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ORIGINAL PAPER

Molecular mapping of two reverse photoperiod-sensitive genic male sterility genes (*rpms1* and *rpms2*) in rice (*Oryza sativa* L.)

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Abstract The reverse photoperiod-sensitive genic male sterility (PGMS) and thermo-sensitive genic male sterility (TGMS) lines have an opposite phenotype compared with normal PGMS and TGMS lines widely used by the two-line system in current hybrid rice seed production. Thus, the application of reverse PGMS and TGMS lines can compensate PGMS and TGMS lines in hybrid rice production. YiD1S is a reverse PGMS line, in which pollen fertility is mainly regulated by day-length, but also influenced by temperature. Genetic analysis indicated that male sterility of YiD1S was controlled by two recessive major genes. An F_2 population from a cross between YiD1S and 8528 was developed and used for molecular mapping of the two reverse PGMS genes which were first named *rpms1* and *rpms2*. Both simple sequence repeat (SSR) markers and bulked segregant analysis (BSA) were used in this study. As a result, one reverse PGMS gene (*rpms1*) was mapped to the interval between SSR markers RM22980 (0.9 cM) and RM23017 (1.8 cM) on chromosome 8. Eight SSR markers, YDS818, RM22984, RM22986, RM22997, YDS816, RM23002, RM339 and YDS810 completely co-segregated with the *rpms1* gene. Another reverse PGMS gene (*rpms2*) was mapped to the interval between SSR markers RM23898 (0.9 cM) and YDS926 (0.9 cM) on chromosome 9. The physical mapping information from publicly available resources shows that the *rpms1* and *rpms2* loci are located in a region of 998 and 68 kb, respectively. The analysis based on marker genotypes showed that the effect of *rpms1* was slightly larger than that of *rpms2* and that the two genes interacted in controlling male sterility.

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Introduction

Hybrid rice breeding has made a tremendous contribution to food security in China. Spontaneous male sterile lines play a pivotal role in large-scale hybrid seed production. Discovery and successful utilization of a wild abortive type of cytoplasmic male sterile (CMS) line resulted in a breakthrough for utilization of heterosis in a self-pollinated crop (Liao and Fu 1995). The three-line system (CMS line, maintainer line, and restorer line) was developed to produce hybrid seed and proved to be effective for increasing rice yield. However, the three-line hybrid breeding system is time-consuming and costly for hybrid seed production. Maintaining the CMS line and choosing an appropriate restorer line for developing the fertile hybrids are the major limitations. The discovery and successful utilization of photoperiod-sensitive genic male sterility (PGMS) and thermo-sensitive genic male sterility (TGMS) led to the development of a simple and highly efficient two-line hybrid breeding system. Compared with the three-line system, the two-line system has many advantages for hybrid

seed production. First, because male sterility expression in PGMS and TGMS rice is mainly regulated by photoperiod and/or temperature, PGMS and TGMS plants can be used not only as male sterile lines, but also as a maintainer lines. Second, any normal fertile plants are able to restore its fertility in the F_1, which providing a broad basis for screening strong heterotic combinations. Third, the PGMS and TGMS trait is usually controlled by one or two major nuclear genes, so it should be relatively easy to transfer to diverse genetic backgrounds (Yuan 1990). These advantages make the two-line system to be widely used to hybrid seed production in rice.

In general, PGMS lines are predominantly photoperiod-sensitive and TGMS lines predominantly temperature-sensitive. Currently, most PGMS or TGMS lines used by two-line system are sterile under long day-length and/or high temperature, but fertile under short day-length and/or low temperature. These lines are usually called normal PGMS or TGMS. To normal PGMS or TGMS system, sudden drop in temperature can be disastrous due to reversion to fertile phase resulting in self seed of the female parent during hybrid seed production. In order to overcome the potential risk caused by unpredicted low temperature below the critical level in hybrid seed production, rice breeders not only have been working for breeding PGMS and TGMS lines with a lower sterility-inducing critical temperature, but also have been focusing on the exploration of new PGMS or TGMS resources for many years. After the 1990s, one kind of new PGMS and TGMS germplasm, which are sterile under short day-length and/or low temperature, but fertile under long day-length and/or high temperature, was discovered and reported in succession, such as IVA (Zhang et al. 1991), N10S and N13S (Li et al. 1991), YDS (Gao 1991), go543S (Yang and Zhu 1996), DiannongS-2 (Jiang et al. 1997), J207S (Jia et al. 2001), and so on. Owning to having an opposite phenotype compared to normal PGMS and TGMS, this kind of new PGMS and TGMS germplasm is called reverse PGMS and TGMS, which can compensate normal PGMS and TGMS lines in hybrid rice production and make two-line hybrid rice used in much large areas.

Since PGMS in rice was reported from China (Shi 1985), there have been considerable advances in different aspects related to theory and technique of two-line system in hybrid rice. However, the PGMS and TGMS mechanism is not yet clear up to now, which became one of the maximum limit to the development of two-line system in hybrid rice. Mapping and cloning of the PGMS and TGMS genes will be especially useful in understanding the PGMS and TGMS mechanism. To date, three normal PGMS genes from 32001S and Nongken58S, *pms1*, *pms2*, and *pms3* have been mapped on chromosomes 7, 3, and 12, respectively (Zhang et al. 1994; Mei et al. 1999). Eight normal TGMS genes, *tms1* from 5460S (Wang et al. 1995), *tms2* from PL-12 (Yamaguchi et al. 1997), *tms3* from IR32364 (Subudhi et al. 1997), *tms4* from TGMS-VN1 (Dong et al. 2000), *tms5* from AnnongS-1 (Wang et al. 2003), *tms6*(t) from 0A15-1(Wang et al. 2004), *TGMS* from SA2 (Reddy et al. 2000), and *ms-h*(t) from Hwacheong ms-h (Koh et al. 1999), have been mapped on the chromosomes 8, 7, 6, 2, 2, 3, 9, and 9, respectively. One reverse TGMS gene, *rtms1* from J207S, has been mapped on chromosome 10 (Jia et al. 2001). One specific TGMS gene from Sokcho-MS, *tms6* has been mapped on chromosome 5 (Lee et al. 2005). Because Sokcho-MS is completely sterile at above 27°C and/or below 25°C, but fertile at 25–27°C regardless of the levels of day-length, *tms6* is neither identical to normal TGMS genes nor identical to reverse TGMS gene. Among these genes, *pms1*, *pms3*, and *tms5*, have been physically mapped to a region of 85, 28.4, and 19 kb, respectively (Liu et al. 2001; Lu et al. 2005; Yang et al. 2007). However, none of the reverse PGMS genes has been mapped as yet. YiD1S is a reverse PGMS line developed from the cross of *indica* variety "B3"and *japonica* variety "Hongjing" in China (Gao 1991). During the past several years, we have conducted exhaustive studies on its characteristic of fertility alteration, inheritance, utilization, and so on. Our previous studies proved the reverse PGMS trait of YiD1S, and showed that male sterility expression in YiD1S was also influenced by temperature (Lu YP et al. 2001). Genetic analysis indicated that the male sterility of YiD1S was controlled by two recessive major nuclear genes, and may be influenced by many minor genes (Lu et al. 2000). On the basis of these results above, this paper reports mapping of the two major reverse PGMS genes by using SSR marker technique combined with bulked segregant analysis. Genetic effects based on the marker genotypes were also estimated for *rpms1* and *rpms2*.

Materials and methods

Development of mapping population

YiD1S is the original source of the two reverse PGMS genes (*rpms1* and *rpms2*) in rice, which was crossed with seven rice cultivars with normal pollen fertility for screening of the mapping population. When YiD1S was completely sterile after the late of September, the pollen fertility of each F_2 individual was examined at this moment. The F_2 population with apparent bimodal distribution of pollen fertility and a relatively high level of polymorphism between the parents was selected as the mapping population. Thus, an F_2 population consisting of 948 individuals derived from the cross YiD1S × 8528 was used for the molecular mapping of two reverse PGMS genes. The mapping population

was planted in the farm of South China Agricultural University in Guangzhou, China.

Examination of pollen fertility

To evaluate the pollen fertility, about 15 spikelets were sampled from three panicles per F_2 plant during anthesis and their anthers were squashed in 1% iodine-potassium iodide (I-KI) solution for examination of pollen fertility. All round, darkly stained pollen were scored as fertile, and the unstained shriveled or spherical pollen or yellow-colored pollen were scored as sterile. The average pollen fertility from three panicles was expressed as percentage.

DNA preparation

DNA of each F_2 individual and both parents was extracted from fresh-frozen leaves. Two DNA bulks, F (fertile) and S (sterile) were constructed by selecting extreme individuals from the F_2 population of the cross YiD1S × 8528. DNA bulk F was made by mixing equal amounts of DNA from ten extremely fertile plants, and DNA bulk S was made by mixing equal amounts of DNA from ten extremely sterile plants.

SSR analysis

A total of 513 SSR markers, including 85 SSR markers designed using the sequence information of the two reverse PGMS genes region in rice genome (http://www.ncbi.nlm.nih.gov), simple sequence repeat identification tool (SSRIT) (http://www.gramene.org) and Premier 5 software, were used to amplify DNA fragment according to the PCR procedure previously described by Panaud et al. (1996). The amplification products were analyzed on the 6% PAGE gel and detected by silver staining.

Linkage analysis and genetic effects analysis

The linkage between molecular markers and reverse PGMS loci were determined with BSA and recessive class approach. The polymorphism between the parents was first detected with SSR markers covering 12 rice chromosomes. Then, the polymorphic markers were further used for identifying possible reverse PGMS genes-containing chromosome regions with sterile and fertile DNA bulks from the F_2 population of the cross YiD1S × 8528. The map locations of reverse PGMS genes were finally determined in 52 highly sterile individuals from mapping population with the polymorphic markers between two DNA bulks. The recombination frequency (c) was calculated with the formula: $c = (N_1+N_2/2)/N$, in which N is the total numbers of sterile plants surveyed, N_1 is the number of individuals with homozygous band from the fertile parent; N_2 is the number of individuals with heterozygous band from the two parents (Zhang et al. 1994). Then the recombination frequency was converted into genetic distance (centiMorgan, cM) using Kosambi function (Kosambi 1944). Linkage maps were made using the software MAPCHART. The genetic effects based on the marker genotypes were estimated for *rpms1* and *rpms2* using a random sample of 484 individuals taken from the mapping population according to the method previously described by Zhang et al. (1994). Using marker genotypes as groups, the genetic effects of *rpms1* and *rpms2* were evaluated by the difference between the average pollen fertility of homozygous dominant allele and that of homozygous recessive allele at its own locus, respectively. SAS 8.0 software was used for a two-way analysis of variance (ANOVA) of *rpms1* and *rpms2*.

Results

Pollen fertility in mapping population

After the late of September in 2005, YiD1S was completely sterile in Guangzhou, China. Similar results were also obtained in 1995, 1996, 1998 (Wan et al. 1998; Lu YP et al. 2001) and 2004 (date not shown). This suggested that YiD1S is a reverse PGMS line with highly sensitive to photoperiod. In sterile stage of YiD1S, the pollen fertility of the F_2 population from the cross YiD1S × 8528 was examined at this moment. The distribution of the pollen fertility frequency of 948 F_2 plants is given in Fig. 1. A bimodal distribution was observed in this F_2 population, and Chi-square analysis showed that breaking at any point in the apparent valley with pollen fertility between 20 and 30% fit to a 15:1 segregation ratio ($\chi^2 = 0.145$, $P > 0.25$), which suggested male sterility of YiD1S was controlled by two recessive major genes under short day-length and low temperature. This result is consistent with the previous reports on genetic analysis of male sterility in YiD1S (Lu et al. 2000).

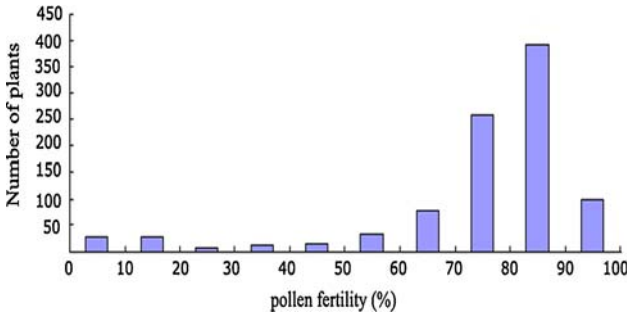

Fig. 1 Distribution of the pollen fertility of 948 F_2 plants from the cross of YiD1S and 8528 in Guangzhou at late cropping season of 2005

Mapping of two reverse PGMS genes

A total of 358 SSR markers distributed on 12 rice chromosomes, were selected to screen polymorphism between the two parental varieties YiD1S and 8528. Of these, 133 SSR markers were polymorphic between the parents. These polymorphic SSR markers were further used to screen polymorphism between the sterile and fertile DNA bulks. Nine SSR markers RM72, RM44, RM339, PSM527, PSM284, PSM399, PSM157, PSM158, and RM105 from chromosomes 8 and 9 were polymorphic between both the bulks as well as the parents. The linkage of nine polymorphic SSR markers to the two reverse PGMS genes were further confirmed using 52 highly sterile plants in the F_2 population. Thus, the two reverse PGMS genes in YiD1S were located on chromosomes 8 and 9, which were designated *rpms1* and *rpms2*, respectively.

Subsequently, 59 SSR markers from chromosome 8 and 96 SSR markers from chromosome 9 were used to fine map the two reverse PGMS genes. As a result, 17 SSR markers from chromosome 8 and 19 SSR markers from chromosome 9 were able to detect clear polymorphism between the bulks as well as the parents. A total of 36 polymorphic SSR markers were used to analyze 52 highly sterile plants in the F_2 population. Based on the SSR marker and the phenotypic segregation data, partial linkage maps for the region around *rpms1* and *rpms2* were constructed, respectively (Fig. 2). The *rpms1* gene was fine mapped between RM22980 and RM23017 at a genetic distance of 0.9 and 1.8 cM on chromosome 8, respectively (Fig. 2a). Eight SSR markers, YDS818, RM22984, RM22986, RM22997, YDS816, RM23002, RM339, and YDS810 completely co-segregated with *rpms1* gene, no recombinant was detected in 52 highly sterile plants. The *rpms2* gene was fine mapped between RM23898 and YDS926 at a genetic distance of 0.9 and 0.9 cM on chromosome 9, respectively (Fig. 2b). The physical mapping information from publicly available resources shows that the markers RM22980 and RM23017 are located in the positions of 17,258 and 18,256 kb on chromosomes 8 (GenBank Accession No. AP008214), and the markers RM23898 and YDS926 are located in the positions of 6,863 and 6,931 kb on chromosome 9 (GenBank Accession No. AP008215). Thus, the *rpms1* and *rpms2* loci have been mapped within the region of 998 and 68 kb, respectively. Table 1 shows partial primer sequences used for fine mapping two reverse PGMS genes.

Genetic effects of two reverse PGMS genes

Two SSR markers, RM339 closely linked to the *rpms1* and YDS926 closely linked to the *rpms2*, were used to further assay the genotypes of 484 individuals taken at random from F_2 population of the cross YiD1S × 8528. The genetic

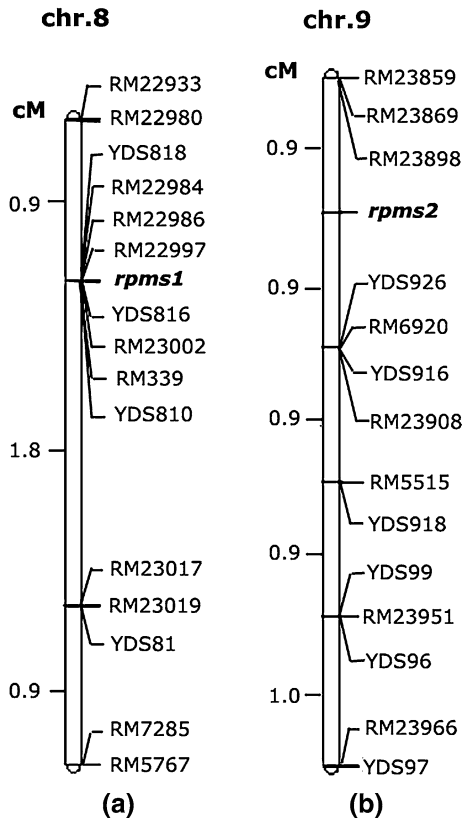

Fig. 2 Genetic maps showing the locations of two reverse photoperiod-sensitive genic male-sterile genes: (**a**) the *rpms1* gene region on chromosome 8; (**b**) the *rpms2* gene region on chromosome 9. The genetic distances (cM) between each gene loci and markers were estimated by using highly sterile F_2 individuals from the cross of YiD1S and 8528

effects based on marker genotypes were estimated for the two loci using a two-locus model (Table 2). The data of Table 2 shows that highly sterile individuals are apparently homozygous for recessive alleles at both loci, whereas all other genotypes appear to produce highly fertile individuals. The result suggests that male sterility in YiD1S is controlled by two interactive reverse PGMS genes (*rpms1* and *rpms2*) together. Further analysis indicate that the effect of *rpms1*(85.14–56.51 = 28.63)is slightly larger than that of *rpms2* (82.77–56.67 = 26.10) in controlling male sterility. A two-way ANOVA, using the marker loci RM339 and YDS926, revealed that *rpms1* and *rpms2* had highly significant effects on male sterility, respectively. Moreover, interaction between *rpms1* and *rpms2* was also highly significant (Table 3).

Discussion

This is the first report on molecular mapping of the reverse PGMS genes in rice. The results of this study showed that two reverse PGMS genes, *rpms1* and *rpms2,* were located

Table 1 Primer sequences used for fine mapping the *rpms1* and *rpms2*

Primer	Chromosome	Forward primer (5′–3′)	Reverse primer (5′–3′)
RM22980	8	ATCGAATTAGACTCGGGCAACG	ATCGTGCTGGGAGACTATCAAGC
YDS818	8	CCGTTGGATCTGGTCGTAT	GCTATTGGGCTATTTCATTTCT
RM22984	8	TTGCCCATCCAAACAAACTGG	ACCATGGTCCTCCTTCCTGTCC
RM22986	8	TGTTGGTGAACATCTCCCATGC	GTCTCAGAAGAATGCACACACAGC
RM22997	8	AGTCATGGTGTTGGACTGTTGG	CAAGATGGATGTGTGAACATGG
YDS816	8	AGATTCAAGTTTCTGCGGT	CCCAGCACAATCAGCAGT
RM23002	8	CGTCGGTTCGGTGAGATATAAAGG	AACCCATTCTCTGGCTACATTGC
RM339	8	GTAATCGATGCTGTGGGAAG	GAGTCATGTGATAGCCGATATG
YDS810	8	CCCTTTGGCTTATCTTGGTG	AAAGAGGATTGGAGGGACAG
RM23017	8	GTCAAACCTTTCACCTTCTCACG	AGGCGAGGCGAGATCTGAGG
RM23898	9	CACAGGGATAGTGTGGTTTGTGG	GGTAAAGCAGGTGTTTCACAAGATCC
YDS926	9	TAGTTATGTTTATGCGTTGTGC	TGGCGACCTACGAGTGTTT
RM6920	9	AATCGTATTGCCAGCGAGAC	AGAGCGTACCACAAATGAGG
YDS916	9	GGGATGAGGCTATTGACT	TGCATGTATCTGTATTGAAAC
RM23908	9	GGTCACCCTTCAAAGATGTCATGG	ATCCCGCTATCGAAGGTGAAACG

Table 2 The genetic effect estimate of the two loci using the average of pollen fertility (%) for each of the two reverse PGMS loci genotypes (11, 12, 22) marked by RM339 and YDS926 on chromosomes 8 and 9, respectively

		RM339			
		11	12	22	Average
YDS926	11	10.04	67.10	82.42	56.67
	12	67.71	78.39	86.17	77.67
	22	80.57	82.36	85.79	82.77
	Average	56.51	76.56	85.14	

11 homozygote for the allele from YiD1S, *22* homozygote for the allele from 8528, *12* heterozygote. The average of pollen fertility is calculated according to a theoretical 1:2:1 ratio within each class of the F_2 population

Table 3 Two-way ANOVA of the effect of two reverse PGMS loci on pollen fertility based on genotypes at the two marker loci RM339 (closely linked to *rpms1*) and YDS926 (closely linked to *rpms2*)

Effect	*df*	Mean square	*F*	*P*
RM339	2	28821.37	166.86	<.0001
YDS926	2	23953.43	138.68	<.0001
RM339 × YDS926	4	10694.95	61.92	<.0001

between RM22980 and YDS810 on chromosome 8 and between RM23898 and YDS926 on chromosome 9, respectively. Among the above-mentioned three PGMS genes and nine TGMS genes and one reverse TGMS genes mapped on the different chromosomes, only *tms1*, *TGMS* and *ms-h*(t) were mapped on chromosomes 8 and 9, respectively. Previous studies indicated that *tms1* locus was located in the region above the RG978 (17,433 kb) at a genetic distance of 8.5 cM on chromosome 8 (Wang et al. 1995; Lu XG et al. 2001), while *rpms1* locus was located in the region containing RG978 (17,433 kb) between RM22980 (17,258 kb) and RM23017 (18,256 kb) on chromosome 8 (GenBank Accession No. AP008214). *TGMS* locus between RM257 (66.1 cM, Cornell SSR 2001) and EAA/MCAG near the central region of chromosome 9 (Reddy et al. 2000) and *ms-h*(t) locus between RG451 (105.5 cM, Cornell SSR 2001) and RZ404 (111.1 cM, Cornell SSR 2001) near the bottom of chromosome 9 (Koh et al. 1999) were located in the region below the RM105 (32.1 cM, Cornell SSR 2001), while *rpms2* locus between RM23898 and YDS926 near the top of chromosomes 9 was located in the region above the RM105 (32.1 cM, Cornell SSR 2001) at a genetic distance of 23.4 cM. Therefore, two reverse PGMS genes (*rpms1* and *rpms2*) are non-allelic to the other mapped PGMS and TGMS genes (including reverse TGMS gene) in rice. The analysis based on marker genotypes showed that the effect of *rpms1* was slightly larger than that of *rpms2* and that the two genes interacted in controlling reverse PGMS. When compared with three normal PGMS genes (*pms1, pms2* and *pms3)* controlling normal PGMS, the genetic effect of two reverse PGMS genes is similar to that of *pms1* and *pms3* from Nongken58S (Mei et al. 1999), but different from that of *pms1* and *pms2* (the effect of *pms1* is 2–3 times larger than that of *pms2*) from 32001S (Zhang et al. 1994) in controlling male sterility.

Male sterility/fertility expression of PGMS and TGMS lines are regulated by photoperiod and/or temperature, this make potential risk of fertility fluctuation exist in hybrid seed production using two-line system. However, tempera-

ture is changeable and photoperiod is changeless on the whole in different years at the same hybrid seed production area. Thus, the PGMS trait mainly regulated by photoperiod should have a more stable expression compared with the TGMS trait mainly regulated by temperature. To reverse PGMS lines, male sterility-inducing environmental factor is predominantly short day-length. Although influenced by temperature in different degrees, male sterility expression of reverse PGMS lines is more thorough and hybrid seed production safer under conditions of unpredicted low temperature. Moreover, owning to a specific characteristic of fertility alteration, the reverse PGMS and TGMS lines have two sterility phases in a year, thus hybrid seed production can be successfully done at early cropping season and late cropping season in the double cropping areas of rice. If the season of fertility alteration is appropriate, hybrid seeds produced at early cropping season can be used at late cropping season, and hybrid seeds produced at late cropping season can be used at early cropping season next year. Therefore, the reverse PGMS or TGMS line is a promising germplasm in the double cropping of rice area and helpful to solve the problem such as fertility fluctuation influenced by low temperature in current hybrid rice seed production (Wan et al. 1998). But reverse PGMS and TGMS system has a potential risk of fertility fluctuation resulted from high temperature in hybrid seed production, so reverse PGMS lines with highly sensitive to photoperiod and low sensitive to temperature are more useful in the double cropping areas of rice. Molecular mapping of two reverse PGMS genes (*rpms1 and rpms2)* in this study provide a basis for cloning the two genes, which will be helpful to understand the PGMS mechanism at the molecular level and facilitate the development of practical reverse PGMS lines used to hybrid rice seed production.

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