 located approximately 2 kb upstream (Fig. 2), including ORF4 which shows high homology to a retrovirus-related pol polyprotein from the transposon *Tnt1*-94 (Grandbastien et al. 1989). Another feature of this fragment was that all putative genes and ORFs, except for one very short ORF, were located in the first 17 kb of this fragment; hence putative gene sequences are unevenly distributed in this fragment.

Comparative sequencing of the homologous DNA fragment from two wild type lines Nongken 58 and DH80 and the mutant Nongken 58S detected three single nucleotide mutations between the two fertile lines and Nongken 58S in the 28.4-kb region (Fig. 2). The first was in the promoter region of ORF1, the second was in one of the introns of Gene 1, and the third was in the intergenic region between ORF4 and ORF5.

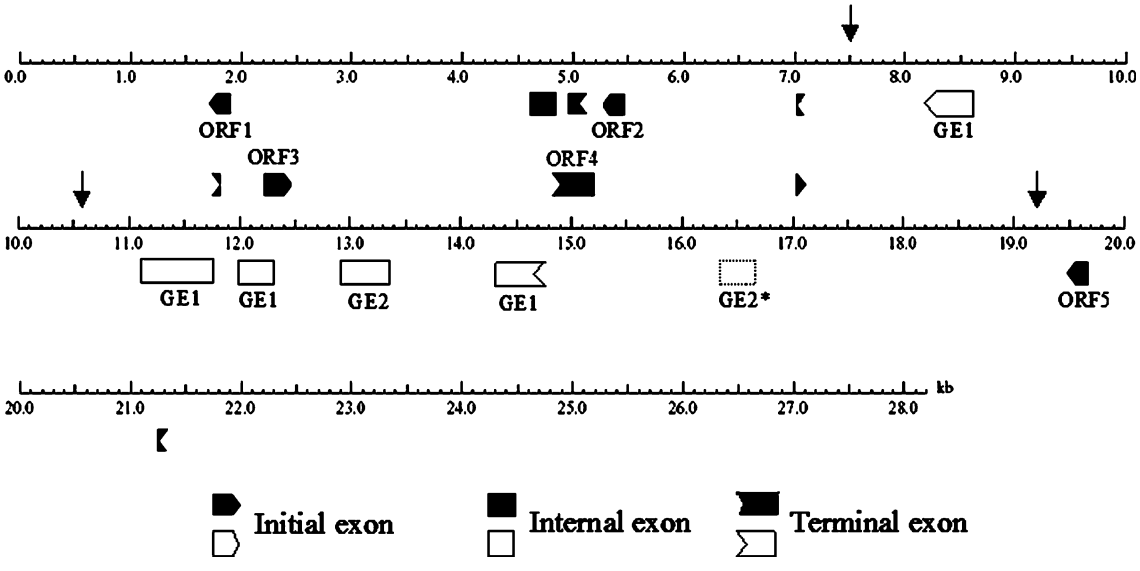
Fig. 2 Putative genes in the 28.4-kb fragment. The *filled symbols* represent predicted ORFs, *open symbols* represent sequences homologous to cDNAs, referred to as Gene 1 (GE1) and Gene 2 (GE2). The GE2* is an incomplete copy of Gene 2. The three *vertical arrows* indicate three SNPs between the two fertile lines and Nongken 58S

Discussion

The most significant result of this work is the localization of the *pms3* locus to a DNA fragment approximately 28.4 kb in length. This represents a major step toward map-based cloning of this gene. By assaying a total of 206 clones with up to 21 restriction enzymes, we also identified 15 RFLP markers surrounding this locus that detected polymorphisms between the parents. These markers should facilitate the transfer of the *pms3* gene in breeding programs designed to develop new PSGMS lines.

Map-based cloning is generally regarded as time-consuming and laborious even for genes that show typical Mendelian inheritance. Sensitivity of trait expression to the genetic background and/or environmental conditions, as in the case of PSGMS in present study (He et al. 1999), complicates the precise gene mapping that is a crucial prerequisite for cloning. In order to reduce the influence of the genetic background, we made a cross between two *japonica* lines, from which a population of DH lines was developed, and identified a line that was similar to Nongken 58S except in the region encompassing the *pms3* locus. The inevitable consequence of adopting such an approach is a lack of polymorphism between the parental lines. In this study that difficulty was circumvented by making use of a large number of subclones in combination with a large number of restriction enzymes, taking advantage of the genomic sequences generated. To minimize the influence of environmental fluctuations on phenotyping of the fertility trait, we made use of the F_3 progeny of the F_2 plants to ensure the sterile F_2 individuals identified in the field were indeed homozygous for the sterility allele at the *pms3* locus.

Coincidently, much of the work was conducted at the same time as the International Rice Genome Sequencing Project (IRGSP). Our work benefited greatly from



publicly available resources such as BAC clones, physical mapping information, and also partially finished sequences, although we still had to sequence a BAC clone due to the unavailability of genome sequence when this work was conducted. It can be expected that the availability of whole genome sequence information in the near future will reshape the procedures involved in map-based cloning. This is because the availability of chromosome-aligned genome sequence information will greatly reduce the need for chromosomal walking, making it possible to quickly associate a target trait with a specific genomic region.

In addition, recent international research efforts in the functional genomics of rice have now generated valuable resources, including large libraries of T-DNA insertion mutants (Hirochika et al. 2004), collections of full-length cDNAs (The Rice Full-Length cDNA Consortium 2003; Xie et al. 2005) and microarrays for global gene expression profiling (http://www.affymetrix.com/products/arrays/specific/rice.affx). The availability of such resources has greatly accelerated the processes of gene isolation and functional characterization, as exemplified in a number of studies (Takano et al. 2001; Kenako et al. 2004). However, map-based cloning will still remain one of the most important forward-genetic approaches for isolating agronomically useful genes. Establishment of associations between large numbers of ESTs and mapped genes and QTLs (Zhang et al. 2005) should also be very useful for identifying candidate genes for map-based cloning.

Acknowledgements We thank Rod Wing for providing a BAC library and for the release of the unpublished physical map information, and the Monsanto Company for releasing the genomic sequences. This research was supported by grants from the National Program of Research and Development of Transgenic Plants, the National Special Key Project of Functional Genomics and Biochips, and the National Natural Science Foundation of China, and a grant from the International Atomic Energy Agency

References

Birren B, Green ED, Klapholz S, Mayers RM, Roskams J (1997) Genome analysis. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp 397–454

Grandbastien MA, Spielmann A, Caboche M (1989) *Tnt1*, a mobile retroviral-like transposable element of tobacco isolated by plant cell genetics. Nature 337:376–380

He YQ, Yang J, Xu CG, Zhang ZG, Zhang Q (1999) Genetic basis of instability of male sterility and fertility reversibility in photoperiod-sensitive genic male-sterile rice. Theor Appl Genet 99:683–693

Hirochika H, Guiderdoni E, An G, Hsing Y, Eun MY, Han C, Upadhyaya N, Ramachandran S, Zhang Q, Pereira A, Sundaresan V, Leung H (2004) Rice mutant resources for gene discovery. Plant Mol Biol 54:325–334

Jin DM (1995) Genetic basis of photoperiod-sensitive genic male sterility. In: Li ZB, Jin ZH, Lu XG, Zhu YG (eds) Studies on photoperiod-sensitive genic male sterility in rice and its utilization in breeding. Hubei Science and Technology Press, Wuhan, China, pp 181–252

Kaneko M, Inukai Y, Ueguchi-Tanaka M, Itoh H, Izawa T, Kobayashi Y, Hattori T, Miyao A (2004) Loss-of-function mutations of the rice GAMBYB gene impair α-amylase expression in aleurone and flower development. Plant Cell 16:33–44

Li XH, Lu Q, Wang FL, Xu CG, Zhang Q (2001) Separation of the two-locus inheritance of photoperiod-sensitive genic male sterility in rice and precisely mapping the *pms3* locus. Euphytica 119:343–348

Li XH, Wang FL, Lu Q, Xu CG (2002) Fine mapping of PSGMS gene *pms3* in rice (*Oryza sativa* L.). Acta Agron Sin 28:310–314

Liu KD, Wang J, Li HB, Xu CG, Liu AM, Li XH, Zhang Q (1997) A genome-wide analysis of wide compatibility in rice and the precise location of the *S5* locus in the molecular map. Theor Appl Genet 95:809–814

Liu N, Shan Y, Wang FP, Xu CG, Peng KM, Li XH, Zhang Q (2001) Identification of an 85-kb fragment containing *pms1*, a locus for photoperiod-sensitive genic male sterility in rice. Mol Genet Genomics 266:271–275

Mei MH, Chen L, Zhang ZH, Li ZY, Xu CG, Zhang Q (1999a) *pms3* is the locus causing the original photoperiod-sensitive genic male sterility mutation of 'Nongken 58S'. Sci China (Ser C) 42:316–322

Mei MH, Dai XK, Xu CG, Zhang Q (1999b) Mapping and genetic analysis of the genes for photoperiod-sensitive genic sterility in rice using the original mutant Nongken 58S. Crop Sci 39:1711–1715

Murray MG, Thompson WF (1980) Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res 8:4321–4325

Peng KM, Zhang HB, Zhang QF (1998) A BAC library constructed to the rice cultivar "Minghui63" for cloning genes of agronomic importance. Acta Bot Sin 40:1108–1114

Shi MS (1985) The discovery and preliminary studies of the photoperiod-sensitive recessive male sterile rice (*Oryza sativa* L. subsp. *japonica*) (in Chinese with English abstract). Sci Agric Sin 2:44–48

Takano M, Kanegae H, Shinomura T, Miyao A, Hirochika H, Furuya M (2001) Isolation and characterization of rice phytochrome A mutants. Plant Cell 13:521–534

The Rice Full-Length cDNA Consortium (2003) Collection, mapping, and annotation of over 28,000 cDNA clones from *japonica* rice. Science 301:376–379

Wang FP, Mei MH, Xu CG, Zhang Q (1997) *pms1* genomic region does not cause fertility difference between the photoperiod-sensitive male sterile rice Nongken 58S and normal Nongken 58. Acta Bot Sin 39:922–925

Xie KB, Zhang JW, Xiang Y, Feng Q, Han B, Chu ZH, Wang SP, Zhang QF, Xiong LZ (2005) Isolation and annotation of 10,828 putative full length cDNAs from *indica* rice. Sci China (Ser C) 36:6–12

Zhang JW, Feng Q, Jin CQ, Qui DY, Zhang LD, Xie KB, Yuan DJ, Han B, Zhang QF, Wang SP (2005) Features of the expressed sequences revealed by a large-scale analysis of ESTs from a normalized cDNA library of an elite *indica* rice cultivar Minghui 63. Plant J. Doi:10.111/j.1365-313X.2005.02408.X

Zhang ZG, Yuan SC (1987) The influence of photoperiod on pollen fertility change of Hubei photoperiod-sensitive genic male sterile rice (in Chinese). Chin J Rice Sci 1:137–143

Zhang Q, Shen BZ, Dai XK, Mei MH, Saghai Maroof MA, Li ZB (1994) Using bulked extremes and recessive classes to map genes for photoperiod-sensitive genic male sterility in rice. Pro Natl Acad Sci USA 91:8675–8679

Zhang JW, Feng Q, Jin CQ, Qiu DY, Zhang LD, Xie KB, Yuan DJ, Han B, Zhang QF, Wang SP (2005) Features of the expressed sequences revealed by a large-scale analysis of ESTs from a normalized cDNA library of an elite *indica* rice cultivar Minghui 63. Plant J (in press)