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Fine mapping and candidate gene prediction of the photoperiod and thermo-sensitive genic male sterile gene *pms1(t)* in rice*#

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Abstract: Pei′ai64S, an *indica* sterile variety with photoperiod and thermo-sensitive genic male sterile (PTGMS) genes, has been widely exploited for commercial seed production for "two-line" hybrid rice in China. One PTGMS gene from Pei′ai64S, *pms1(t)*, was mapped by a strategy of bulked-extreme and recessive-class approach with simple sequence repeat (SSR) and insert and deletion (In-Del) markers. Using linkage analysis for the F_2 mapping population consisting of 320 completely male sterile individuals derived from a cross between Pei′ai64S and 93-11 (*indica* restorer) lines, the *pms1(t)* gene was delimited to the region between the RM21242 (0.2 cM) and YF11 (0.2 cM) markers on the short arm of chromosome 7. The interval containing the *pms1(t)* locus, which was co-segregated with RM6776, is a 101.1 kb region based on the Nipponbare rice genome. Fourteen predicted loci were found in this region by the Institute for Genomic Research (TIGR) Genomic Annotation. Based on the function of the locus LOC_Os07g12130 by bioinformatics analysis, it is predicted to encode a protein containing a Myb-like DNA-binding domain, and may process the transcript with thermosensory response. The reverse transcription-polymerase chain reaction (RT-PCR) results revealed that the mRNA levels of LOC_Os07g12130 were altered in different photoperiod and temperature treatments. Thus, the LOC_Os07g12130 locus is the most likely candidate gene for *pms1(t)*. These results may facilitate not only using the molecular marker assisted selection of PTGMS genes, but also cloning of the *pms1(t)* gene itself.

Key words: Super hybrid rice, *pms1(t)*, Photoperiod and thermo-sensitive male sterile gene, Genetic mapping
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1 Introduction

Discovery and successful application of male sterility in hybrid rice breeding have made a tremendous contribution to food security in the world. A photoperiod and thermo-sensitive genic male sterile (PTGMS) rice, named Nongken 58S, was first found as a spontaneous mutant in the *japonica* variety Nongken 58 in China (Shi, 1985). PTGMS rice has a unique trait distinguishing it from the other varieties, in which its pollen fertility is altered by day-length and temperature (Zhang and Yuan, 1987). Pollens of the PTGMS line show sterility under long day and high temperature (LD/HT), whereas they become fertile during short day and low temperature (SD/LT). Consequently, as a desirable germplasm in developing ''two-line'' hybrid rice, the PTGMS strain serves as a sterile line under LD/HT conditions to produce hybrid seeds, whereas various degrees of male fertility under SD/LT conditions allow it to be used as a maintainer to propagate itself. Therefore, large-scale hybrid rice seeds can be developed by this "two-line" seed production system less expensively and more efficiently. In addition, regardless of the relationship of restoration and maintenance, PTGMS rice has a broad spectrum of restoration compared with the "three-line" system. A further advantage is that the performance of PTGMS hybrids avoids the negative

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effects of male sterile cytoplasm (Virmani *et al*., 2003).

The fertility trait shows a typical single-locus segregation in the F_2 population from a cross between Nongken 58S and Nongken 58. This locus was designated as *pms3* (Mei *et al*., 1999a; 1999b), and was delimited into a 28.4-kb interval on chromosome 12 (Lu *et al*., 2005). In contrast, the fertility trait of 32001S, an *indica* PGMS line also derived from Nongken 58S, displays a two-locus segregation pattern in the progeny resulting from a cross with the *indica* cultivar Minghui 63 (Zhang *et al*., 1994), indicating that the fertility trait segregation is dependent on the genetic backgrounds of the materials used in the crosses. The two loci were designated as *pms1* and *pms2*, and were localized on chromosomes 7 and 3, respectively. The effect of *pms1* was found to be two to three times larger than that of *pms2* (Zhang *et al*., 1994). Liu *et al*. (2001) constructed a physical map of the *pms1* region and further localized the *pms1* locus to an 85-kb region on chromosome 7. Compared with the TGMS, the genetic mechanism of PTGMS is far more complex. The sterile lines derived from Nongken 58S show diversity of photoperiod and thermo-sensitive characteristics, and the thermo-sensitive genes are not always allelic under different genetic backgrounds (Chen and Lu, 1996; Xiang *et al*., 2002; Zhou *et al*., 2005). Recently cloned PGMS genes *OSMYOXIB* (Jiang *et al*., 2007) and *Ugp1* (Chen *et al*., 2007) co-suppressing plants show TGMS phenotype, but are different from the PTGMS genes of Nongken 58S.

Pei′ai64S as a widely-applied PTGMS line, which shows significant thermo-sensitive characteristics, was also derived from the *japonica* variety Nongken 58S. It contains complex germplasm backgrounds such as *indica* and *javanica* (Luo *et al*., 2000). A new elite "two-line" hybrid rice variety named Liangyoupeijiu (Pei′ai64S/93-11) with Pei′ai64S as a male sterile line has been widely extended as a super hybrid rice variety in the southern provinces of China. However, the genetic factor(s) responsible for the PTGMS trait in the above line is still unclear. Thus, it is necessary to further investigate and map the sterile genes responsible for PTGMS in Pei′ai64S. In this study, we constructed a larger F_2 population with a total of 320 sterile plants, and attempted to make fine genetic mapping of PTGMS genes in Pei′ai64S. Here, we show that the *pms1(t)* gene is located in a 101.1-kb region between RM21242 (0.2 cM) and YF11 (0.2 cM) markers, and was predicted to encode a protein containing a Myb-like DNA-binding domain. The results of this study will greatly facilitate the final isolation of the *pms1(t)* gene by a map-based cloning approach and would be useful for molecular marker-assisted selection for new PTGMS lines in hybrid rice breeding.

2 Materials and methods

2.1 Plant materials and mapping population

The maternal parent Pei′ai64S, as an *indica* type of the PTGMS variety with a low critical temperature for fertility transfer, is widely applied in China (Luo *et al*., 2000). The paternal parent 93-11 is a typical *indica* variety, and its whole genome has been sequenced (Yu *et al*., 2002). The F_1 (Pei′ai64S/93-11) and F_2 populations were planted in the field under LD/HT conditions on the experimental farm at Zhejiang University, Hangzhou, China in 2005. The heading stage was arranged from late July to mid-August, during which the day length and average daily temperature were favorable for the identification of sterile plants in the F_2 population. The sterile control (Pei′ai64S) was sowed five times at 10 d intervals from the first 10 d interval of May to the second 10 d interval of June.

2.2 Pollen fertility examination

Plants were classified as sterile based on the anther's shape (not dehiscing) and without dispersed pollens during the heading stage. For pollen fertility, three panicles per sterile plant were sampled after the heading stage, but before flowering, and were fixed in 37% (v/v) formaldehyde/glacial acetic acid (FAA) solution. Pollen grains from broken anthers were stained with a 0.01 g/ml iodine and iodine-potassium solution (I_2-KI). More than 600 pollen grains from each individual were examined under a microscope to estimate the percentage of fertile stainable pollen. Plants with 100% sterile pollens were considered to be completely male sterile. Pollen fertility of each sterile plant was tested at least three times during the heading stage. All sterile individuals were planted in the field during the summer of 2006 to reconfirm their sterility.

2.3 Molecular marker development and assay

Whole genomic sequences of *Oryza sativa* subspecies *indica* (93-11) and *japonica* (Nipponbare) were downloaded from ftp://ftp.genomics.org.cn/pub/ricedb/SynVs9311/9311/Sequence/Chromosome/ and http://rgp.dna.affrc.go.jp/IRGSP/Build4/build4.html websites, respectively. Information about 18828 simple sequence repeats (SSRs) was obtained from the supplementary Table 18 of Matsumoto *et al.* (2005). Other SSR loci in the target genomic region were identified by the online software Simple Sequence Repeat Identification Tool (SSRIT; http://www.gramene.org/gramene/searches/ssrtool) (Temnykh *et al*., 2001). Polymerase chain reaction (PCR) based insert and deletion (In-Del) markers were developed for the regions in defect of SSR markers. Sequence differences between the corresponding genomic regions of 93-11 and Nipponbare were identified by bl2seq (http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi).

Primers for the candidate SSR or In-Del markers were designed based on the flanking sequence of SSR loci or In-Dels using the Primer3 program (http://frodo.wi.mit.edu/primer3/) (Rozen and Skaletsky, 2000). In order to predict the polymorphism of markers between 93-11 and Nipponbare, all primers were analyzed by electronic PCR (e-PCR) (ftp://ftp.ncbi.nlm.nih.gov/pub/schuler/e-PCR/) before synthesizing.

Plant DNA used for constructing the DNA pool of fertility or sterility was extracted from 5 g of fresh leaf tissues using the hexadecyltrimethy ammonium bromide (CTAB) method described by Murray and Thompson (1980). DNA extraction from other sterile plants was carried out following a rapid one-tube genomic DNA extraction method described by Steiner *et al*. (1995).

PCR was performed in a 15-μl reaction volume containing 25 ng of the template DNA, 1.5 μl 10× PCR buffer, 0.2 mmol/L deoxy-ribonucleoside triphosphate (dNTP), 0.2 μmol/L primer pairs, and 0.5 U *Taq* DNA polymerase. The amplification protocol included an initial denaturation at 94 °C for 5 min, followed by 35 cycles of 30 s denaturation at 94 °C, 30 s annealing at 55 °C, and extension of 1 min at 72 °C, and a final extension step of 5 min at 72 °C in a DNA Engine Thermal Cycler (MJ Research, USA). *Taq* DNA polymerase and dNTP were purchased from Dingguo Biotech Co., Ltd. (Beijing, China). Primers were synthesized by Sangon Co., Ltd. (Shanghai, China). PCR products were separated on a 0.06 g/ml polyacrylamide gel, and the amplified DNA fragments were silver-stained for visualization.

2.4 Genetic mapping and linkage analysis

Linkage between molecular markers and the PTGMS gene was determined following bulked-extreme and recessive-class approaches (Zhang *et al*., 1994). Fertile (BF pool) and sterile (BS pool) DNA bulks were prepared by mixing equal amounts of DNA from 10 highly fertile (>95% fertile pollen) plants and 10 completely sterile (100% sterile pollen) plants, respectively. Bulked segregation analysis was used to identify all of the markers linked to the PTGMS gene (Michelmore *et al*., 1991). Markers that could detect polymorphism between the two bulks were further used to analyze the completely sterile population.

2.5 Expression analysis

The plants tested for sterile traits were grown under two natural conditions: LD/HT and SD/LT. TRIZOL™ kit (Invitrogen, Shanghai, China) was used for RNA isolation with the manufacturer's recommended protocol. The total RNA was extracted from the developing young panicles with length of 2, 10, and 20 cm at the boot stage of the plants. Semi-quantitative reverse transcription (RT)-PCR experiments were performed with PrimeScript™ 1st strand cDNA synthesis kit (TaKaRa, Dalian, China). Total RNA was reverse transcribed into single-stranded cDNA. The cDNA transcribed from the total RNA (20 μl reaction volume) was used as the template according to the manufacturer's instructions. A 1-μl cDNA solution of each sample was used for RT-PCR. Base on information from the Institute for Genomic Research (TIGR) rice genome (http://rice.plantbiology.msu.edu/cgi-bin/gbrowse/rice/), sequences of five putative genes located in the target region were used for primer design to study their expressions. Primers were designed using the Primer3 program (http://cbr-rbc.nrc-cnrc.gc.ca/cgi-bin/primer3_www.cgi) (Rozen and Skaletsky, 2000). To exclude contamination of DNA, all primer pairs were designed to span at least one intron. Details of primers specific for these genes are shown in Table 1. RT-PCR amplification

with all the primers was carried out at an annealing temperature of 55 °C. The ubiquitin gene was used as a control. The PCR reactions consisted of an initial denaturation for 5 min at 95 °C followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 40 s. The PCR products were electrophoresed on 0.01 g/ml agarose gels. The experiments were repeated in triplicate.

Table 1 Primers for the RT-PCR analysis

Gene name	Primer name	Primer sequence (5′→3′)
LOC_Os07g12130	G2130F	AGTGGGCAGAGATTGCAAAG
	G2130R	CTTGCACCTATCACCCCACT
LOC_Os07g12140	G2140F	GGTCCCAGTTTCCTGCAATA
	G2140R	TCCGTGCAATCAAATCAAGA
LOC_Os07g12160	G2160F	CCTGAAGATGCAAGGAAAGC
	G2160R	GCCTCCAAATACCCCTTGAT
LOC_Os07g12170	G2170F	CTGTTCGCGAAGAAGGAGAT
	G2170R	TCATTCAGCATCCTGTGGAG
LOC_Os07g12240	G2240F	TGTTGGTACTGGGACCATTG
	G2240R	TGATCCTCCCATCTCTGTCC
Ubiquitin	UbiqF	AGAAGGAGTCCACCCTCCAC
	UbiqR	CACGGTTCAACAACATCCAG

2.6 Sequencing and data analysis

To calculate the linkage distance between molecular markers and *pms1(t)* locus, the data were analyzed with MAPMAKER/EXP 3.0 (Lincoln *et al*., 1992) at a limit of detection (LOD) threshold of 3.0. A linkage map was constructed using MapDraw V2.01 (Liu and Meng, 2003). The candidate gene sequencing was made by Invitrogen Corporation. The obtained gene sequences were used for gene prediction through Genscan (http://genes.mit.edu/GENSCAN.html) and FGENESH (http://mendel.cs.rhul.ac.uk/mendel.php?topic=fgen), and subjected to multiple sequence alignment with CLUSTALW (http://clustalw.genome.jp/).

3 Results

3.1 Construction of a completely sterile population and genetic analysis of male fertility

The day length and temperature are suitable for PTGMS gene expression during the heading stage from late July to mid August in Hangzhou. By pollen grain fertility test, a total of 320 completely sterile plants were obtained from a large F_2 population consisting of 6384 individuals. These plants were used to construct a completely sterile population for PTGMS gene mapping.

A total of 1008 plants were randomly selected from the F_2 population for genetic analysis. Pollen fertility of each plant was investigated under the LD/HT conditions. In this population, 59 plants classified as completely sterile showed no dehiscing anther (A3, B3 in Fig. 1) with 100% sterile pollen as the maternal parent Pei′ai64S (Table 2; C3, C4 in Fig. 1). Another 193 plants classified as partially sterile had a thin anther with some dehiscing occurring at the top (A2, B2 in Fig. 1) and contained about 6.20% of dark stained fertile pollens (Table 2; C2 in Fig. 1). The anther shape and pollen fertility of the rest of the 756 fertile plants were similar to those of the paternal parent 93-11 (A1, B1, C1 in Fig. 1). The numbers of fertile plus partially sterile and completely sterile individuals fit the expected 15:1 ratio (χ^2=0.21, P>0.9), indicating that the PTGMS trait of Pei′ai64S was controlled by two recessive genes. The expected segregation ratio was also confirmed by distribution of seed setting rate (data not shown).

Table 2 Pollen fertilities of F_2 sterile individuals and control by the observation under light microscope

F_2 sterile individuals and control	*n*	Pollen fertility (%)			
		TS	RS	SS	NF
Extreme sterility	59	99.23	0.01	0.77	0.00
Partial sterility	193	77.56	0.30	15.94	6.20
Pei′ai64S (CK)	150	98.14	0.76	1.10	0.00

On the basis of their shapes and staining patterns, pollen grains could be classified into four categories, viz. typical sterile or unstained withered sterile (TS), round sterile or unstained spherical sterile (RS), stained sterile or stained round sterile (SS), and normal fertile or stained round fertile (NF), respectively

3.2 Analyses of linkage molecular markers using bulked segregant analysis (BSA) method

The sterile genes of Pei′ai64S were linked with the SSR marker on chromosome 7 based on previous study (data not shown). After analysis of the predicted amplification by e-PCR, 58 pairs of primers based on specific sequences of chromosome 7 were designed for screening the polymorphism between two parents (Pei′ai64S and 93-11), and between two bulks (fertile pool and sterile pool). Twenty-two pairs of primers were identified as polymorphic markers, which showed a linkage relationship with the PTGMS gene (Table 3; Fig. 2).

Table 3 Primer sequences and sizes of polymorphic bands for the SSR and In-Del markers in the *pms1(t)* region

Marker name	Synonym	Motif	No. of Repeat	Chr.	Primer sequence (5′→3′)	PCR product size (bp)	
						Nipponbare	93-11
RM21136		AAG	13	7	Forward: GAAGCCAAACGCAACCAAGG Reverse: TCGGTGAATTGTCCTGTATCAGC	108	108
RM21216		AT	41	7	Forward: GTGGCCACCTGTGGATACAA Reverse: TATCTCATGCGAGCCAGATG	385	
YF48		AT	8	7	Forward: GGCTCTTAGCCATCAAGATACAA Reverse: CAGTATGGAACAAAGAGCCAAG	152	
RM21238		CCG	10	7	Forward: GAGCTTCTCCTCACCCATCACC Reverse: CTTCTGCAGAGGGTGTTCAACG	189	273
RM21242		GTC	7	7	Forward: GAGAGGAATGGAATGGAATGAGG Reverse: GAACAGGCATGGTGAAGAGTGC	131	134
RM6776	RM21244	TCC	9	7	Forward: AGCCCGGACATGCAAAAC Reverse: GAAGCAGGCGAAATCTCCTC	169	
YF11		A	17	7	Forward: ATACCTCACTTGGCCTGTGC Reverse: TACCCTATACCGAACCGTGC	190	194
YF15		In-Del		7	Forward: TGGAGGCAAACCTAAAAAGG Reverse: TAATCCAACCGCACGTGAT	351	343
YF22		In-Del		7	Forward: TCGAGTCCGCTTATCTCCAG Reverse: AGGGAGAGGGGAGAGGAGTA	210	205
YF25		In-Del		7	Forward: TGGCGACATAGGAGTGTTTG Reverse: GAGAGATATCCGCCAGTTGC	386	377
RM21247		AG	10	7	Forward: GACGTCTCATCCTCCATGGTTCC Reverse: TTGCGATGCCGAGGAGATAAGG	236	
YF6		In-Del		7	Forward: ACTTCAGATGAGAGTTCCCAACA Reverse: GTCAGACCAGCGGCTATACTGT	307	224
YF10		TC, TA	8, 22	7	Forward: ACGCCAGAGACCACTCACTC Reverse: TGGGAGTAACGAACACCACA	205	239
RM1253	RM21249	AG	16	7	Forward: CTGAACTTGCCTGAGAACTC Reverse: GACGACCTCTCCATGCTCG	175	
RM7153	RM21310	ACAT	8	7	Forward: ATCCTAAAGCAGTTGCACGG Reverse: GATCAGCAACCATCCAAAGG	154	
RM542	RM21421	AG	22	7	Forward: TGAATCAAGCCCCTCACTAC Reverse: CTGCAACGAGTAAGGCAGAG	114	92
RM214	RM21423	AG	32	7	Forward: CTGATGATAGAAACCTCTTCTC Reverse: AAGAACAGCTGACTTCACAA	149	113
RM7338	RM21502	ATCC	9	7	Forward: CTTATCTCTCGGCAAGCAGC Reverse: CTCACACGCATGGATCAATC	164	160
RM445	RM21582	AG	12	7	Forward: CGTAACATGCATATCACGCC Reverse: ATATGCCGATATGCGTAGCC	251	259
RM432	RM21652	ATCC	9	7	Forward: TTCTGTCTCACGCTGGATTG Reverse: AGCTGCGTACGTGATGAATG	187	179
RM3691	RM21671	AG	15	7	Forward: GCTGATGGTCAAAGATCAGG Reverse: ATGTGTCTGCTGGCACAGAG	117	145
RM11	RM21672	AG	15	7	Forward: TCTCCTCTTCCCCCGATC Reverse: ATAGCGGGCGAGGCTTAG	126	146

3.3 Fine mapping of the *pms1(t)* gene

The *pms1(t)* locus was mapped using several polymorphic SSR markers that distributed randomly on chromosome 7 and were used to assay a population consisting of 218 completely sterile individuals separately one by one. The genotype data of the sterile population were obtained by reading the band of PCR products from the polyacrylamide gel electrophoresis. The recombination events between the *pms1(t)* locus and closely linked markers indicated that a marker closer to the gene had a smaller number of recombinants in the panel (Table 4). There are 42 recombinants between the farthest marker RM11 and *pms1(t)* with the genetic distance of 11.5 cM. For the closer linked marker RM7153, 10 recombinants occurred between the *pms1(t)* locus and RM7153, so the distance from RM7153 to *pms1(t)* was only 2.6 cM. On the other hand, 37 recombination events occurred between RM21136 and the *pms1(t)* locus with the genetic distance of 9.8 cM (Fig. 3).

In an additional 102 completely sterile individuals, 3 and 16 recombinants were detected at RM7153 and RM21136, respectively. Altogether, the data comprising 320 completely sterile individuals showed that the number of recombinant individuals was 13 at RM7153 and 53 at RM21136, respectively.

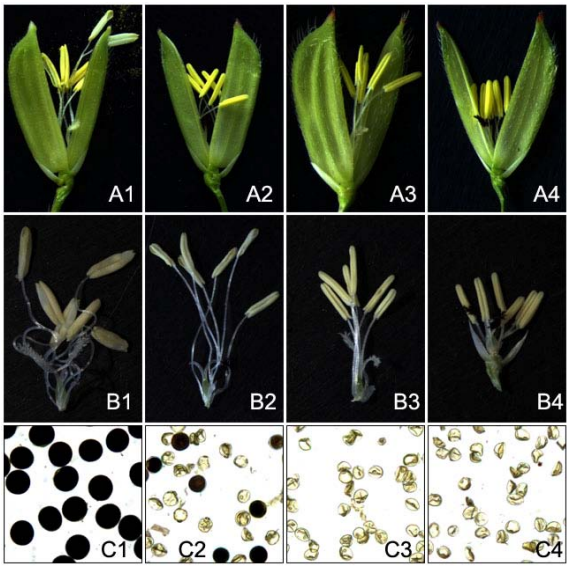

Fig. 1 Anthers observed by naked eyes and pollen grains observed under light microscope

A1–A4 showed the shapes of anthers from two parents and their F_2 individuals. A1: 93-11 as the fertile control with dispersed normal fertility pollens; A2: F_2 individual with partially dehiscent anther; A3: F_2 individual with no dehiscent anther; A4: Pei′ai64S as the sterile control under the LD/HT condition. B1–B4 and C1–C4 showed anthers and pollen grains stained with I_2-KI solution, corresponding to column specimens of A1–A4. The black-stained pollen grains are considered viable, while shrink and unstained sterile pollen grains are abortive

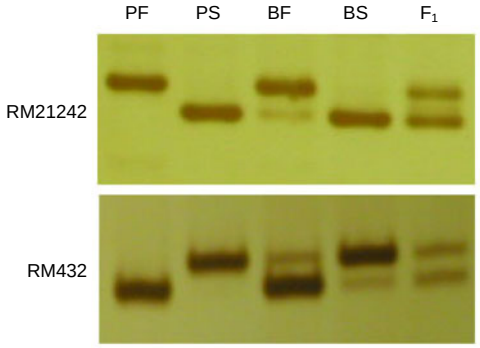

Fig. 2 BSA analysis for SSR markers

PF, PS, BF, BS, and F_1 represent 93-11, Pei′ai64S, fertile bulk, sterile bulk, and F_1 hybrid of a cross of Pei′ai64S/93-11, respectively. The PCR products were separated in non-denatured 0.06 g/ml polyacrylamide gel electrophoresis, and visualized by quick silver-staining. The bands were amplified by two SSR primers (RM21242 and RM432)

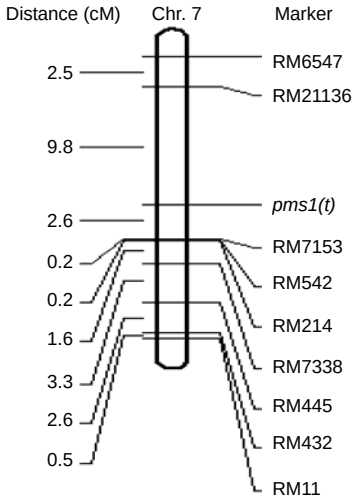

Fig. 3 Location of the *pms1(t)* locus on the molecular linkage map of chromosome 7

Table 4 Genotypes of sterile individuals for recombinant between sterile genes and molecular markers

Marker	Genotype													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
RM21136	B	B	B	B	B	B	B	B	B	B	A	H	H	H
YF48	B	B	B	B	B	B	B	B	B	B	B	B	B	H
RM21238	B	B	B	B	B	B	B	B	B	B	B	B	B	H
RM21242	B	B	B	B	B	B	B	B	B	B	B	B	B	H
RM6776	B	B	B	B	B	B	B	B	B	B	B	B	B	B
YF11	H	B	B	B	B	B	B	B	B	B	B	B	B	B
YF22	H	B	B	B	B	B	B	B	B	B	B	B	B	B
YF25	H	H	B	B	B	B	B	B	B	B	B	B	B	B
YF6	H	H	H	B	B	B	B	B	B	B	B	B	B	B
RM1253	H	H	H	B	B	B	B	B	B	B	B	B	B	B
RM7153	H	H	H	H	H	H	H	H	H	H	A	H	H	B

A, B, and H represent genotypes of 93-11, Pei′ai64S and their heterozygote, respectively. 1–14 represent the selected individuals

For fine physical mapping of *pms1(t)*, a series of markers was further developed. As a result, we obtained 11 polymorphic molecular markers, including 7 SSR markers and 4 In-Del markers between RM21136 and RM7153 (Table 4). These markers were further used to screen the recombinants detected by RM7153 and RM21136. No recombination event was detected between the marker RM6776 and *pms1(t)*, and only one recombination occurred between *pms1(t)* and its closest linked markers RM21242 and YF11 on both sides. Thus, the genetic distance decreased to 0.2 cM between *pms1(t)* and RM21242 (Table 5; Fig. 4).

3.4 Candidate genes of *pms1(t)*

Genomic sequence analysis indicated that RM21242 and YF11 were located on BAC clones AP005197 and AP003740, respectively. Based on the

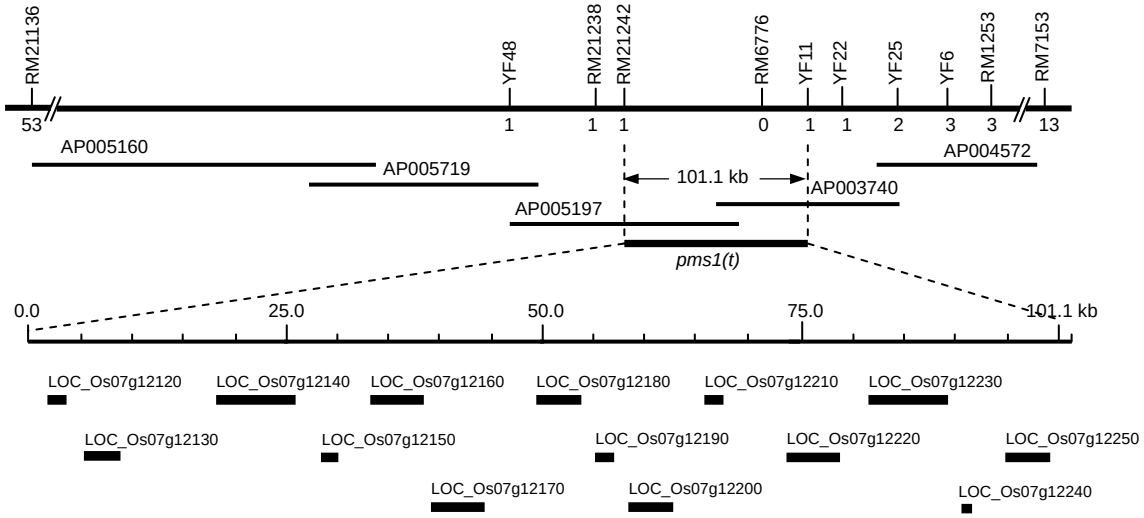

Fig. 4 High-resolution genetic and physical map of the *pms1(t)* locus

In the partial genetic map of the molecular markers, the numbers under the long horizontal line indicate the times of recombination events detected between the *pms1(t)* locus and its corresponding markers. The bold bars represent the BAC clones. The *pms1(t)* locus was covered by the thick horizontal line between the two vertical dashed lines from RM21242 to YF11, in which the genomic information was based on Nipponbare genome sequence (released 4.0), and some gene annotations of the gene loci represented with black bars were from TIGR database

Table 5 Gene annotations and positions of start and end points of 14 gene loci within the 101.1-kb region from RM21242 to YF11 on chromosome 7

No.	Gene locus	Gene annotation	Start	End
1	LOC_Os07g12120	Hypothetical protein	6775171	6776190
2	LOC_Os07g12130	Myb-like DNA-binding domain containing protein, expressed	6779818	6777673
3	LOC_Os07g12140	DNA-binding protein, putative, expressed	6790010	6794406
4	LOC_Os07g12150	Acyl carrier protein, mitochondrial precursor, putative, expressed	6797368	6797839
5	LOC_Os07g12160	KHG/KDPG aldolase, putative, expressed	6801745	6804236
6	LOC_Os07g12170	ADP-ribosylation factor 1, putative, expressed	6807971	6810407
7	LOC_Os07g12180	Retrotransposon protein, putative, Ty1-copia subclass	6816373	6813623
8	LOC_Os07g12190	Retrotransposon protein, putative, unclassified	6818596	6817502
9	LOC_Os07g12200	ADP-ribosylation factor 1, putative, expressed	6821343	6823745
10	LOC_Os07g12210	Conserved hypothetical protein	6832221	6833291
11	LOC_Os07g12220	Conserved hypothetical protein	6839803	6843124
12	LOC_Os07g12230	Hypothetical protein	6846320	6851694
13	LOC_Os07g12240	Calmodulin TaCaM2-1, putative	6854921	6855484
14	LOC_Os07g12250	60S ribosomal protein L24, putative, expressed	6863637	6864932

The above information is cited from the TIGR Rice Genome Annotation website http://rice.plantbiology.msu.edu/cgi-bin/gbrowse/rice/

genome sequence of Nipponbare, the physical distance between these two markers is approximately 101.1 kb. Based on the gene annotation information (TIGR Rice Genome Annotation Release 5, Jan. 24, 2007; http://www.tigr.org/tdb/e2k1/osa1/pseudomolecules/info.shtml), about 14 predicted loci were found to be present in the above region, which may be considered as the candidate genes for *pms1(t)* (Fig. 4; Table 5). Among these loci, LOC_Os07g12130 encodes a Myb-like protein containing a DNA-binding domain, which hits 16 gene ontology (GO) terms. One of the gene ontology (GO) terms is GO:0009628, which responds to abiotic stimulus. Interestingly, GO:0009266, a child term for GO:0009628, responds to thermal stimulus. Two other second child terms, GO:0009408 and GO:0040040, also respond to heat stimulus and have the characteristics of thermosensory behavior, which are very closely related to the thermo-sensitive genic male sterile characteristics. However, for the other 13 candidate genes, we did not find a direct relationship with the photoperiod or thermo-sensitive response. These results suggest that the locus of LOC_Os07g12130 is more likely a thermo-sensitive candidate gene for *pms1(t)*.

3.5 Expression analysis

To investigate whether the expression levels of the candidate genes located in the target region change under different conditions, five putative genes annotated as expressed proteins were tested for their expressions in rice panicles at 2, 10, and 20 cm stages. These tissues were harvested from Pei′ai64S plants grown under two natural conditions, LD/HT and SD/LT. The results showed that expressions of LOC_Os07g12140, LOC_Os07g12160, and LOC_Os07g12170 were detected in all tested panicles, and their mRNA levels displayed similar expression patterns in different photoperiod and temperature treatments (Fig. 5). The band intensities of LOC_Os07g12130 were higher at 2 and 10 cm panicles than that at 20 cm panicles under LD/HT conditions, but the band intensities from 2 and 10 cm panicles were lower under LD/HT conditions than those from same stage panicles under SD/LT conditions (Fig. 5), indicating that the expression of LOC_Os07g12130 is altered under different environmental conditions and/or different panicle growth stages. These expression patterns of LOC_Os07g12130 further support that the *pms1(t)* is a promising candidate gene. Similar diversities of the expression levels for LOC_Os07g12240 were observed under different environments and/or panicle developmental stages. The band intensities of 20 cm panicles under LD/HT conditions and 10 cm panicles under SD/LT conditions were almost undetectable. Our results imply that LOC_Os07g12140 may also associate with photoperiod and thermo-sensitive male sterile traits, and its expression may be controlled by other factor(s).

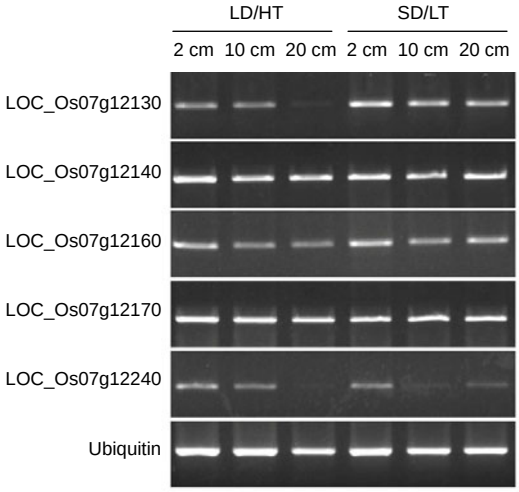

Fig. 5 Semi-quantitative RT-PCR results of five candidate genes for Pei′ai64S

The individuals were planted under LD/HT and SD/LT conditions, respectively. Also, samples were taken at three different stages of the young panicle sizes (2, 10, and 20 cm). LOC_Os07g12130, 12140, 12160, 12170, and 12240 were used as the candidate genes, and ubiquitin as a control in our experiment

4 Discussion

4.1 Genetic diversity of PTGMS rice

Nongken 58S, which was a photosensitive male sterile line discovered by the Shi (1985), has played a crucial role in "two-line" rice breeding in China; however, the studies on its photosensitive sterile gene and genetic mapping remain controversial. Early research suggested that the sterility trait for *japonica* PTGMS lines, involving Nongken 58S, is controlled by a single nuclear gene (Shi, 1985), which was named as *pms3* (Mei *et al*., 1999a; 1999b), and mapped on chromosome 12 (Lu *et al*., 2005). However, subsequent studies showed that the sterile trait for

PTGMS varieties derived from Nongken 58S was controlled by two recessive genes. The PGMS genes of the line 32001S consist of the two gene loci (*pms1* and *pms2*), of which the *pms1* gene was located on chromosome 7 (Zhang *et al*., 1994; Mei *et al*., 1999b; Liu *et al*., 2001; Lu *et al*., 2005), and another *pms3* gene on chromosome 12 (Lu *et al*., 2005).

Localization studies showed that the TGMS genes from different sources may be located in different chromosomes. Examples include reports that *tms1* (Wang *et al*., 1995), *tms2* (Lopez *et al*., 2003), *tms3* (Subudhi *et al*., 1997), *tms4* (Dong *et al*., 2000), *tms5* (Yang *et al*., 2007a), *rtms1* (Jia *et al*., 2001), *ms-h* (Koh *et al*., 1999), and *tms6* (Lee *et al*., 2005) were located on chromosomes 8, 7, 6, 2, 2, 10, 9, and 5, respectively. However, these TGMS materials are not to be applied in rice production because of the non-stability of their sterility. The Pei′ai64S used in this study has been widely used in the seed production of hybrid rice in China. This PTGMS line is developed through subspecies hybridization, and has a complex genetic background containing rice germplasms from *indica*, *japonica*, and *javanica*, although its sterility is also derived from the Nongken 58S. So it is of great significance in theory and practice to clarify the localization of the sterile gene.

4.2 Distribution of PTGMS genes on rice chromosome 7

In this study, we showed that the sterility trait for Pei′ai64S is controlled by two recessive genes. In our linkage analysis of SSR markers, we showed that the *pms1(t)* gene for the Pei′ai64S located on the short arm of chromosome 7 between two markers (RM7153 and RM21136) with a 12.4-cM genetic distance. Further we found that the *pms1(t)* gene was within the interval of the RM21242 and YF11 with a 0.4-cM distance, and was co-segregated with the RM6776. Based on sequence information from the Nipponbare genome, the gene range between RM21242 and YF11 covers close to 101.1 kb, in which there are 14 loci. By combining the above bioinformatics and gene expression analyses, we hypothesized that the LOC_Os07g12130 locus is most likely a candidate gene of *pms1(t)*.

So far, two other TGMS genes, *tms2* (Lopez *et al*., 2003) and *tms5* (Zhou *et al*., 2006), were mapped on the long arm of rice chromosome 7 while the PTGMS gene *pms1* was also mapped on the short arm of this chromosome (Liu *et al*., 2001). Therefore, we hypothesized that there may exist a "hotspot" of the TGMS genes on chromosome 7. Interestingly, the RG477 linked to *pms1* gene with the interval of 0.25-cM (Liu *et al*., 2001), and was just located at the second intron position of the LOC_Os07g12130 locus. Further analysis showed that our positioning of the *pms1(t)* gene coincided with the location of the *pms1* gene in the same BAC clone, but in the opposite direction (GenBank accession No. AP005197). We suggest that there are two following possible explanations:

The first one is the different genetic background between both tested PTGMS varieties (Pei′ai64S in this study and 32001S tested in Liu *et al*. (2001)). The former was developed from the complex crosses of *indica*/*japonica*, while the latter was derived from the cross of *indica*/*indica*, although both sterile varieties contained commonly the sterile genes from Nongken 58S. Therefore, we assume that a gene cluster (a set of two or more genes) may play the different effects on the male sterility, which was also found in the fertility restorer genes. There is a set of the *Rf-1* homologous genes on chromosome 10 (Akagi *et al*., 2004; Komori *et al*., 2004).

The second one is that our experimental methods and identification standard of the sterile plants are different from those used by Liu *et al*. (2001), which will have an impact on the positioning results. Liu's sterile standard was based on the seed setting rate under the natural conditions, in which the plants with seed setting rate lower than 10% were regarded as the sterile type. However, this estimated number of sterile plants was generally higher than that from pollen fertility (data not shown). Therefore, the adoption of identification standards with the pollen fertility was superior to that with the seed fertility, which can accurately calculate the crossing-over value of the sterile plants. In order to ensure the accuracy of the investigation, we also conducted two years of repeated testing and reproduced consistent results. Meanwhile, we also developed an optimal DNA silver staining method for SSR marker analysis on 0.06 g/ml polyacrylamide gel, which ensures gene mapping accuracy of *pms1(t)*.

4.3 Correlation between Myb-like protein and PTGMS gene

Based on RT-PCR results, we sequenced the two candidate genes LOC_Os07g12130 and LOC_Os07g 12240 (supplementary Data 1–4), which displayed different expression levels under different environmental conditions. LOC_Os07g12130, as a *pms1(t)* candidate gene, encodes a Myb-like protein with a DNA binding domain. Myb proteins are the largest superfamily of transcription factors that play a regulating role in the process of development and defense reaction, such as regulation of plant cell shape, growth regulators, and responses to stress (Meissner *et al*., 1999). A new *Myb*-like gene (*AtMYB103*) showed a high level of mRNA expression in the early development of anther tapetum and middle-level performance in *Arabidopsis thaliana* (Li *et al*., 1999). The fertility of the mutant with double defects of anther development in *myb33* and *myb65* was enhanced in higher light and lower temperature conditions (Millar and Gubler, 2005). The over expression of *AtMYB24*, as a member of the R2R3 *Myb* gene family, usually causes abnormal pollen grains and anther indehiscence during the microspore development in *Arabidopsis* (Yang *et al*., 2007b). Zhu *et al*. (2004) identified an anther dehiscence 1 (*aid1*) gene, which encodes a new protein containing single Myb domains from a mutant of rice spikelet fertility. In addition, *OsGAMYB* gene knockout mutants produced abnormal flowers more frequently under high temperature conditions than under low temperature conditions (Kaneko *et al*., 2004). Thus, LOC_Os07g 12130 that may be regarded as a member of a signal transduction pathway affects anther development by regulating gene expression at different temperatures. In young panicles of Pei′ai64S, Myb transcription factors showed a significantly lower transcriptional abundance under the LD/HT than under the SD/LT conditions, which may lead to a significant gene inhibition in the normal process of transcription (Guo *et al*., 2006).

In this study, we hypothesized that there might be different expression patterns of *pms1(t)* gene under different photoperiod and temperature conditions. The RT-PCR results demonstrated that the mRNA level of *pms1(t)* (LOC_Os07g12130) was dramatically reduced under the LD/HT conditions, which suggests an association between male sterility and this gene. However, the detail on the *pms1(t)* expression pattern under different photoperiod and temperature conditions remains unclear. Further work is required to elucidate whether this gene product is similar to the UGP-like enzyme (Chen *et al*., 2007) in the rice spikelet.

The mutations that occurred in the promoter/ untranslated regions (UTR) may lead to changes in gene expression profiles. Recently, by sequencing of the upstream of *BnGID1* gene of a dwarf mutant from *Brassica napus*, a tripartite base mutant in the pyrimidine box of the *BnGID1* promoter was found (Li *et al*., 2010), based on which we need to further confirm sequence mutations in the promoter/UTR of LOC_Os07g12130 in our further research.

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List of electronic supplementary materials

Supplementary Data 1 Multiple DNA sequence alignment for predicted protein of LOC_Os07g12130

Supplementary Data 2 Multiple DNA sequence alignment for LOC_Os07g12130 in Pei′ai64S S1, S2, and S3

Supplementary Data 3 Multiple DNA sequence alignment for LOC_Os07g12130 in Pei′ai64S, 93-11, and Nipponbare

Supplementary Data 4 Multiple DNA sequence alignment for LOC_Os07g12240 in Pei′ai64S, 93-11, and Nipponbare