Mol Breeding (2010) 26:275–292
DOI 10.1007/s11032-010-9397-1

Genic markers for wild abortive (WA) cytoplasm based male sterility and its fertility restoration in rice

Umakanta Ngangkham · Swarup K. Parida · Sandip De · K. Anand Raj Kumar · Ashok K. Singh · Nagendra K. Singh · Trilochan Mohapatra

Received: 18 July 2009 / Accepted: 21 January 2010 / Published online: 25 February 2010

Abstract Commercial exploitation of heterosis is essential for enhancing productivity of rice. The use of cytoplasmic male sterility (CMS) and fertility restoration system greatly facilitates large scale production of hybrid seed. The wild abortive (WA) cytoplasm is most widely used for hybrid seed production in rice. The present study was undertaken to develop molecular markers for both WA cytoplasm based male sterility and its fertility restoration for use in efficient hybrid breeding. High degree of genetic differentiation of WA-cytoplasm from its normal fertile counterpart was observed due to DNA rearrangements involving five (*coxI*, *coxIII*, *cob*, *atp6* and *rps3*) mitochondrial genes. Cleaved amplified polymorphic sequence (CAPS) markers based on five mitochondrial genes namely, *coxIII*, *cob*, *atp9*, *rps3* and *18SrRNA* polymorphic between CMS and maintainer line were developed. The utility of these informative markers was demonstrated in purity testing of the CMS line Pusa6A being used in commercial hybrid seed production. Fertility restoration was found to be controlled by a major locus in the Basmati restorer line PRR78, which was mapped to a short marker interval of 0.8 cM and a physical interval of 163.6 kb on rice chromosome 10. A total of 13 pentatricopeptide repeat (PPR) motif containing genes were predicted in a 1.66 Mb region on the long-arm of this chromosome of which, four were present in the marker interval containing the fertility restorer gene. High degree of conservation of gene order was observed between *japonica* and *indica* for the predicted PPR genes. A sequence tagged site (STS) and a genic non-coding microsatellite (GNMS) marker were designed based on one of the candidate PPR motif containing genes present in the marker interval, which were validated using F_2 population and other known restorer lines. The candidate gene based marker identified in the present study would be useful in marker assisted selection (MAS) for fertility restorer gene in hybrid breeding programme based on WA-CMS of rice.

Keywords RFLP · Wild abortive (WA) · Cytoplasmic male sterility (CMS) · Rice · Mitochondrial genes · Fertility restoration · PPR · CAPS · STS · GNMS

The authors Umakanta Ngangkham, Swarup K. Parida, Sandip De and K. Anand Raj Kumar have contributed equally to the work.

Electronic supplementary material The online version of this article (doi:10.1007/s11032-010-9397-1) contains supplementary material, which is available to authorized users.

U. Ngangkham · S. K. Parida · S. De · K. Anand Raj Kumar · N. K. Singh · T. Mohapatra (✉)
National Research Centre on Plant Biotechnology, Indian Agricultural Research Institute, New Delhi 110012, India
e-mail: tm@nrcpb.org

A. K. Singh
Division of Genetics, Indian Agricultural Research Institute, New Delhi 110012, India

Introduction

Rice (*Oryza sativa* L.) is the most important staple food crop, providing food for more than half of the world population. Breeding rice for higher yield remains the first priority for many rice growing regions around the world particularly Asia. For breaking yield barrier in rice, large-scale exploitation of heterosis is a feasible alternative. Cytoplasmic male sterility (CMS) system has been widely used in hybrid seed production in a number of economically important food crops, which eliminates the need for hand emasculation, which is highly tedious in crops like rice. CMS is characterized by production of normal female gamete with non-functional pollen grains that fail to fertilize the female gamete. This makes an excellent model system to study interaction between nuclear and cytoplasmic factors. Restoration of male fertility by nuclear genes allows commercial exploitation of CMS system in production of hybrid seeds (Newton 1988; Kazama et al. 2008). All commercial rice hybrids in India are based on three line system namely, A (male sterile line), B (maintainer line), and R (restorer line). Plant breeders produce fertile hybrid seeds by crossing female lines that carry male sterilizing cytoplasm (A line) with a male line that carries the gene for fertility restoration (R line) in its nucleus. The male sterility is maintained by crossing CMS (A) line taken as female with an isonuclear fertile (B) line without sterilizing cytoplasm. Considering its importance, the CMS systems have been identified and characterized in many crop species, including *Petunia*, maize, sunflower, *Brassica*, *Sorghum* and rice (Vedel et al. 1994; Schnable and Wise 1998).

The most widely used CMS in rice is based on wild abortive (WA) cytoplasm. Characterization of this cytoplasm is essential for designing markers for its precise identification and also for understanding the molecular basis of male sterility expression. The success of hybrid seed production in rice largely depends on the genetic purity of the CMS line. Due to isonuclear situation between CMS and maintainer lines, the admixture of these lines during hybrid seed production is expected. Differentiation of these two lines at early stages of plant growth is difficult. Development and use of molecular markers is therefore very important to distinguish a CMS line from its maintainer in hybrid seed production and purity testing (Ichii et al. 2003; Narayanan et al. 1996; Rajendrakumar et al. 2007; Sane et al. 1997; Yashitola et al. 2004). The CMS is a maternally inherited trait caused by mutations and rearrangements of mitochondrial genes (Schnable and Wise 1998; Chase 2007; Fujii and Toriyama 2008). Considering the role of chimeric *orf* associated with the CMS phenotype, the mitochondrial gene sequence specific DNA markers would be more suitable for efficient differentiation of the CMS line from its maintainer line (Liu et al. 2007; Kazama et al. 2008). A limited effort has been made so far to identify the candidate mitochondrial gene(s) having role in expression of male sterility and development of gene based markers for the WA-cytoplasm of rice.

The utility of CMS lines is determined by availability of well characterized and effective fertility restoration systems. Recently, the nuclear genes for male fertility restoration have been studied in detail for Lead Rice (LD, Itabashi et al. 2009) and Boro II (BT, Kazama et al. 2008) cytoplasms of rice. Despite the importance of WA-CMS, its fertility restoration is poorly understood at molecular level. Genetically, the gene for restoration of fertility of the BT cytoplasm based male sterility has been mapped on the long arm of rice chromosome 10 and isolated by positional cloning strategy (Akagi et al. 2004; Komori et al. 2004; Wang et al. 2006). It is reported to encode a protein having pentatricopeptide repeat (PPR) motifs in addition to mitochondrial transit peptide, which indicates its transport to mitochondria (Schmitz-Linneweber and Small 2008). A major locus for fertility restoration of the WA-CMS has also been located on the long-arm of rice chromosome 10 (Jing et al. 2001; Zhang et al. 2002; Ahmadikhah and Karlov 2006; Sheeba et al. 2009), though it has not been cloned and characterized yet. Molecular characterization of restoration of fertility of WA-CMS requires fine mapping and isolation of this major restorer gene. Development of robust sequence based molecular markers in the target gene region is therefore essential. A detailed molecular analysis of this region using the male sterile and restorer lines would be useful in identifying the most probable candidate and developing a tightly linked/candidate gene based marker for an efficient marker aided selection (MAS) for male fertility restoration in rice.

The objectives of the present study were to carry out molecular differentiation of the WA cytoplasm

from its normal counterpart, develop mitochondrial gene-based markers and evaluate their efficiency in assessing purity of CMS line, map the gene for fertility restoration in the Basmati restorer line PRR78 and design markers using the available sequence information about the candidate genes on the rice chromosome 10.

Materials and methods

Plant materials

Eight rice lines namely, CMS Pusa6A and IR262829A, maintainer lines Pusa4B, Pusa5B, Pusa6B, and Pusa9B, restorer PRR78 (Pusa Rice Restorer78) having the WA cytoplasm, and hybrid PRH10 (Pusa Rice Hybrid10) developed by crossing Pusa6A and PRR78 for commercial cultivation in India were selected for molecular analysis. Besides, nine additional restorer lines namely, IR24, BR827, KMR3, MTU9992, NDR3026, AjayaR, IR66, C20R and UPRI93133 were used for validation of candidate gene-based markers for fertility restoration. A F_2 segregating population consisting of 1,200 plants derived from the cross between Pusa6A and PRR78 was phenotyped for pollen fertility and used for genetic mapping of the restorer gene and validation of the candidate restorer gene-based markers.

Phenotyping of F_2 segregants for pollen fertility

Pollen fertility was tested using potassium iodide (KI) as staining agent (Rosamma and Vijayakumar 2005). Darkly stained pollens under compound microscope were considered as fertile ones. Pollens which were shriveled, partly stained or unstained were considered sterile. Pollen fertility count was taken in three different microscopic fields to reduce the chance of misclassification. Plants having 0, 0–30, 30–60 and 60–100% pollen fertility were considered as completely sterile, partially sterile, partially fertile and completely fertile ones (Govinda Raj and Virmani 1988), respectively. However, F_2 plants used for genetic mapping of the major fertility restorer gene in the present study were classified into two major categories namely, sterile with less than 40% pollen fertility and fertile with more than 40% pollen fertility.

Prediction of PPR motifs containing rice genes

The genomic sequences of the long-arm of rice chromosome 10 from the marker R2303 to the telomeric region of the *japonica* rice cultivar Nipponbare (Pseudomolecule 6.1, http://rice.plantbiology.msu.edu/pseudomolecules) and *indica* cultivar 93–11 (Beijing Rice Information System, http://rice.genomics.org.cn/rice) were downloaded and annotated using FGENESH (http://sun1.softberry.com/berry.phtml) gene prediction software to identify protein encoding genes. Aminoacid sequences of the predicted genes were used to determine the presence of PPR motifs using the software Pfam (http://www.sanger.ac.uk). The genes that contained PPR motifs were further analyzed for the presence of mitochondrial transit peptide using the software Target P (http://www.cbs.dtu.dk/services/TargetP).

Primer design, PCR amplification and sequencing of mitochondrial and PPR motif containing genes

Primers were designed from the available (http://www.ncbi.nlm.org) coding sequences of 10 rice mitochondrial genes namely, *atp*6 and *atp9* (encoding gene product F_0–F_1 ATPase complex), *coxI* and *coxIII* (cytochrome C oxidase), *cob* (apocytochrome b subunit), *18SrRNA* (ribosomal RNA), *rps3* (ribosomal proteins), and *nadI, nadIB and nadIV* (NADH ubiquinone oxidoreductase complex) using PRIMER 3 (http://frodo.wi.mit.edu/primer3) and oligonucleotides were custom synthesized for molecular analysis of WA-CMS in rice. Besides, primers for the predicted PPR motif containing rice genes were designed and synthesized for molecular studies related to fertility restoration.

The designed mitochondrial and PPR gene based primers were used to amplify the target sequences from the CMS, maintainer, restorer and hybrid rice lines included in this study. Standard PCR constituents and cycling conditions except for annealing temperature, which varied from 50 to 64°C depending on the primers, were used for PCR amplification. To confirm that the primers amplified the expected target sequences, the amplified products were purified and sequenced in both forward and reverse directions twice using a capillary-based Automated DNA Sequencer (MegaBACE 4000, Amersham Biosciences, USA)

following the manufacturer's instructions. The trace files were base called, checked for quality and assembled into contigs (Ewing and Green 1998). The high quality sequences thus obtained were BLASTN searched against universal nr nucleotide database of NCBI (http://www.ncbi.nlm.org) to verify the origin of amplicons.

The amplicons obtained for the PPR gene based STS marker that differentiated the CMS line Pusa6A from its restorer PRR78, were eluted from agarose gel, purified, cloned and sequenced as described above. Twenty random positive clones were sequenced twice in both forward and reverse directions. The high quality sequences were assembled and the derived consensus sequences compared with the sequences of *indica* cultivar 93–11 and *japonica* cultivar Nipponbare using CLUSTALW multiple sequence alignment tool employing BIOEDIT software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Using these sequences of Pusa6A and PRR78, primers were redesigned for specific amplification of the STS marker.

Southern analysis using mitochondrial gene probes

DNA was isolated from the young leaves of CMS, maintainer, restorer and hybrid lines, purified, quantified and diluted to 2 µg/µl. About 10 µg of total cellular DNA was digested separately using *Eco*RI and *Hin*dIII at 37°C overnight, separated on 0.8% agarose gel in 0.5× TBE buffer for 14–16 h and visualized under UV light. The gel was treated with depurination (0.25 N HCl), denaturation (0.5 N NaOH + 1.5 M NaCl) and neutralization (1.5 M NaCl + 1 M Tris–Cl, pH 8.0) solutions, and DNA was transferred to nylon membrane (Millipore, USA) using LKB Vacuagene vacuum blotting apparatus (60 cm. H_2O) following manufacturer's instructions. The membrane was air dried properly at room temperature followed by UV cross-linking of DNA by exposing the membrane at 5,000 mV for 1.5 min, prehybridized at 65°C overnight and hybridized with mitochondrial gene specific probes. Probes were obtained by random primer labeling of gene specific PCR amplicons. After overnight hybridization at 65°C, the blot was washed, exposed to the X-ray film (Kodak, India) at −80°C for 2–3 days and the autoradiograms developed. The size of the hybridizing restriction fragments was determined by comparison of their mobility with the DNA size standard.

CAPS analysis of mitochondrial gene specific amplicons

CAPS analysis was carried out to find out polymorphism in the monomorphic PCR products amplified using gene specific primers. The amplified product for each gene was quantified, digested with 20 restriction enzymes at 37°C for 2 h and resolved on 1.5% agarose gel using 0.5× TBE buffer. The fragment size of the digested products was determined by comparing with 100 bp DNA ladder plus as size standard. The CAPS markers for the rice mitochondrial genes showing polymorphism between CMS 'Pusa6A' and maintainer 'Pusa6B' lines were further used to genotype a set of 48 random Pusa6A plants from the seed production plot of Pusa Rice Hybrid 10 for testing the purity of the 'A' line. For evaluating the efficiency of CAPS assay in detection of the levels of contamination of 'A' line with its 'B' line, three different known DNA admixtures (2, 5 and 10%) of Pusa6B in Pusa6A were constituted and genotyped using one of the informative CAPS markers. The intensity of fragments obtained in admixture and control lines was compared and the minimum admixture level that can be detected with CAPS assay was inferred.

Genotyping of F_2 mapping population

For mapping the fertility restoration gene in the Basmati restorer line PRR78, a total of 95 markers including 36 rice microsatellite markers (RM series, McCouch et al. 2002), 50 STS markers designed from the EST sequences and another nine genic non-coding microsatellite (GNMS) markers (Parida et al. 2009) derived from the 3′ and 5′ UTR sequence components (Table S1) of the PPR genes present in the fertility restorer gene region of the long-arm of rice chromosome 10 (Kazama and Toriyama 2003; Akagi et al. 2004; Komori et al. 2004; Wang et al. 2006) were used for survey of polymorphism between the two parental lines, Pusa 6A and PRR78. The informative markers showing polymorphism between the parents were used to genotype 138 random F_2 plants of the mapping population. The PCR amplified microsatellite and gene

specific products were resolved in 3 or 4% metaphor and 1% agarose gel, respectively in 0.5× TBE buffer, stained using ethidium bromide, visualized under UV light and analyzed for the segregation. Linkage analysis was carried out using MAPMAKER/EXP 3.0 (Lander et al. 1987). A subset of 66 including 42 highly fertile (>80% pollen fertility) and 24 highly sterile (<10% pollen fertility) F_2 plants were genotyped using the candidate PPR gene based STS and GNMS markers polymorphic between the parental CMS and restorer lines for their validation.

Results

Differentiation of WA-cytoplasm using RFLP markers

Five (*coxI, rps3*, *atp6, cob* and *coxIII*) of the 10 mitochondrial gene specific probes used in Southern hybridization showed polymorphic pattern between the CMS 'Pusa6A' and the maintainer 'Pusa6B' lines in both *Eco*RI and *Hin*dIII enzyme combinations. The restorer line PPR78 and the hybrid PRH10 having the WA cytoplasm gave the same pattern as Pusa6A with all the probes. The remaining five (*atp9*, *nadI*, *nadIB*, *nadIV* and *18SrRNA*) gene probes showed monomorphic pattern (Table 1). Of these, three (*atp9*, *nadI* and *nadIB*) probes hybridized to single restriction fragment each in both CMS and the maintainer lines whereas two (*nadIV* and *18SrRNA*) probes showed hybridization with multiple restriction fragments. Of the five polymorphic probes, *coxI* gave single fragment hybridization (Fig. 1a) and showed fragment length polymorphism between the CMS and maintainer lines in both *Eco*RI and *Hin*dIII digestion. In case of *rps3*, 4.3 kb *Eco*RI and 3.5 kb *Hin*dIII fragments being specific to the maintainer line, differentiated this line from the other three lines (CMS, restorer and hybrid), which had 5.0 and 3.0 kb *Eco*RI and *Hin*dIII fragments, respectively. The other three gene specific probes (*atp6, cob* and *coxIII*) hybridized to more than one restriction fragments (Table 1) in the CMS and the maintainer lines (Fig. 1c–e). In addition to common fragment of size 2.5 kb which was present in all the lines, the *atp6-Eco*RI combination gave two additional

Table 1 Hybridization pattern of mitochondrial gene specific probes

Mitochondrial gene specific probes	Hybridization pattern (fragment size in Kb)			
	*Eco*RI		*Hin*dIII	
	Pusa6A/PRR78	Pusa6B	Pusa6A/PRR78	Pusa6B
atp6	–	5.0	5.0	4.8
	2.5	2.5	–	4.3
	–	1.5	–	–
cob	–	6.0	3.0	3.0
	4.3	4.3	–	2.3
	–	–	1.7	1.7
coxI	6.5	4.9	7.0	7.0
rps3	5.0	–	–	3.5
	–	4.3	3.0	3.0
coxIII	23.1	23.1	3.5	–
	11.1	–	2.2	2.2
atp9	9.4	9.4	1.4	1.4
nadI	2.3	2.3	1.1	1.1
nadIB	3.5	3.5	2.0	2.0
nadIV	5.0	5.0	6.0	6.0
	0.6	0.6	–	–
18SrRNA	16.2	16.2	9.0	9.0
	8.0	8.0	5.5	5.5
	4.5	4.5	3.5	3.5

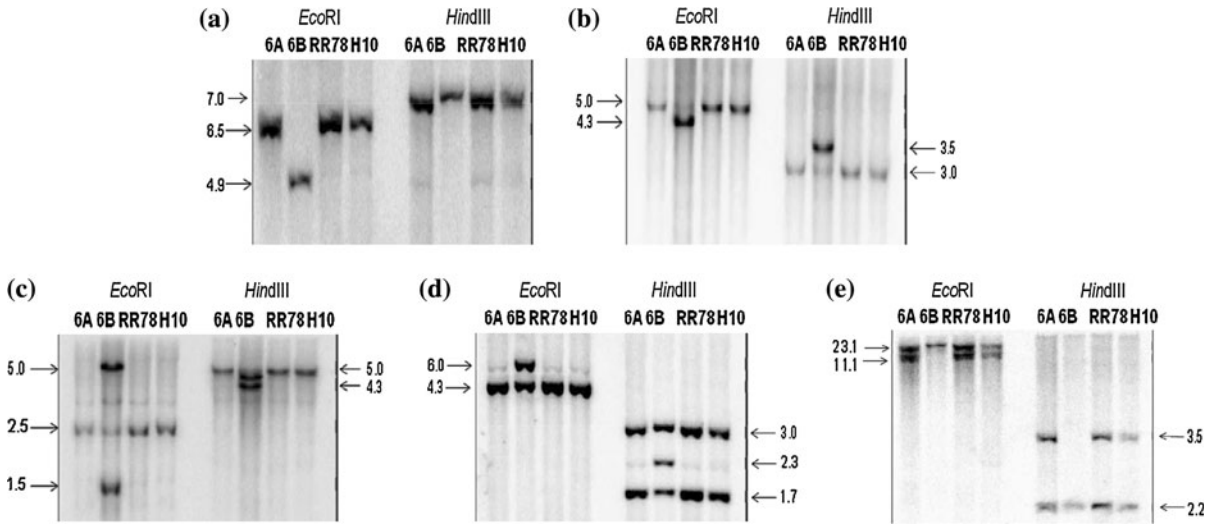

Fig. 1 Polymorphic Southern hybridization pattern of mitochondrial gene specific probes (**a** *coxI*, **b** *rps3*, **c** *atp6*, **d** *cob* and e: *coxIII*) obtained in combination with *Eco*RI and *Hin*dIII restriction enzymes. *Lanes* *6A*-CMS line Pusa6A, 6B maintainer line Pusa6B, *RR78* restorer line PRR78 and *H10* Pusa Rice Hybrid 10. The size (kb) of the restriction fragments hybridizing with the probe is indicated

bands (5.0 and 1.5 kb) in the maintainer line. The *atp6*-*Hin*dIII combination gave two maintainer specific fragments (4.8 and 4.3 kb) which were different from the WA-cytoplasm specific 5.0 kb fragment. With *cob* gene, a 6.0 kb *Eco*RI fragment and 2.3 kb *Hin*dIII fragment were specific to the maintainer line, besides one 4.3 kb *Eco*RI fragment and two *Hin*dIII fragments (3.0 and 1.7 kb), which were shared by all the lines used. In case of *coxIII*, an additional band of size 11.1 kb in *Eco*RI and 3.5 kb in *Hin*dIII enzyme combination was observed in the CMS, restorer and hybrid lines, while 23.1 kb *Eco*RI and 2.2 kb *Hin*dIII fragments were common to all the four lines. Absence of *Eco*RI and *Hin*dIII restriction sites in the probe sequence of *atp6* and *coxIII* revealed the presence of two copies of *coxIII* gene in the male sterilizing WA cytoplasm of rice, while two or more copies of *atp6* in the fertile cytoplasm. The presence of one *Hin*dIII and absence of *Eco*RI restriction site in the probe sequence of *cob* suggested occurrence of two copies of *cob* gene in the fertile cytoplasm.

Development of CAPS markers for the rice mitochondrial genes

Restriction analysis of monomorphic amplicons of the mitochondrial genes revealed polymorphism between the CMS and maintainer line (Table 2) in different enzyme combinations with five mitochondrial genes namely, *cob*, *atp9*, *coxIII*, *rps3* and *18SrRNA*. CAPS analysis for five gene-enzyme combinations are depicted in Fig. 2. As clearly evident from this figure, variation in multiple restriction enzyme target sites in the PCR amplicons of the five mitochondrial genes clearly differentiated the WA cytoplasm present in the CMS, restorer and the hybrid lines from the normal cytoplasm present in the maintainer line. The genotyping of a set of 48 random Pusa6A plants from the hybrid seed production plot of PRH10 using five rice mitochondrial gene based CAPS markers revealed no polymorphism (Fig. S1) and confirmed high degree of purity of the CMS line Pusa6A without any contamination with its maintainer line (Pusa6B). The *cob*-*Nla*III CAPS marker was used for detection of contamination of 'A' line (Pusa6A) with its 'B' line (Pusa6B) in different known admixtures (2, 5 and 10%) showed the presence of low intensity Pusa6B-specific fragment at 2% admixture level. The intensity of contaminant specific restriction fragment was more pronounced and clear with increase in admixture level from 5 to 10% (Fig. 3). Therefore, the mitochondrial gene based CAPS marker developed in this study was efficient enough to detect a minimum level of 2% or more contamination of maintainer line.

Table 2 CAPS analysis of mitochondrial gene specific amplicons

S. no.	Mitochondrial gene specific primers	Nucleotide sequences	Amplicon size (bp)	Enzymes showing polymorphism between CMS and maintainer line	Size of major scorable restriction fragment (bp)	
					Pusa6A	Pusa6B
1	*atp6*_F	5′-ATGGGTTTGAATCAGAGAGA-3′	999	Monomorphic	999	999
	atp6_R	5′-ATTCAATTATGAAATTACTC-3′				
	(F_0–F_1 ATPase)					
2	*atp9_F*	5′-GGTGTGGTGTTCAGTCTACC-3′	406	*Dpn*II	380	406
	atp9_R	5′-GGGCCTCGTATCTCTATTTG-3′		*Mbo*I	350 & 56	406
	(F_0–F_1 ATPase)					
3	*coxI_F*	5′-TATTACCAGCCATTCTGGAG-3	1,008	Monomorphic	1,008	1,008
	coxI_R	5′-CTACGAAGAAACGACGAATC-3				
	(cytochrome C oxidase)					
4	*coxIII_F*	5′-GGAGAGGGCATGATAAAGAC-3′	810	*Hae*III & *Pvu*II	300 & 250	400
	coxIII_R	5′-AAATAGTGGAGGGTGCTTG-3′		*HpyCH*4IV	400	300 & 250
	(cytochrome C oxidase)					
5	*cob_F*	5′-AAGGAACCAACGATTCTCTC-3′	1,000	*Nla*III & *Mbo*II,	1,000	550, 220, 140 & 90
	cob_R	5′-CGGTCGAAAACTTGAACTAC-3′		*Eco*RI & *Eco*RV	520 & 480	1,000
	(apocytochrome b)					
6	*rps3_F*	5′-AAACCCTCTCTAAGGTGGAG-3′	1,002	*Dpn*II & *Hae*III	500 & 250	602 & 400
	rps3_R	5′-AGATAGTCACCCATCACACG-3′		*Pvu*II & *Mbo*I	602 & 400	500 & 250
	(ribosomal proteins)					
7	*18srRNA_F*	5′-GTGTTGCTGAGACATGCGCC-3	749	*Nla*III & *Hin*dIII	320 & 170	500 & 130
	18srRNA_R	5′-ATATGGCGCAAGACGATTCC-3′		*HpyCH*4IV & *Hae*III	500 & 130	320 & 170
	(ribosomal RNA)					
8	*nadI_F*	5′-ATACACCAGGGCAACTAATG-3′	790	Monomorphic	790	790
	nadI_R	5′-AGGGAGTAGGGTGAGTAAGC-3′				
	(NADH ubiquinone oxidoreductase complex)					
9	*nadIB_F*	5′-GCATTACGATCTGCAGCTCA-3′	1,614	Monomorphic	1,614	1,614
	nadIB_R	5′-GGAGCTCGATTAGTTTCTGC-3′				
	(NADH ubiquinone oxidoreductase complex)					

Table 2 continued

S. no.	Mitochondrial gene specific primers	Nucleotide sequences	Amplicon size (bp)	Enzymes showing polymorphism between CMS and maintainer line	Size of major scorable restriction fragment (bp)	
					Pusa6A	Pusa6B
10	*nadIV_F* *nadIV_R* (NADH ubiquinone oxidoreductase complex)	5′-CAGTGGGTTGGTCTGGTCTG-3′ 5′-TCATATGGGCTACTGAGGAG-3′	2,073	Monomorphic	2,073	2,073

Segregation analysis for pollen fertility in F_2 population

The sterile pollen grains were found shriveled and smaller in size without any stain or lightly stained with KI, whereas the fertile pollens were larger in size and darkly stained. The pollen fertility ranged from 0 to 100%. Considering the plants with 0–30% fertility under sterile class as recommended earlier (Mishra et al. 2003), 138 F_2 plants segregated into 117 fertile: 21 sterile as against an expected Mendelian segregation ratio of 103.5 fertile: 34.5 sterile based on 3:1 ratio. When we included the plants having up to 40% pollen fertility under sterile class, 102 plants were recorded as fertile and 36 as sterile, giving a segregation ratio of 2.83:1. The analysis of goodness of fit to the expected Mendelian segregation ratio of 3:1 showed that the trait was inherited in the expected pattern at 1% level of significance with the latter phenotypic classification. Thus the effect of a major gene on male fertility in the rice restorer line PRR78 was evident.

Molecular mapping of fertility restoration gene

Three microsatellite markers namely, RM6100, RM6737 and RM258 showed polymorphism between the parents (Fig. S2A). Segregation analysis of these markers in the F_2 mapping population revealed normal Mendelian segregation (Fig. S3). Based on the linkage analysis, the fertility restorer gene could be placed between the markers RM6737 and RM6100 at 0.3 and 0.5 cM distance, respectively on the long-arm of rice chromosome 10 (Fig. 4). The genetic map information was combined with the available DNA sequence of the region (IRGSP 2005) to establish correspondence between the genetic map and sequence based physical map. The region from 53.3 to 61.7 cM of the long-arm of chromosome 10 was about 1.66 million base pair (Mb) in size. One centimorgan in this 8.4 cM interval thus corresponded to about 198.12 kb. The integration of the genetic and physical maps suggested location of the restorer gene to a physical interval of 163.6 kb between markers RM6737 (18,653.71 kb) and RM6100 (18,817.28 kb) on the long-arm of chromosome 10 of rice (Fig. 4).

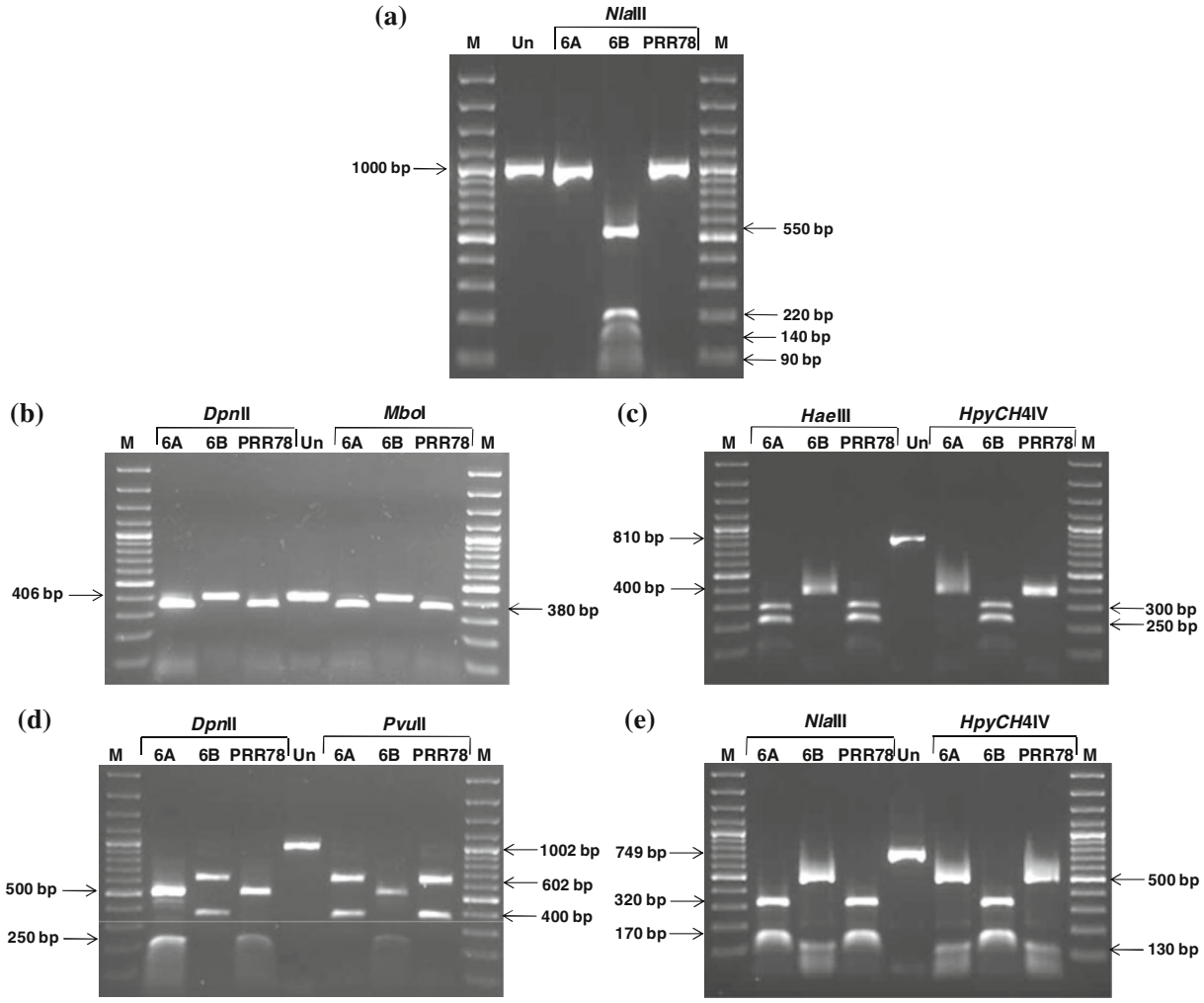

Fig. 2 CAPS analysis of mitochondrial gene specific amplicons (**a** *cob*, **b** *atp9*, **c** *coxIII*, **d** *rps3*, and **e** *18SrRNA*) using different restriction enzymes. The digested products were separated in 1.2% agarose gel. *Lanes 6A-CMS* line Pusa6A, *6B* Maintainer line Pusa6B, *PRR78* Restorer line PRR78, Un-Undigested amplicon and M-100 bp DNA ladder plus. The size (bp) of the polymorphic restriction DNA fragments is indicated

Identification of PPR motifs containing candidate rice genes

A total of 115 protein coding genes were predicted in 1.66 Mb sequence (between 53.3 and 61.7 cM positions), of which 13 contained PPR motifs as well as mitochondrial transit peptides (Fig. 4). Within the marker interval containing the restorer locus, we did not find any genes other than those having PPR motifs that possessed the mitochondrial transit peptides. The number of PPR motifs ranged from 1 to 16 in different genes (Fig. S4). The number of exons in these genes varied from 1 to 8. Based on their physical order on the chromosome they were named as PPR 1 to 13 (Table 3). The orientation of these genes varied within the genome of the *japonica* cultivar Nipponbare, which is a known maintainer of male sterility. Four of these PPR motif-containing genes (PPR1, PPR2, PPR3, and PPR4) were placed on the targeted fertility restorer gene region with a physical interval of 163.6 kb between the microsatellite markers RM6737 and RM6100. Similar analysis on the long arm of *indica* rice chromosome 10 of genotype 93–11, which is a known restorer of HongLian (HL) type CMS (Liu et al. 2004) indicated the presence of 13 PPR containing genes (Fig. 4). In

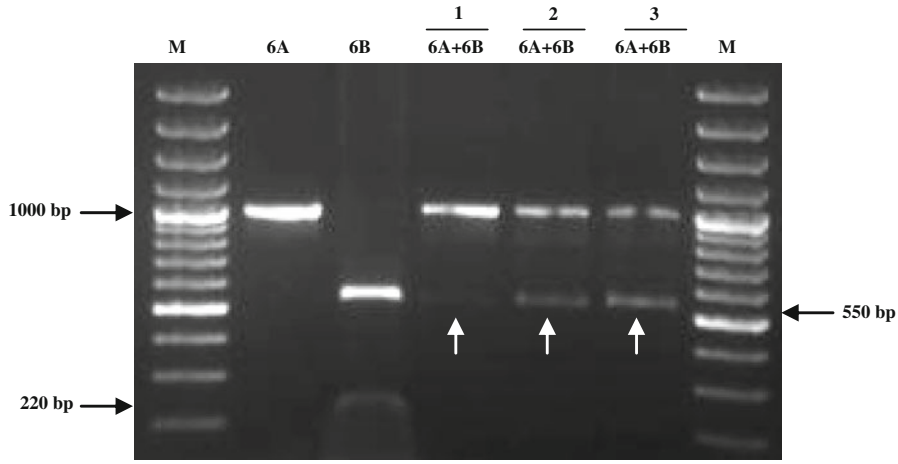

Fig. 3 Evaluation of efficiency of a CAPS marker based on rice mitochondrial gene *cob* for checking contamination of CMS line Pusa6A with maintainer line Pusa6B during hybrid seed production of Pusa Rice Hybrid10 using *Nla*III restriction enzyme. The PCR fragments were resolved in 1.2% agarose gel. *Lanes 6A-CMS* line Pusa6A, *6B* Maintainer line Pusa6B (*1–3*): 2, 5 and 10% admixtures of Pusa6B with Pusa6A (2:98, 5:95 and 10:90, by volume in a total of 100 µl, respectively) and M-100 bp DNA ladder plus. With increase in admixture level of maintainer line 'Pusa6B' with its CMS line 'Pusa6A' from 2 to 10%, the intensity of maintainer specific 550 bp fragment increased gradually. *White arrows* indicate the maintainer line Pusa6B specific fragment in admixture samples

these genes, the number of exons ranged from 1 to 7 and the number of PPR motifs varied from 1 to 15 (Table 3 and Fig. S5). Based on their physical order of occurrence on the chromosome, they were named as PPR 1–13. When the mitochondrial transit peptide was analyzed, the PPR8 of *japonica* and PPR8 and PPR9 of *indica* showed low probability of having such a peptide. The PPR2 of *indica* also had a lower probability of having mitochondrial transit peptide (Table 3). The PPR genes predicted from the long arm of rice chromosome 10 of the *japonica* and *indica* cultivars were compared at the nucleotide as well as predicted aminoacid level and phylogenetic trees (Fig. S6A and B) were constructed. High degree of correspondence between the two sub-species at both nucleotide and aminoacid sequence levels for the PPR genes was evident.

Development and validation of candidate PPR gene based marker for fertility restoration

For developing candidate gene based markers for fertility restoration, primers designed from 13 PPR motif containing candidate rice genes were amplified in the CMS line Pusa6A and the restorer line PRR78. Ten PPR genes namely, 1, 4, 5, 6, 7, 9, 10, 11, 12 and 13 gave amplicons of expected fragment size and showed no fragment length polymorphism between the CMS and restorer line for these genes. No amplification was observed in the restorer line in case of PPR2 whereas PPR8 amplified two fragments in both CMS and restorer line. However, fragment length polymorphism was clearly evident between the CMS and restorer lines for the PPR3 gene that provided a candidate gene based sequence tagged site marker. The STS marker designed based on the *japonica* sequence (Forward 5′-AGAACAGAAGCCAAGAA GC-3′ and reverse 5′-AGCAGCAAACAAATCCT TAG-3′ primers) gave a 1,756 bp amplicon in CMS line Pusa6A and a 1,640 bp allelic fragment in the fertile parent PRR78. Cloning, sequencing and sequence comparison of multiple clones for each of the two parents revealed presence of a mixture of amplicons of same size due to cross-amplification of different PPR genes. Therefore, the primers were redesigned based on the PPR3 gene sequence (Forward 5′-CTGCGTTATGCAAGGCTCAAGCTA-3′ and reverse 5′-AGCAGCAAACAAATCCTTAG-3′) and used to generate parent specific pattern that gave a 1,488 bp amplicon specific to CMS line Pusa6A and a 1,383 bp allelic fragment in the fertile parent PRR78.

Co-segregation analysis of this gene based STS marker with fertility restoration in 42 highly fertile and 24 highly sterile F_2 plants showed the presence of

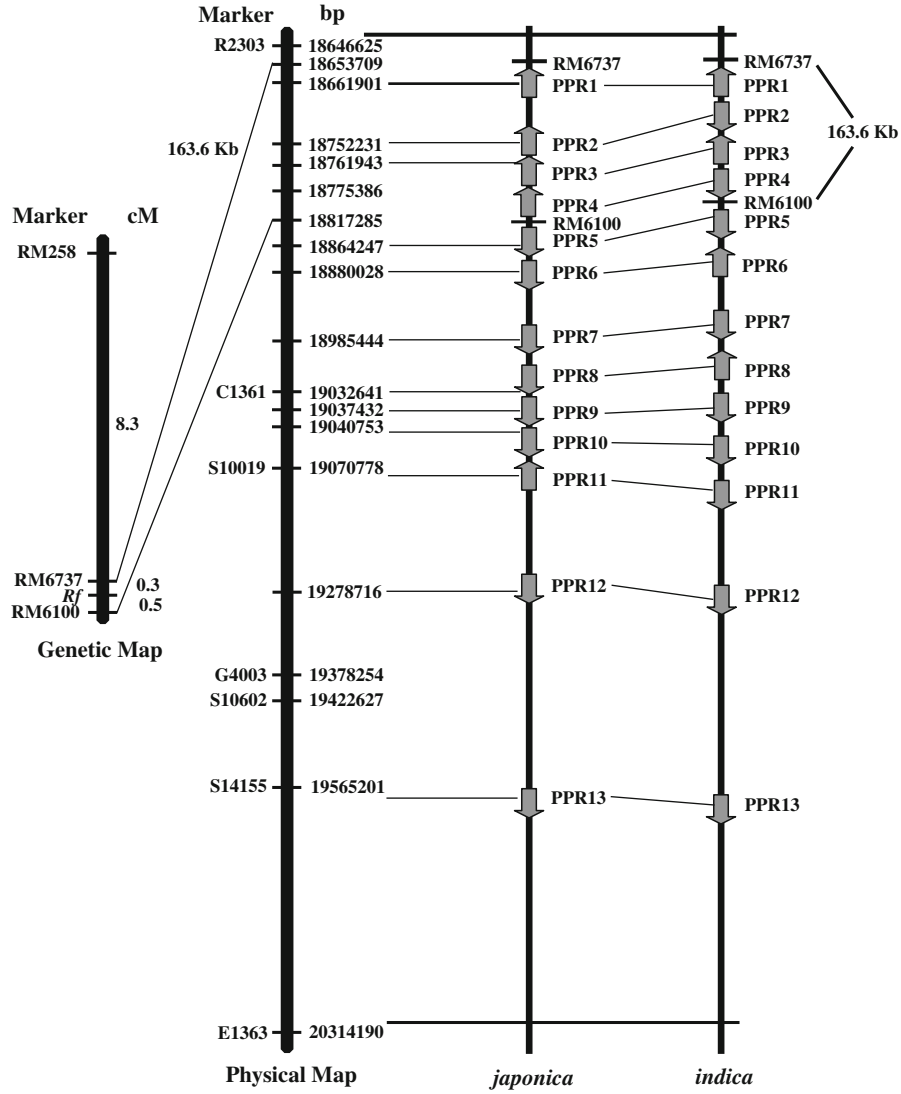

Fig. 4 Integration of the genetic and physical maps of the fertility restorer gene region on the long-arm of rice chromosome 10 showing the physical location and orientation of the PPR genes predicted in this region. The names of the markers are given on the *left side*, and the genetic distances in centimorgan and physical distances in base-pairs are given on the *right side* of the map. Correspondence between the PPR genes of *japonica* and *indica* based on nucleotide and aminoacid sequence homology is indicated by *horizontal lines*. For instance, PPR1 and PPR2 of *japonica* corresponded to PPR1 and PPR2 of *indica*, respectively

CMS line Pusa6A specific 1,488 bp fragment in all the 24 male sterile plants. The highly fertile segregants had the restorer line PRR78 specific 1,383 fragment either in homozygous or in heterozygous condition (Fig. 5). Identical result was obtained with a GNMS marker containing $(GGC)_4$ repeat-motif designed from the 5′UTR sequence component of PPR3 gene located in the targeted fertility restorer gene region using the same set of highly sterile and highly fertile F_2 individuals (Fig. S7). The STS and GNMS markers were also validated in nine other restorer lines which are known to restore the WA based cytoplasmic male sterility. The restorer line PRR78 specific fragment for both the markers was present in all the nine restorers, whereas the CMS line IR262829A carrying the WA cytoplasm and the maintainer lines Pusa4B, Pusa5B, Pusa6B and Pusa9B showed Pusa6A specific pattern (Fig. 6). High quality sequence alignment (Fig. S8) revealed presence of 16 and 15 PPR motifs in the PPR3 gene in the CMS Pusa6A and restorer PRR78 lines, respectively. However, the PPR3 gene contained 15 PPR motifs in both the *japonica* cultivar Nipponbare and *indica* cultivar 93–11. Thus the presence of an additional 105 nucleotide (35 aminoacid) long PPR motif in the CMS line Pusa6A and its absence in the restorer line PRR78 was the basis of fragment length polymorphism between the two parental rice lines.

Table 3 The genes predicted to have PPR motifs in the long arm of rice chromosome 10 and the STS markers designed from these gene sequences

Gene identity	TIGR gene locus IDs	Length in bp		No. of exons		Length (aminoacids)		No. of PPR motifs		Mitochondrial targeting peptides (mTP) predicted using Target P		Forward Primer (5′-3′)	Reverse Primer (5′-3′)	Amplicon size (bp)	Actual annealing temperature (°C)
		japonica	*indica*	*japonica*	*indica*	*japonica*	*indica*	*japonica*	*indica*	*japonica*	*indica*				
PPR1	LOC_Os10g35090	4,287	3,790	5	4	174	174	1	1	0.732	0.712	AGAACAGAAGCC AAGAAGC	GTCCTTCATTGA TCATCTGC	896	60
PPR2	LOC_Os10g35230	1,654	1,079	1	1	462	355	10	5	0.685	0.180	AGAACAGAAGCC AAGAAGC	GAGCATGCATC TCAACAAG	1,648	58
PPR3	LOC_Os10g35240	3,873	2,252	1	3	783	750	15	15	0.900	0.908	CTGCGTTATGCAA GGCTCAAGCTA	AGCAGCAAACAA ATCCTTAG	1,383	62
PPR4	LOC_Os10g35260	2,255	1,667	4	3	518	371	7	7	0.885	0.808	AGAACAGAAGCC AAGAAGC	GAGAGAGCTGCA AACAAATC	1,661	60
PPR5	LOC_Os10g35436	6,884	3,940	8	7	795	787	16	14	0.897	0.891	AGAACAGAAGC CAAGAAGC	CAGGTCTTAGTC CTTCATCG	785	57
PPR6	LOC_Os10g35440[a]	2,259	1,195	1	4	267	267	3	4	0.727	0.764	AGAACAGAAGCC AAGAAGC	CTAAGTTCAATC TGCGTTCC	770	56
PPR7	LOC_Os10g35640[a]	2,797	1,818	2	2	507	507	10	10	0.764	0.795	AGAACAGAAGCC AAGAAGC	GTGCTAAAGCA TTCTCCATC	827	55
PPR8	LOC_Os10g35730	1,667	796	2	2	342	213	3	3	0.073	0.083	CATACCATGAAA TGCTGGAC	CCAGCTAAGCA ATATCCATC	866	60
PPR9	LOC_Os10g35750	2,170	1,091	2	6	546	289	3	1	0.727	0.156	GATTTTGTTTTGTT TTCTTTTCTC	GTCAGTGATATGTT TTATCCAATC	1,295	64
PPR10	LOC_Os10g35760	5,430	1,475	2	2	514	411	3	3	0.783	0.713	GCTCCCTTCTG AGATGCTAC	CATAATTTCAAAT AACCATACGAG	1,288	60
PPR11	LOC_Os10g35790	1,365	1,365	1	1	455	455	12	12	0.758	0.887	GGTGTCGTATAA TACCATCATTG	GAGGATTATGTTGTA TGTGCTAAG	1,270	63
PPR12	LOC_Os10g36190	1,791	1,347	1	1	508	449	6	6	0.756	0.688	AGGTTCTTTGA GGAGCTGAG	ATCTTCTATATCTC CTGAACCTTG	1,219	62
PPR13	LOC_Os10g40920	3,383	3,381	1	3	906	906	9	4	0.889	0.702	ATATCCAGCACTCT TGTAGATATG	CAAAGTTTCTCATA AAATATCAGG	1,296	62

[a] LOC_Os10g35440 and LOC_Os10g35640 correspond to *Rf1a* and *Rf1b*, respectively

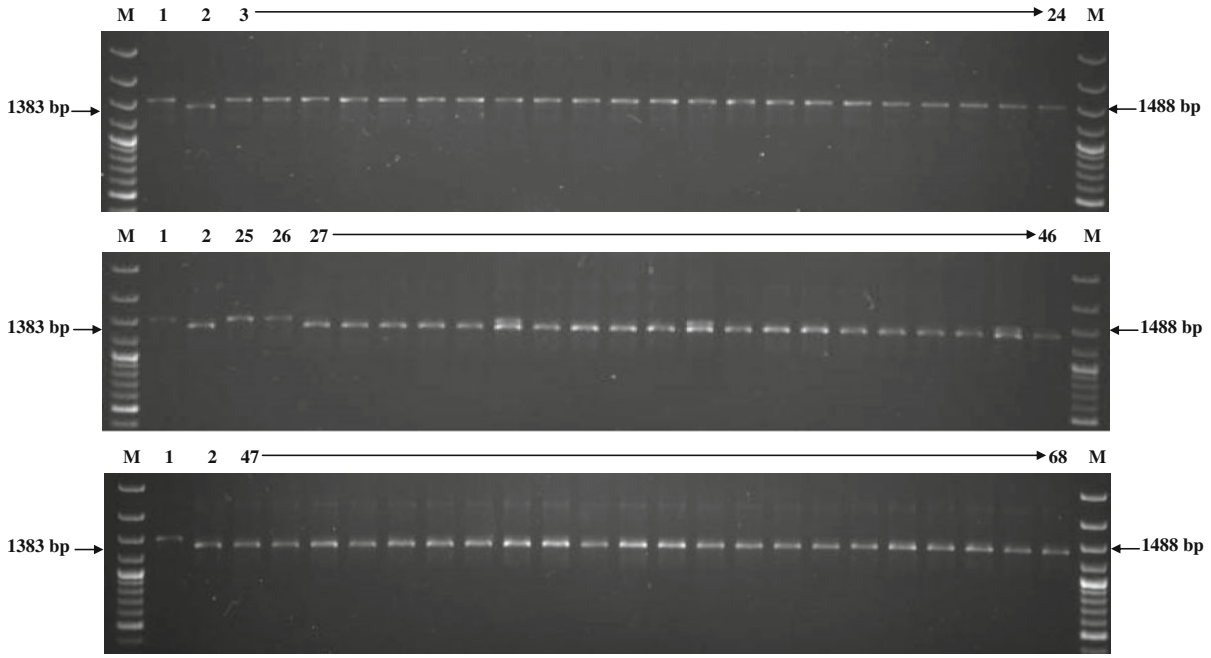

Fig. 5 A STS marker based on PPR3 gene polymorphic between two parents Pusa6A (*lane 1*) and PRR78 (*lane 2*). The CMS line specific 1,488 bp fragment co-segregates with male sterility in sterile F_2 plants (*lanes 3–26*) and the restorer specific 1,383 fragment co-segregates with fertility in the fertile F_2 plants (*lanes 27–68*) from the cross Pusa6A × PRR78. The fragments were separated in 1.2% agarose gel. *Lane M*-100 bp ladder plus. The sizes (bp) of the polymorphic fragments are indicated

Discussion

CMS has been observed in many plant species, including *Petunia*, maize and rice. It is a maternally inherited trait caused by mutations and rearrangements of mitochondrial genes (Forde and Leaver 1980; Vedel et al. 1994; Wang et al. 2006; Chase 2007; Fujii and Toriyama 2008; Rajendrakumar et al. 2007). In the present study, polymorphic Southern hybridization pattern obtained between the sterile WA cytoplasm and normal fertile cytoplasm for five (*coxI*, *rps3*, *atp6*, *cob* and *coxIII*) of the 10 mitochondrial gene specific probes in combination with *Eco*RI and *Hin*dIII revealed high degree of differentiation of the WA cytoplasm from its normal counterpart. The fragment size variation observed particularly in case of *coxI* between CMS and maintainer lines in both *Eco*RI and *Hin*dIII enzyme combinations suggested involvement of DNA rearrangements in the genic region (Pathania et al. 2007). Such type of variation in RFLP profiles between the sterile and fertile cytoplasm has also been reported earlier in the Boro II and WA-CMS of rice (Huang et al. 2006), and T-cytoplasm of maize (Forde and Leaver 1980). The presence of single copy *coxI* gene in rice mitochondrial genome was in contrast to those reported previously in the alloplasmic *B. juncea* (Pathania et al. 2007) and *Sorghum* (Bailey-Serres et al. 1986), where two copies with alteration of 303 nucleotides at the 3′ end were observed. The identification of more than one copy of *atp6* gene in the fertile maintainer line and a single copy in the sterile WA-CMS line is unlike the Boro II cytoplasm of rice, where two copies of *atp6* genes were observed in the sterile line (Wang et al. 2006). This observation thus reflected a significant difference between the gametophytic male sterility inducing BT cytoplasm and the WA cytoplasm imparting sporophytic male sterility (Huang et al. 2006). Similarly, the presence of two copies of *cob* gene in the fertile maintainer line was further confirmed by the absence of *Eco*RI and presence of one *Hin*dIII restriction site in the gene specific probe sequence. The absence of *cob* psuedogene in the WA cytoplasm and differential organization of this gene region in the fertile lines has already been observed (Narayanan et al. 1995).

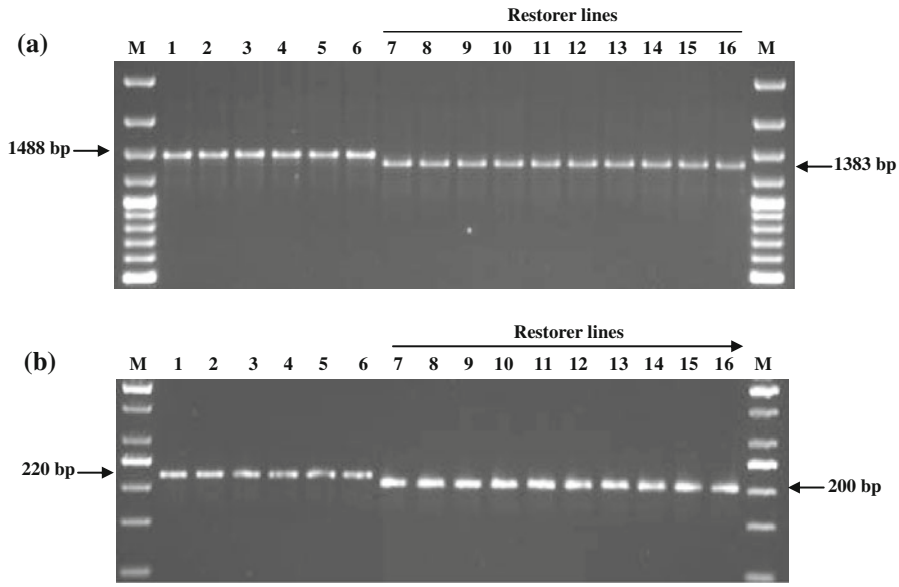

Fig. 6 Validation of restorer specific alleles of the PPR3 gene based STS (**a**) and GNMS (**b**) markers in 10 different restorer lines (*lanes 7–16*). *Lanes 1* Pusa6A, *2* Pusa29A, *3* Pusa4B, *4* Pusa5B, *5* Pusa6B, *6* Pusa9B, *7* PRR78, *8* UPRI93133, *9* C20R, *10* IR24, *11* MTU9992, *12* IR66, *13* AjayaR, *14* NDR3026, *15* BR827 and *16* KMR3. The fragments were separated in 1.2% agarose gel. *Lane M* 100 bp ladder plus. The size (bp) of the polymorphic fragments is indicated

Differential gene organization of *cob* might have resulted from recombination or insertion events involving this gene in the mitochondrial genome of male fertile rice line. Presence of recombination hot spots in the *cob* and *atp6* gene regions possibly contributed to rearrangements not affecting male fertility and clearly differentiated the male fertile line from the WA-CMS line (Iwabuchi et al. 1993; Huang et al. 2006). Occurrence of additional copies of *coxIII* gene in the WA cytoplasm of rice indicated differential mode of rearrangement in evolution that might in turn have contributed to difference in the expression of male sterility (Narayanan et al. 1995). These results provide leads for further analysis to identify gene(s) responsible for WA cytoplasm based male sterility in rice.

The informative PCR based markers with proven superiority over the hybridization based RFLP markers necessitated development of such markers from the mitochondrial gene sequences for differentiation of maintainer and CMS lines. CAPS analysis of PCR amplicons of mitochondrial gene segments using different restriction enzymes including hexacutters and tetracutters revealed presence of single nucleotide polymorphism (SNPs) at the restriction enzyme target sites in the coding regions of five mitochondrial genes (*18SrRNA*, *atp9*, *cob*, *coxIII* and *rps3*) of the CMS and maintainer lines. Presence of SNPs in the mitochondrial genes has been reported earlier (Tian et al. 2006). This further suggests differential mitochondrial genome evolution, which might be associated with differential editing/trans-splicing (Iwabuchi et al. 1993; Liu et al. 2007). From an applied angle, the mitochondrial gene specific CAPS markers developed in this study would be of immense use in efficient differentiation of the WA-based CMS line from its maintainer line. Successful hybrid rice seed production based on "three line system" largely depends on genetic purity of parental lines. CMS lines often get contaminated with iso-nuclear maintainer line during multiplication. The efficiency of polymorphic mitochondrial gene sequence specific CAPS markers for testing the purity of Pusa6A parental lines and detection of maximum acceptable limit of 2% or more contamination level of maintainer 'Pusa6B' line in CMS Pusa6A line during seed production of Pusa Rice Hybrid 10 as demonstrated in this study suggested their immediate use in marker assisted production and testing of pure hybrid seed. Validation of these markers across other commercial rice hybrids would expand the scope of use of the designed CAPS markers.

The restoration of fertility for WA as well as BT cytoplasm in rice is controlled by a major locus that mapped on the long-arm of rice chromosome 10 (Mishra et al. 2003; Wang et al. 2006). Considering the monogenic inheritance of fertility restoration, a set of 138 random F_2 individuals derived from the cross between CMS line Pusa6A and restorer line PRR78 were used in the present investigation, which was well in excess of the minimum population size required (Mather 1951). The test of goodness of fit of the observed ratio of fertile plants to sterile plants to the expected segregation of 3 fertile: 1 sterile for pollen fertility in F_2 revealed a clear monogenic inheritance pattern when the plants having up to 40% pollen fertility were included under the sterile class. In contrast, when the recommended 30% limit was applied, the observed ratio did not fit to the expected ratio of 3:1. These results thus suggested that although there is a major locus for the trait, the additional loci did influence its expression. Influence of more than one locus on pollen fertility has also been reported for BT as well as WA cytoplasm based male sterility (Govinda Raj and Virmani 1988) in rice. Further genetic characterization of the segregants is thus needed to determine the number and location of other genes responsible for fertility restoration in the restorer line PRR78 used in this study.

A major locus for male fertility restoration of WA cytoplasm based male sterility present in the Basmati Restorer line PRR78 was earlier mapped on rice chromosome 10 at a distance of 9.9 cM from the microsatellite marker RM258 (Mishra et al. 2003). Based on this result, the present study focused on identification of additional markers tightly linked with gene of interest. Out of several rice microsatellite and EST based STS markers used, only two (RM6737 and RM6100) additional informative markers could be identified, which were found tightly linked and flanking the gene of interest. The marker RM6100 is also reported to be tightly linked to the gene for fertility restoration of BT-cytoplasm (Akagi et al. 2004; Komori et al. 2004; Wang et al. 2006). Recently, Sheeba et al. (2009) have also validated its tight linkage with fertility restoration of WA cytoplasm based male sterility using two different mapping populations. These results suggested that RM6100 is a common marker for fertility restoration of both BT and WA cytoplasm and thus should be useful in marker assisted breeding of improved restorers in rice.

Considering the role of PPR motif-containing genes located on the long-arm of rice chromosome 10 for fertility restoration of BT-CMS in rice (Kazama and Toriyama 2003; Akagi et al. 2004; Komori et al. 2004; Wang et al. 2006), a physical region of 1.66 Mb on the long-arm of rice chromosome 10 was analyzed for such genes. Though the target gene was genetically mapped to a 0.8 cM interval (163.6 kb physical interval), effort was made to analyze a longer region of 1.66 Mb to gain insight into the location, orientation and distribution of all the PPR genes. A total of 13 PPR motif-containing genes including four (PPR1, 2, 3 and 4) in the target fertility restorer gene region were identified in both *indica* and *japonica* sequences. Similarity in both nucleotide and aminoacid sequences among the PPR genes of the two rice sub-species suggested their evolution through duplication. The PPR3 gene in *indica* as well as in *japonica*, was most similar to PPR5 gene and thus have remained more conserved as compared to others. Such duplication within the rice genome is, however, known based on whole genome sequence information (RCSC 2005; Lin et al. 2008). High-degree of correspondence of the order of PPR genes between *indica* and *japonica* was clearly evident. Such conservation of gene order is, however, expected keeping in view that the two subspecies of *Oryza sativa* possibly diverged from each other about one million years ago (Sang and Ge 2007). Conservation of the PPR motifs although suggested a common origin, great variation was observed in their organization with regard to their size, number of exons and the number of PPR motifs. It is not surprising in view of the earlier observation that duplication followed by sequence divergence is an established mode of evolution of gene families like R-genes (RCSC 2005) and PPR genes (Geddy and Brown2007; O'Toole et al. 2008) in crop plants.

Integration of the genetic map constructed in the present study with the sequence based physical map enabled identification of the candidate genes having the RNA binding PPR motifs and mitochondrial transit peptides, which suggested their possible role in RNA processing in mitochondria as reported earlier for the other CMS systems (Akagi et al. 2004; Komori et al. 2004; Wang et al. 2006; Schmitz-Linneweber and Small 2008). The PPR3 gene located

in the marker interval RM6737-RM6100 in the long-arm of rice chromosome 10 was identified as the most likely candidate involved sporophytically in fertility restoration of WA cytoplasm based male sterility in *indica* line PRR78. This possibly corresponds to the *Rf4* gene that maps in the same genomic region in another *indica* line Amol1 and restores fertility of WA-CMS in sporophytic mode (Ahmadikhah and Karlov 2006). However, PPR3 candidate gene appears different from another two fertility restorer genes namely, *Rf6(t)* present in the *indica* cultivar 93–11 for HongLian (HL) CMS (Liu et al. 2004) and *Rf1* carried in the *japonica* line MTC-10R for BT-CMS (Akagi et al. 2004), which have been mapped on the long arm of rice chromosome 10 but function gametophytically. It is supported by our observation that PPR3 has different sequence and physical location as compared to *Rf1a* and *Rf1b*, which were cloned and characterized earlier (Kazama and Toriyama 2003; Akagi et al. 2004; Komori et al. 2004). However, further experiment is required to functionally validate the identified candidate PPR gene and establish its precise role in restoration of fertility of WA-CMS. Recent identification and functional validation of a non-PPR gene on the rice chromosome 4 having role in fertility restoration of Chinese wild (CW) type CMS (Fujii and Toriyama 2009), however, suggests desirability of widening the search for candidates beyond PPR genes. Nevertheless, the integrated genetic and the physical map data guided development of a robust STS marker based on one of the candidate genes, which has several PPR motifs. Interestingly, informativeness of this marker being based on the presence of 105 nucleotides that encoded a single PPR motif in the CMS line and their absence in the restorer provides an allele specific marker for the trait. Besides, the polymorphic GNMS marker identified in the 5′UTR of the same PPR gene with more number of microsatellite repeats in the CMS than the restorer is of significance. The fact that the STS and GNMS markers identified precisely the sterile and fertile segregants in the F_2 population and all the restorers which are being used in hybrid breeding in India without any ambiguity, these would be useful in marker assisted breeding for new restorers in rice.

In summary, the present study clearly revealed high degree of differentiation of male-sterility inducing WA-cytoplasm from its normal fertile counterpart most likely through DNA rearrangements involving mitochondrial genes such as *coxI*, *coxIII*, *cob*, *atp6* and *rps3*. CAPS marker could be successfully designed and efficiently used for clear differentiation of the CMS line from its maintainer as well as in purity testing of the CMS line. The major locus for fertility restoration was localized to a physical interval of 163.3 kb that led to identification of four PPR motif containing candidate genes with possible role in fertility restoration of WA based CMS. STS and GNMS markers based on one of the candidate genes were designed and validated that would be of use in efficient marker assisted selection for fertility restoration of WA-CMS in rice.

Acknowledgments The work was partly funded under the NAAS-TATA Fellowship scheme awarded to the corresponding author by the National Academy of Agricultural Sciences, New Delhi. Part of the research work carried out by the first and the third authors for their M.Sc. degrees is included. Funding support to the first and the third authors in the form of fellowship by the Indian Agricultural Research Institute (IARI) and research facilities provided by the Project Director, National Research Centre on Plant Biotechnology, New Delhi are duly acknowledged.

References

Ahmadikhah A, Karlov GI (2006) Molecular mapping of the fertility-restoration gene *Rf4* for WA-cytoplasmic male sterility in rice. Plant Breed 125:363–367

Akagi H, Nakamura A, Yokozeki-Misono Y, Inagaki A, Takahashi H, Mori K, Fujimura T (2004) Positional cloning of the rice *Rf-1* gene, a restorer of BT-type cytoplasmic male sterility that encodes a mitochondria-targeting PPR protein. Theor Appl Genet 108:1449–1457

Bailey-Serres J, Dixon LK, Liddell AD, Leaver CJ (1986) Nuclear-mitochondrial interactions in cytoplasmic male-sterile sorghum. Theor Appl Genet 73:252–260

Chase CD (2007) Cytoplasmic male sterility: a window to the world of plant mitochondrial-nuclear interactions. Trends Genet 23:81–90

Ewing B, Green P (1998) Base calling of automated sequencer traces using Phred II. Error probabilities. Genome Res 8:186–194

Forde BG, Leaver CJ (1980) Nuclear and cytoplasmic genes controlling synthesis of variant mitochondrial polypeptides in male-sterile maize. Proc Natl Acad Sci USA 77: 418–422

Fujii S, Toriyama K (2008) Genome barriers between nuclei and mitochondria exemplified by cytoplasmic male sterility. Plant Cell Physiol 49:1484–1494

Fujii S, Toriyama K (2009) Suppressed expression of *RETROGRADE-REGULATED MALE STERILITY* restores pollen fertility in cytoplasmic male sterile rice plants. Proc Natl Acad Sci USA 106:9513–9518

Geddy R, Brown GG (2007) Genes encoding pentatricopeptide repeat (PPR) proteins are not conserved in location in plant genomes and may be subject to diversifying selection. BMC Genomics 8:130

Govinda Raj K, Virmani SS (1988) Genetics of fertility restoration of 'WA' type cytoplasmic male sterility in rice. Crop Sci 28:787–792

Huang W, Wang L, Yi P, Tan X-L, Zhang X-M, Zhang Z-J, Li Y-S, Zhu Y-G (2006) RFLP analysis for mitochondrial genome of CMS-rice. Acta Genetica Sinica 33:330–338

Ichii M, Hong DL, Ohara Y, Zhao CM, Taketa S (2003) Characterization of CMS and maintainer lines in *indica* rice (*Oryza sativa* L.) based on RAPD marker analysis. Euphytica 129:249–252

International Rice Genome Sequencing Project (IRGSP) (2005) The map based sequence of rice genome. Nature 436:793–800

Itabashi E, Kazama T, Toriyama K (2009) Characterization of cytoplasmic male sterility of rice with Lead Rice cytoplasm in comparison with that with Chinsurah Boro II cytoplasm. Plant Cell Rep 28:233–239

Iwabuchi M, Kyozuka J, Shimamoto K (1993) Processing followed by complete editing of an altered mitochondrial atp6 RNA restores fertility of cytoplasmic male sterile rice. EMBO J 12:1437–1446

Jing R, Li X, Yi P, Zhu Y (2001) Mapping fertility-restoring genes of rice WA cytoplasmic male sterility using SSLP markers. Bot Bull Acad Sin 42:167–171

Kazama T, Toriyama K (2003) A pentatricopeptide repeat-containing gene that promotes the processing of aberrant apt6 RNA of cytoplasmic male-sterile rice. FEBS Lett 544:99–102

Kazama T, Nakamura T, Watanabe M, Sugita M, Toriyama K (2008) Suppression mechanism of mitochondrial *ORF79* accumulation by Rf1 protein in BT-type cytoplasmic male sterile rice. Plant J 55:619–628

Komori T, Ohta S, Murai N, Takakura Y, Kuraya Y, Suzuki S, Hiei Y, Imaseki H, Nitta N (2004) Map-based cloning of a fertility restorer gene, *Rf-1*, in rice (*Oryza sativa* L.). Plant J 37:315–325

Lander ES, Green P, Abrahamson J, Barlow A, Daly MJ, Lincoln SE, Newburg LA (1987) MAPMAKER: An interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics 1:174–181

Lin H, Ouyang S, Egan A, Nobuta K, Haas BJ, Zhu W, Gu X, Silva JC, Meyers BC, Buell CR (2008) Characterization of paralogous protein families in rice. BMC Plant Biol 8:18

Liu X-Q, Xu X, Tan Y-P, Li S-Q, Hu J, Huang J-Y, Yang D-C, Li Y-S, Zhu Y-G (2004) Inheritance and molecular mapping of two fertility-restoring loci for Honglian gametophytic cytoplasmic male sterility in rice (*Oryza sativa* L.). Mol Gen Genomics 271:586–594

Liu Z-L, Xu H, Guo J-X, Liu Y-G (2007) Structural and expressional variations of the mitochondrial genome conferring the Wild Abortive type of cytoplasmic male sterility in rice. J Integr Plant Biol 49:908–914

Mather K (1951) The measurement of linkage in heredity, 2nd edn. Wiley, New York, p 149

McCouch SR, Teytelman L, Xu Y, Lobos KB, Clare K, Walton M, Fu B, Maghirang R, Li Z, Xing Y, Zhang Q, Kono I, Yano M, Fjellstrom R, DeClerck G, Schneider D, Cartinhour S, Ware D, Stein L (2002) Development and mapping of 2, 240 new SSR markers for rice (*Oryza sativa* L.). DNA Res 9:199–207

Mishra GP, Singh RK, Mohapatra T, Singh AK, Prabhu KV, Zaman FU, Sharma RK (2003) Molecular mapping of a gene for fertility restoration of wild abortive cytoplasmic male sterility using a Basmati rice restorer line. J Plant Biochem Biotech 12:37–42

Narayanan KK, Senthilkumar P, Sridhar VV, Thomas G, Thomas J (1995) Organization of the mitochondrial *cob2* pseudogene in different lines of rice. Theor Appl Genet 90:1087–1093

Narayanan KK, Senthikumar P, Venmadhi S, Thomas G, Thomas J (1996) Molecular genetics studies on the rice mitochondrial genome. In: Khush GS (Ed) Rice genetics III. Proceedings of third international rice genet symposium, international rice research institute, Los Bonos, Manila, The Philippines, pp 689–695

Newton KJ (1988) Plant mitochondrial genomes: organization, expression and variation. Annu Rev Plant Physiol Plant Mol Biol 39:503–532

O'Toole N, Hattori M, Andres C, Iida K, Lurin C, Schmitz-Linneweber C, Sugita M, Small I (2008) On the expansion of the pentatricopeptide repeat gene family in plants. Mol Biol Evol 25:1120–1128

Parida SK, Dalal V, Singh AK, Singh NK, Mohapatra T (2009) Genic non-coding microsatellites in the rice genome: characterization, marker design and use in assessing genetic and evolutionary relationships among domesticated groups. BMC Genomics 10:140

Pathania A, Kumar R, Kumar DV, Ashutosh, Dwivedi KK, Kirti PB, Prakash S, Chopra VL, Bhat SR (2007) A duplex *coxI* gene is associated with cytoplasmic male sterility in an alloplasmic *Brassica juncea* line derived from somatic hybridization with *Diplotaxis catholica*. J Genet 86: 93–101

Rajendrakumar P, Biswal AK, Balachandran SM, Ramesha MS, Viraktamath BC, Sundaram RM (2007) A mitochondrial repeat specific marker for distinguishing wild abortive types cytoplasmic male sterile rice lines from their cognate isogenic maintainer lines. Crop Sci 47: 207–211

Rice Chromosomes 11, 12 Sequencing Consortia (RCSC) (2005) The sequence of rice chromosomes 11 and 12 rich in disease resistance genes and recent gene duplications. BMC Biol 3:20

Rosamma CA, Vijayakumar NK (2005) Maintainers and restorers for CMS lines of rice. J Trop Agric 43:75–77

Sane AP, Seth P, Ranade SA, Nath P, Sane PV (1997) RAPD analysis of isolated mitochondrial DNA reveals heterogeneity in elite wild abortive (WA) cytoplasm in rice. Theor Appl Genet 95:1098–1103

Sang T, Ge Song (2007) The puzzle of rice domestication. J Int Plant Biol 49:760–768

Schmitz-Linneweber C, Small I (2008) Pentatricopeptide repeat proteins: a socket set for organelle gene expression. Trends Plant Sci 13:663–670

Schnable PS, Wise RP (1998) The molecular basis of cytoplasmic male sterility and fertility restoration. Trends Plant Sci 3:175–180

Sheeba NK, Viraktamath BC, Sivaramakrishnan S, Gangashetti MG, Khera P, Sundaram RM (2009) Validation of molecular markers linked to fertility restorer gene(s) for WA-CMS lines of rice. Euphytica 167:217–227

Tian X, Zheng J, Hu S, Yu J (2006) The rice mitochondrial genomes and their variations. Plant Physiol 140:401–410

Vedel F, Pla M, Vitart V, Guteirres S, Chetrit P, De Paepe R (1994) Molecular basis of nuclear and cytoplasmic male sterility in higher plants. Plant Physiol Biochem 32: 601–618

Wang Z, Zou Y, Li X, Zhang Q, Chen L, Wu H, Su D, Chen Y, Guo J, Luo D, Long Y, Zhong Y, Liu YG (2006) Cytoplasmic male sterility of rice with Boro II cytoplasm is caused by a cytotoxic peptide and is restored by two related PPR motif genes via distinct modes of mRNA silencing. Plant Cell 18:676–687

Yashitola J, Sundaram RM, Biradar SK, Thirumurugan T, Vishnupriya MR, Rajeshwari R, Viraktamath BC, Sarma NP, Sonti RV (2004) A sequence specific PCR marker for distinguishing rice lines on the basis of wild abortive cytoplasm from their cognate maintainer lines. Crop Sci 44:920–924

Zhang QY, Liu YG, Zhang GQ, Mei MT (2002) Molecular mapping of the fertility restorer gene *Rf4* for WA cytoplasmic male sterility in rice. Acta Genet Sin 29: 1001–1004