Available online at http://www.journalcra.com

International Journal of Current Research
Vol. 5, Issue, 04, pp.784-792, April, 2013

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

ISSN: 0975-833X

RESEARCH ARTICLE

GENOME ANALYSIS AND DEVELOPMENT OF DNA MARKER FOR THE DETECTION OF RESTORER LINES OF WILD ABORTIVE CYTOPLASMIC MALE STERILE SOURCE IN RICE (*Oryza sativa* L.)

[1,2,*]Nirmala, R., [1,3]Gnanesh A. U., [1]Anshu Rani, *[,1,4]Shashidhar, H. E.

[1]Barwale Foundation, # 3-6-666, Street No. 10, Himayath Nagar, Hyderabad, Andhra Pradesh, India
[2]Directorate General of Agriculture and Livestock Research, Ministry of Agriculture, Muscat, Sultanate of Oman
[3]College of Agriculture, Dharwad, Karnataka, India
[4]Department of Biotechnology, University of Agriculture, GKVK, Bangalore, India

ARTICLE INFO

Article History:
Received 29th January, 2012
Received in revised form
22nd February, 2013
Accepted 03rd March, 2013
Published online 13th April, 2013

Key words:
WA-CMS,
Fertility restoration,
DNA marker,
Rice.

ABSTRACT

The aim of the present study was to develop candidate gene specific polymorphic DNA marker(s) to detect the restorer lines of wild abortive cytoplasmic male sterility source in rice. To suppress the male sterility inducing components of mitochondria, nuclear genome encoded proteins need to be targeted to mitochondria with the help of signal peptide. Fertility restoration in crop species is governed by the pentatricopeptide proteins and proteins involved in the cellular oxidoreductase processes. Therefore, the present study was focused on (i) identifying the genomic regions code for these genes using rice genome database, (ii) listing the number of genes possess mitochondrial targeting signal peptide sequence, (iii) develop primer pairs to amplify the region of signal peptide sequence regions, (iv) assess the polymorphism if any, and (v) validate the polymorphic markers among germplasm lines known for their fertility restoration ability. Earlier reports on molecular mapping of fertility restorer genes (*Rf*s) in rice indicated *Rf* genes are located on chromosome 1 and 10 and therefore these two chromosomes were chosen for the genome analysis. A set of 132 loci out of 393 clones from chromosome 1 and 84 loci out of 202 clones from chromosome 10 were found to possess the select genes of interest. Using Mitoprot software, genes containing the mitochondrial signal sequences were identified and their sequences were retrieved. A set of 59 sequence tagged site (STS) markers to amplify at and around the region of mitochondrial signal sequence were developed. Amplification check of these primers was performed initially with two cytoplasmic male sterile lines namely IR58025A and Pusa6A and two restorer lines KMR3 and PRR78. Out of 59 markers analyzed, two markers namely CGS2 and CGS36 were polymorphic among CMS and restorer lines. Therefore, these markers were analyzed further among a set of 30 CMS lines, 34 maintainer lines, 54 restorer lines and two F_2 segregating populations for the validation. The efficiency of the marker to detect the restore lines was found to be 87.5%. Marker-trait association analysis revealed STS marker CGS36 was associated with the restoration trait at the significance level of $p < 0.001$ and its phenotypic variance was 71.74%. This result indicated the STS marker CGS36 as informative to detect the restorer lines.

INTRODUCTION

Rice, a major cereal crop, cultivated on about 150 million hectares with an annual production of 600 million tonnes and feeds more than 2/3 of the world population. The expanding population size demands to produce 1.7 million tones more rice every year. Therefore, to enhance the rice productivity, one of the success strategies adopted was to exploit heterosis through hybrid breeding. Use of cytoplasmic male sterility (CMS) in hybrid production, popularly known as three line hybrid breeding in rice and other crop species enabled to evaluate thousands of hybrids at once. This system not only eliminated hand emasculation but also ensured 100% genetically stable sterile line production by utilizing near isogenic non restorer male fertile lines called maintainer lines; and hybrid rice is produced by crossing CMS lines with the fertility restorer lines. Presently, about 27 CMS based rice hybrids are under commercial cultivation in India. Though several cms sources were obtained in rice to maintain the genetic variability, Wild Abortive (WA) cytoplasm based CMS system is widely utilized due to the availability of broader genetic base for the fertility restoration and excellent out crossing potential (Virmani and Kumar, 2004). Restorer lines are the critical factors for successful development of hybrid rice. To identify restorer lines, conventionally, line x tester analysis is being performed by crossing with cms lines. With the scalable CMS-WA hybrid rice released for commercial production, the inheritance and mapping of *Rf* alleles have been extensively studied using various restorer lines with different origins. Different genetic models of *Rf* alleles such as one gene (Shen *et al*., 1996), two linked genes (Li and Zhu, 1986) and two independent genes (Li and Yuan 1986; Virmani *et al*., 1986; Teng and Shen, 1994; Bharaj *et al*., 1995) were proposed by different groups. Fu and Xue (2004) found that *indica* restorer lines Milyang46 and H804 possess two dominant restorer alleles, but *japonica* restorer lines H921 and T984 have one restorer allele. Although the results are controversial in different reports, it is a tendency that the fertility of CMS-WA is controlled by one or two pairs of restorer alleles corresponding to different restorer lines (Li and Yuan, 1986; Virmani, 1996) and they function as independent or dependent fashions in various restorer lines (Yuan, 2002). Fast growing genomics research in rice enabled to design molecular tools to precisely estimate the genetic distance, gene locations for traits of interest etc. For *Rf* genes of WA-CMS, eight chromosomal loci have been proposed in earlier published studies, one on chromosome 1 (Zhang *et al*., 1997), two on chromosomes 7 and 10 (Bharaj *et al*., 1995), four QTLs on chromosomes 2, 3, 4, and 5 (Zhu *et al*., 1996; Zhuang and Zheng, 2000) and two major QTLs on

Corresponding author: heshashidhar@rediffmail.com

chromosome 10 (Tan *et al*., 1998). The availability of rice saturated molecular maps, especially the map of the rice genome with saturated SSR markers (McCouch *et al*., 2002) and efficient DNA marker detection techniques are useful in finding the location of fertility restorer alleles in rice more precisely. Jing *et al.*, (2001) found that *Rf4* locus in IR24 was flanked by RM171 (OSR33) and RM228 on the long arm of chromosome 10 with a genetic distance of 3.7 cM and 3.4 cM respectively. Further, *Rf4* in the restorer line Minghui 63 was flanked by RM258 and RM304 with a distance 2.9 and 0 cM respectively, and *Rf3* was mapped on Chromosome 1 and linked to RM1 with the distance of about 1.9 cM (He *et al.*, 2002).

The identification of fertility restorer gene in plants such as brassica, petunia, rice and raphanus revealed that the restorer alleles contained a pentatricopepetide motif (PPR) (Bentolila *et al.*, 2002; Brown *et al*., 2003; Kazama and Toriyama, 2003; Koizuka *et al*., 2003). Small and Peeters (2000) described the pentatricopeptide repeat (PPR) peptide motif as a degenerate tandem repeats of 35 amino acid sequence, present exclusively in eukaryotic genome. Geddy and Brown (2007) showed that PPR genes possess a novel, "nomadic" character and their positions are highly variable among closely related genomes. These proteins are known to mediate specific RNA processing events including RNA editing (Kotera *et al.*, 2005), transcript processing (Nakamura *et al.*, 2004) and translation initiation (Schmitz-Linneweber *et al*., 2005), thus, thought to be capable of specific binding to both protein and RNA molecules. In cms-T maize, restoration was encoded by *aldehyde dehydrogenase* which interacts with chimeric URF13, the mitochondrial protein associated with male sterility. Here, we report the development and validation of candidate gene based markers for fertility restoration of WA-cms using rice genome sequence database (www.rice.plantbiology.msu.edu) for use in detecting restorer lines among elite rice germplasm as well as hybrid seed purity analysis.

METHODOLOGY

Plant materials and DNA isolation

A set of one hundred and eighteen rice germplasm accessions comprising 30 wild abortive-cytoplasmic male sterile lines, 34 maintainer lines and 54 restorer lines were used in this analysis (Table1). In addition, two F_2 segregating populations derived from F_1 hybrids of IR58025A x KMR3 and IR58025A x PRR78 with 250 plants per population were also utilized. All the rice lines were grown in the field at Maharajpet farm, Barwale Foundation experimental station, Hyderabad, India and their genomic DNA was extracted from four-week old rice seedlings using modified Dellaporta method (Dellaporta *et al*., 1983).

Phenotyping of F_1 hybrids and F_2 populations

By crossing the cms (IR58025A) with two commercial restorer lines (KMR3 and PRR78), two F_1 hybrids (IR58025A X KMR3 and IR58025AxPRR78) were developed. The F_1 hybrid plants were evaluated for the percentage pollen and spikelet fertility according to Yui *et al*. (2003). For pollen fertility analysis, 10-15 spikelets/plant were collected in 70% alcohol, anthers were crushed, stained with 2% IKI solution and visualized under microscope. The unstained, irregular pollen grains were recorded as sterile and completely stained, round pollen grains as fertile. Pollen fertility percentage was generated based on the number of fertile pollens over total number of pollen grains analyzed. To record the percentage spikelet fertility, at the time of plant flowering, three panicles per plant were bagged and at plant maturity, bagged panicles were collected and counted for the number of filled grains over total number of spikelet per panicle. F_1 hybrid plants were selfed to produce F_2 population and a total of 250 plants per population were grown in the field and their pollen and spikelet fertility data was recorded as described.

Genome database analysis

Using rice genome database (www.rice.plantbiology.msu.edu) developed by the Institute for Genome Research (TIGR), genome sequences of chromosome 1 and 10 were analyzed for the presence of pentatricopeptide (PPR) genes and genes involved in oxidative processes. Genomic sequences of these genes were analyzed for the mitochondrial targeting signal peptide sequence using Mitoprot algorithm, (http://ihg2.helmholtz-muenchen.de/ihg/mitoprot.html), and their probability of transferability to the mitochondria were recorded.

Development of STS primers

Based on the probability value of Mitoprot analysis, the loci containing the probability value of above 80% were selected (Table 2). For these loci, genomic DNA sequences of mitochondrial targeting signal peptide sequences along with their flanking regions were retrieved from rice genome database. Using Gene Tool software (Bio tools Inc., Edmonton, Canada), a set of 59 primer pairs were developed to amplify the signal peptide sequence region of select loci (Table 3) and designed at Sigma-Bangalore, India. These primers were tested for the polymorphism among sterile and fertile lines involving two cms lines such as IR58025A and Pusa6A and two restorer lines such as KMR3 and PRR78. PCR amplification was performed in MJ Research thermal cycler with 15µl reaction volume containing 10–15ng genomic DNA, 10 mM Tris–HCl (pH8.4), 50mM KCl, 1.5mM $MgCl_2$, 400µM dNTPs, 2.5pM each primer and 0.5U*Taq* polymerase. Amplification profile was standardized with the initial denaturation at 95°C for 5 min, followed by 30 cycles of cyclic denaturation at 94°C for 8 s, annealing at 62°C for 5 s, extension at 72°C for 40 s and the final extension at 72°C for 7 min. PCR products were subjected to electrophoresis on 2% agarose gel followed by ethidium bromide staining and visualized under UV gel documentation system (Figure 1a and1b).

Validation of STS markers

The STS markers showed polymorphism among cms and restorer lines under study were further analyzed among a set of 118 rice lines of known maintainer and restorer ability by involving 30 WA-cms lines, 34 maintainer lines and 54 restorer lines. PCR amplification analysis of these samples was performed using the same PCR composition and thermo profile condition and resolved on 2.0% agarose gel followed by ethidium bromide staining and visualized under UV gel documentation system. Polymorphic markers clearly distinguishing maintainer and restorer lines were further screened among two F_2 segregating populations with 250 samples each. Phenotype-genotype dataset was studied for the marker-trait association analysis using TASSEL (Trait Analysis by Association, Evolution, and linkage) version 3.0.145 (Bradbury *et al.*, 2007).

RESULTS

Identification of chromosomal regions for fertility restoration

With the scalable WA-CMS rice hybrids released for commercial production, the inheritance and mapping of *Rf* alleles have been extensively studied using various restorer lines of different origins. Although the results are controversial in different reports, it is a tendency that the fertility of CMS-WA is controlled by one or two pairs of restorer alleles (Rf_3Rf_3 and Rf_4Rf_4) (Li and Yuan, 1986; Virmani, 1996) located on chromosome 1 and 10. Therefore, present study involved screening of genomic DNA sequences of these two chromosomes using rice genome database (www.rice. plantbiology. msu.edu).

Screening genomic DNA sequences and signal peptide sequence analysis

Scanning for the pentatricopeptide (PPR) genes and genes involved in oxido-reduction processes on chromosome 1 and 10 revealed 132 loci from 393 clones of chromosome 1 possessed the genes of interest.

Table 1. List of plant material used in this study

S.No	Genotype	Cytoplasm	Sl.No	Genotype	Cytoplasm
CMS lines			Maintainer lines		
1	IR58025A	Sterile	31	IR58025B	Fertile
2	IR62429A	Sterile	32	IR62429B	Fertile
3	IR67884A	Sterile	33	IR67884B	Fertile
4	IR68888A	Sterile	34	IR68275B	Fertile
5	IR68897A	Sterile	35	IR68885B	Fertile
6	IR69628A	Sterile	36	IR68888B	Fertile
7	IR70369A	Sterile	37	IR68897B	Fertile
8	IR70372A	Sterile	38	IR68902B	Fertile
9	IR70959A	Sterile	39	IR69628B	Fertile
10	IR69624A	Sterile	40	IR70362B	Fertile
11	IR72078A	Sterile	41	IR70369B	Fertile
12	IR72080A	Sterile	42	IR70372B	Fertile
13	IR72081A	Sterile	43	IR70959B	Fertile
14	IR73323A	Sterile	44	IR69624B	Fertile
15	IR73327A	Sterile	45	IR72078B	Fertile
16	IR75596A	Sterile	46	IR72080B	Fertile
17	IR68895A	Sterile	47	IR72081B	Fertile
18	IR79128A	Sterile	48	IR73320B	Fertile
19	IR80151A	Sterile	49	IR73323B	Fertile
20	IR80154A	Sterile	50	IR73323B	Fertile
21	IR80155A	Sterile	51	IR73793B	Fertile
22	IR80156A	Sterile	52	IR75596B	Fertile
23	IR80555A	Sterile	53	IR64608B	Fertile
24	IR80559A	Sterile	54	IR68886B	Fertile
25	IR80561A	Sterile	55	IR68895B	Fertile
26	48A	Sterile	56	IR79128B	Fertile
27	49A	Sterile	57	IR79156B	Fertile
28	50A	Sterile	58	IR80151B	Fertile
29	52A	Sterile	59	IR80154B	Fertile
30	PUSA6A	Sterile	60	IR80155B	Fertile
			61	IR80156B	Fertile
			62	IR80555B	Fertile
			63	IR80559B	Fertile
			64	IR80561B	Fertile
65	KMR-3	Fertile	92	IR62036-222-3-3-1-2R	Fertile
66	PRR-78	Sterile	93	IR62037-12-1-2-2-2R	Fertile
67	BR 827-35	Fertile	94	IR62037-129-2-3-3-3R	Fertile
68	NDR 3026	Fertile	95	IR62037-93-1-3-1-1R	Fertile
69	IR40750	Fertile	96	IR62048-47-3-3-2R	Fertile
70	UPRI-92-133	Fertile	97	IR62161-184-3-1-3-2R	Fertile
71	C-20R	Fertile	98	IR62171-122-3-2-3-3R	Fertile
72	IR10198R	Fertile	99	IR63875-196-2-2-1-3R	Fertile
73	IR10198-66-2R	Fertile	100	IR63877-43-2-1-3-1R	Fertile
74	IR23352-7R	Fertile	101	IR63879-195-2-2-3-2R	Fertile
75	IR33509-26-2-2R	Fertile	102	IR65483-14-1-4-13R	Fertile
76	IR42266-29-4-2-2-2R	Fertile	103	IR65489-H-AC2-2R	Fertile
77	IR43342-10-1-1-3-3R	Fertile	104	IR65514-5-2-19R	Fertile
78	IR55838-B2-2-3-2-3R	Fertile	105	IR65515-56-1-3-19R	Fertile
79	IR56381-139-2-2R	Fertile	106	IR65622-151-1-2-2-2R	Fertile
80	IR57298-174-2-2R	Fertile	107	IR65629-22-3-3-3-1R	Fertile

Continue……

81	IR58082-126-1-2R	Fertile	108	IR65912-90-1-6-3-2-3R	Fertile
82	IR58103-62-3R	Fertile	109	IR68427-8-3-3-2R	Fertile
83	IR59624-34-2-2R	Fertile	110	IR68445-62-1-3-1R	Fertile
84	IR59669-93-1-3R	Fertile	111	IR69502-6-SRN-3-UBN-2-R	Fertile
85	IR59682-132-1-1-2R	Fertile	112	IR69701-22-1-2R	Fertile
86	IR60199-B-B-2-1R	Fertile	113	IR69702-52-3-3R	Fertile
87	IR60819-34-2-1R	Fertile	114	IR69707-10-2-2-3-3R	Fertile
88	IR60919-150-3-3-3-2R	Fertile	115	IR69715-123-1-3R	Fertile
89	IR60997-16-2-3-2-2R	Fertile	116	IR71604-4-1-4-4-4-2-2-2R	Fertile
90	IR61614-38-19-3-2R	Fertile	117	IR72R	Fertile
91	IR62030-54-1-2-2R	Fertile	118	IR75243-15-13-1R	Fertile

Table 2. List of loci selected on rice chromosome 1 and 10 based on the probability of mitochondrial targeting signal peptide sequence

Locus Identifier	Putative Function	Amino acid cleavage site	Cleaved Sequence	Probability
Chr. 1				
LOC_Os01g11946	ATPase, coupled to transmembrane movement of substances, putative, expressed	80	MGLPTAAAPACCFPSTSSSSPRLLLLPLQP PPPQPPRRRRRLVSPGVCFGSPLPLHARFH WPHAVASSSMRRRGRRRRA	0.9964
LOC_Os01g74520	Ubiquinone biosynthesis methyltransferase COQ5, mitochondrial precursor, putative, expressed	27	MALRSAAGRLASSSRRRLLSPPTSIH	0.9938
LOC_Os01g61410	Rotenone-insensitive NADH-ubiquinone oxidoreductase, mitochondrial precursor, putative, expressed	18	MAASSLLRSLSRISRRG	0.993
LOC_Os01g51390	Mitochondrial-processing peptidase beta subunit, mitochondrial precursor, putative, expressed	38	MAATSIVRSKRRLALPYLHRLLHSGPATP SPNRFLRH	0.9917
LOC_Os01g22520	Dihydrolipoyl dehydrogenase, mitochondrial precursor, putative, expressed	28	MALAILARRRAAEALLRRPLGAAGVSA	0.9885
LOC_Os01g22520	Dihydrolipoyl dehydrogenase, mitochondrial precursor, putative, expressed	28	MALAILARRRAAEALLRRPLGAAGVSA	0.9885
LOC_Os01g72049	Mitochondrial SBP40, putative, expressed	34	MRHLARLLNNRILLPASSSPAAAFSKRTY ARRT	0.984
LOC_Os01g25270	Pentatricopeptide repeat protein PPR986-12, putative, expressed	15	MRSAGTISQQLTRY	0.9835
LOC_Os01g53700	Mitochondrial-processing peptidase alpha subunit, mitochondrial precursor, putative, expressed	21	MYRIAGSHLRSLKRYSYSRF	0.9809
LOC_Os01g52720	NADH dehydrogenase 1 alpha subcomplex, assembly factor 1, putative, expressed	27	MSRLRALWQASVNATRRAIVWNSEDL	0.9639
LOC_Os01g66000	NADH dehydrogenase I subunit N, putative, expressed	38	MWSAAAARTVTPLPAASPLQQHQQRRG AWARVGNGRA	0.9316
LOC_Os01g55700	Import inner membrane translocase subunit TIM50, mitochondrial precursor, putative, expressed	30	MDGVARSRLLVPLLPRISARSFSAASPAS	0.9277
LOC_Os01g40720	Pentatricopeptide repeat protein PPR1106-17, putative	45	MHRKLPPLPPLTLRRSSSSSSSAAAAASPP PPPPRRLPPPVPLR	0.9198
LOC_Os01g33070	Pentatricopeptide repeat protein PPR868-14, putative	46	MRNAAAAAARRAAPPPLLPPVRLSRSPC YPHQVFLPLQPYPGHRP	0.919
LOC_Os01g71180	Pentatricopeptide repeat protein PPR1106-17, putative	21	MLRRTPRLLAAVNPATAVRS	0.9144
LOC_Os01g52214	NADH-ubiquinone oxidoreductase 20 kDa subunit, mitochondrial precursor, putative, expressed	34	MALLPRTARLALLSAPRAYSAAATGAGA APARY	0.9127
LOC_Os01g08120	Pentatricopeptide repeat protein PPR868-14, putative, expressed	23	MVRALSRARSLLDGIPHRRGRA	0.911
LOC_Os01g70960	Cytochrome c1, heme protein, mitochondrial precursor, putative, expressed	18	MAAGRGISQLLKKAFRP	0.91
LOC_Os01g48410	Pollen-specific kinase partner protein, putative, expressed	53	MVRRHLLRGHSLDRFLPIRSLIMSSSSSFS SSSPSPPPPSSSSSRGSSSGRW	0.8835
LOC_Os01g41650	Pentatricopeptide repeat protein PPR986-12, putative, expressed	36	MASLPLPALHHEPLLSRSHRRLPPSPPPPP LPSRL	0.8641
LOC_Os01g07910	NADH-cytochrome b5 reductase-like protein, putative, expressed	17	MAALLLRRLAGTHRGR	0.8434
LOC_Os01g06454	Mitochondrial import inner membrane translocase subunit TIM14, putative, expressed	41	MAAPLIAGLAVAAAALASRYSIQAWHAY KARPIVPRMRKF	0.8341

Continue……

LOC_Os01g49190	ATP synthase beta chain, mitochondrial precursor, putative, expressed	48	MATRRALTSVLRSASRLRAASPSPCPRR APLHPHRRPSPAGFLLNRA	0.999
LOC_Os01g72730	Cytochrome P450, putative, expressed	34	MATPGHRQHALTARLAAAPRPACAVE LLSPRRA	0.787
LOC_Os01g73020	Mitochondrial import inner membrane translocase subunit TIM16, putative, expressed	48	MAARLLANLLVIGGTVLGRAAVQAYR QAIVNANKTGAAQEAINGIRR	0.77
LOC_Os01g58630	Pentatricopeptide repeat, putative, expressed	92	MSHLQHLAAGELVTALRGASCPSSALR LYSLIRIHARPSDPALFAWRPAVLALKP LSAAASLPLLSHFHAHLIRSNLLAYPHV ASSLLRGY	0.759
LOC_Os01g72740	Cytochrome P450 71D8, putative, expressed	93	MAGIVDTAAFCTLLCLLLTLVVFKLKT ATSSRHNAGVNLPPGPWALPVIGSIHCL LGSLPHHAMRELSRRYGPVMLLRLGH VRTLVLSSPEA	0.759
LOC_Os01g68620	Signal peptide peptidase-like 2B, putative, expressed	33	MAFPAPSSSSPRRRGRGLAYLLVSVLLL ASRV	0.751
LOC_Os01g24780	Cytochrome P450 72A1, putative, expressed	58	MDVPSVVIPILVVLVSRLLTSALVHLLW KPYAITKLFRGQGITGPKYRLFVGSLPEI	0.748
LOC_Os01g72760	Cytochrome P450 71D10, putative, expressed	99	MAGIMDSTTASYYTTLLCGALLLAAVV FKLKTAAAFSRHNAGVNLPPGPWALPV IGSIHCLLGSLPHHAMRELSRRYGPVML LRLGHVRTLVLSSPEA	0.743
LOC_Os01g61380	Malate dehydrogenase, mitochondrial precursor, putative, expressed	61	MASTVAINLIGAQAGIISKLRNCDITSYS GLKARSSISFESRSSFLGQNASLRSSISPR I	0.739
LOC_Os01g61880	Respiratory burst oxidase, putative, expressed	77	MASPYDHQSPHAQHPSGLPRPPGAGAG AAAGGFARGLMKQPSRLASGVRQFAS RVSMKVPEGVGGMRPGGGRMTRM	0.7
LOC_Os01g05060	Mitochondrial glycoprotein, expressed	47	MAFVSAAAAATAAAAFLPGVSSASRA GTPLLSLQRQPLAGSLRA	0.676
LOC_Os01g23610	Dihydrolipoyl dehydrogenase, putative, expressed	24	MYSTAISLSAAATAAAAVGGARP	0.667
LOC_Os01g53020	Electron transporter/ heat shock protein binding protein, putative, expressed	54	MAPLLSPPLLADSVAKFHCSSTPTPCSG SVRRWAITRFAGAGRRRDWHRRRRT	0.664
LOC_Os10g41740	Pentatricopeptide repeat protein PPR868-14, putative, expressed	92	MSLPVGSPPPPPTSPPYTGILAALHRSIA GGHAAAAVALLPELSRAGLRPPFPLLSS LARLLLLRRATAPCFPSLAGRLLLYVRL	0.9827
LOC_Os10g17280	ATP synthase gamma chain, mitochondrial precursor, putative, expressed	55	MAMAALRRDGRRVLLSSTPSPAAAMA ARSPAAAHQEIAPLGARSVSTQVVRTR M	0.9788
LOC_Os10g35960	NAD-dependent malic enzyme 59 kDa isoform, mitochondrial precursor, putative, expressed	17	MWRHAARRSSAQIRRS	0.9772
LOC_Os10g34310	rf1 protein, mitochondrial precursor, putative, expressed	115	MPLATLLGHLAAGRFGLVQALTGAAT AAAAHRLLHLLLRTAPPPPLPDLVSLAR WSRAHFRAPLPLRLHGLLLARLASKGL Y PLLRSELHVLAAARLHSPASILRALPSPS ASA	0.9676
LOC_Os10g35090	rf1 protein, mitochondrial precursor, putative, expressed	24	MARRAASRALRSEGSIQGRGGRA	0.9646
LOC_Os10g37180	Glycine cleavage system H protein, mitochondrial precursor, putative, expressed	42	MALRLWASSAANALKISCSGATRAAPA YSISRYFSTVLDGL	0.9512
LOC_Os10g37180	Glycine cleavage system H protein, mitochondrial precursor, putative, expressed	42	MALRLWASSAANALKISCSGATRAAPA YSISRYFSTVLDGL	0.9512
LOC_Os10g35640	rf1 protein, mitochondrial precursor, putative, expressed	20	MARRVAARARARAGGVPRS	0.9497
LOC_Os10g35436	rf1 protein, mitochondrial precursor, putative, expressed	31	MARRAASRVRAGAVGALRSEGSTQGR GGRT	0.9494
LOC_Os10g40360	Proline oxidase, mitochondrial precursor, putative, expressed	20	MAIASRIQKRVLASFAAAA	0.949
LOC_Os10g40920	Pentatricopeptide repeat protein PPR986-12, putative, expressed	45	MGKCAARQRQWRWPLLHRSPRPTPPPP HGLHPPRRALAE HARMP	0.9464
LOC_Os10g35440	rf1 protein, mitochondrial precursor, putative, expressed	27	MARRAASRAVGALRSDGSIQGRGGRA	0.8799
LOC_Os10g35230	rf1 protein, mitochondrial precursor, putative, expressed	24	MARRAASRAVGSEGSIQGRGGRA	0.8639
LOC_Os10g37330	aldo-keto reductase/ oxidoreductase, putative, expressed	40	MALPVTTRAAPAMPFAPQPRTAGGGLL RRPSPPAAALRI	0.805
LOC_Os10g35240	rf1 protein, mitochondrial precursor, putative, expressed	32	MARRVPTRPRGGGGGGVPRSEGSIQGR GGRA	0.6054
LOC_Os10g42840	NADH-ubiquinone oxidoreductase subunit B17.2, putative, expressed	15	MAAVVRGVLNGIRE	0.582
LOC_Os10g30290	Anther-specific proline-rich protein APG precursor, putative, expressed	37	MASSRSSLVVAMAVVILHRWCCAAAP AAAAANRTRT	0.5667

Continue……

LOC_Os10g30290	Anther-specific proline-rich protein APG precursor, putative, expressed	37	MASSRSSLVVAMAVVILHRWCCAAAP AAAAANRTRT	0.5667
LOC_Os10g21270	ATP synthase beta chain, putative, expressed	11	MRTNPTTSRP	0.4815
LOC_Os10g35260	rf1 protein, mitochondrial precursor, putative	12	MARRGRRYCRA	0.4644
LOC_Os10g40000	Oxidoreductase, putative, expressed	12	MAVRNVAAGRN	0.3932
LOC_Os10g21230	ATP synthase C chain, putative	43	MNPLIAAASVIAAGLAVGLASIGPGVG QGTAAGQAVEGIARQ	0.3124
LOC_Os10g17910	OsWAK114 - OsWAK receptor-like cytoplasmic kinase (OsWAK-RLCK)	42	MHTENHTIFVGITLGISFLIVGLLFILMM RQKRRMNEYFRK	0.2898
LOC_Os10g36390	Monocopper oxidase precursor, putative	19	MWRALAAAAAAVAVAVVVAARP	0.263

Table 3. Sequence tagged site primers generated for the genes of interest on rice chromosome 1 and 10

Primer ID	Forward sequence 5'-3'	Reverse sequence 5'-3'	PCR Product (bp) size	T_A (°C)
Chr 1.				
CGS1-F/R	GCACGGCGAGAACTCTTACACGA	TGGGAAGGACCGTACACGATCAC	400	52
CGS2-F/R	GAAATCCCCTCTGTGGCTTGTCA	CCCATCAATTCAGCAGAGGACAA	395	52
CGS3-F/R	GCGGTGTGAGGCAGCTGAGAG	TGGGCAATCGAGCTTTCTGAGTT	158	52
CGS4-F/R	TGGCGACGTAGCTCAAAATTCAC	TCCTGGTCAGCAAGCACACTCTC	194	52
CGS5-F/R	GAGGGGAGGCTTCAGGTAATC	CGGTGACAAAAGTATGGAGGG	337	50
CGS6-F/R	GCCGCCAGCCACAACCAG	GCAGGGGTCGCCAATGTCAC	366	52
CGS7-F/R	GCGTTCCGAGCACACACTTT	GGAGCAAACAAAGCCACCAC	240	48
CGS8-F/R	TGGAGGGTTTGGTTTTTTGAA	TGGGGCAACTGCATATTTTTC	143	48
CGS9-F/R	TGCCTTTTGGTGCTTTGAGAT	ATCCGGAAAGCAAAATGAAGG	267	48
CGS10-F/R	GAACCTCCAGGCACCCGAAAA	GCATGGATGGAGGGGGTTAGC	185	52
CGS11-F/R	TTGGCCGATACGATGATTTAA	TGCGACACGTCCTACCACTC	444	47
CGS12-F/R	CCTTGTCCTTCATCGCGTAGT	TCGCGGCTCCTGGGTAC	296	48
CGS13-F/R	TGCCTTAGAACCTCGGACGAT	GGGCCTAAGCATCCAACACAC	194	50
CGS14-F/R	CGCTGAACGTGACGACCAAC	CGGCGAGGAAGAGGGTGAT	327	50
CGS15-F/R	GCAGGAGGGGAGGCTTCA	CGGTGACAAAAGTATGGAGGG	341	48
CGS16-F/R	TGCGTCAGCTATTGGTGGAGA	CGTCCTATCCGGTTTTCTGGT	394	48
CGS17-F/R	CCGCTCGGCTTCATTTTTGT	CGAGCTCACCCATGTCCTTTG	250	50
CGS18-F/R	CCCTTTCAATAGTTCCGCATA	CAAGAAACGAAGTCCCATGAA	237	45
CGS19-F/R	GGCACGCTTTTTAAACCGCTA	CCGGCAAAAAATATGAAAACG	325	50
CGS20-F/R	CGCCGAGGCACCTTCCC	GGCGGGGGGCTTCCAG	163	52
CGS21-F/R	CCCGGCGGCAATGGAC	GGCGGAGGAAGAAAATCACAA	186	50
CGS22-F/R	CAGCAGCAGCAGTAACAGTTT	GGCGCAGGAAGGCAAC	311	45
CGS23-2F/R	GGCATGTGAACTTTATACTCCCT	TGTTCCCTCCGATCATAAATATT	281	45
CGS24-2F/R	TCCGCTCTTTTTCTCCTTTCC	CCGGAGCAGCACCAGAAA	324	48
CGS25-2F/R	TGCAAAACCCTATGGATGGA	TGCAAAGATTCAGAGGTCAACA	523	45
CGS26-2F/R	ATTGCTCATCATCTTCCCTAGTC	CTCCGTCTTTCATTCCTCCTAC	340	45
CGS27-2F/R	CTGGCATAGAAGCAAACCCT	CGGGGACGAGTGGATTTT	298	45
CGS28-2F/R	TGGCTAAAACCTCACTGACAAAA	CGCCAAGCTCCCAAAAAG	342	48
CGS29-2F/R	TCGAAACATGCAAACCAACTCA	GGGATAAAAACGGAGCGGATAG	418	49
CGS30-2F/R	GCCCAGCCTTTCTCCCTCTT	CCGAGCCCCCCTTCAATAA	391	50
CGS31-2F/R	CCGAACACCCCATACGACA	GCGCTGGGTAAAAACTCACTC	141	48
CGS32-2F/R	CCCCGTCCCTCCAATTTTTA	CAGCGGCAGCAGTGTCAGA	546	50
CGS33-2F/R	ATCGGAATCGGCATGTGGA	CCGGCTAGAGGAGGAAGACG	348	50
CGS34-2F/R	CCCTGAGGACGTGCGTAC	CCCTGGGGAGTCCTGAAC	195	45
CGS35-2F/R	CCTGCAAAGAAGTGGGATGA	TGGAACAGAAAACTCAGCCTCA	327	48
Chr 10				
CGS23F/R	CCGCCATCCGCAGCCAC	GCGGCCGGAGTAGTGAAAGAGG	333	55
CGS24F/R	GGGGCTGTGTCTTGTGGGTAA	CGGAACAGGGGGTATGATGC	319	50
CGS25F/R	ATGGTCATTGTGTTTTTAGCT	AACGACTTTCTACCAACCTAA	499	40

Continue……

CGS26F/R	GCAGAAAGCAGAGGATGTAGA	GCGCAGGAGGCAAATT	473	42
CGS27F/R	GCCGATTCCGATGATTATTT	TGTGGAGAAGCCGATTTGTAT	329	45
CGS28F/R	AAGGCATCGGCGTGTC	CTGAAATTGGAGGTGAAAGAT	377	42
CGS29F/R	CGTGGTTGGGTTTTGCTAG	CCGGGACAGGTAGGCAG	284	45
CGS30F/R	ACGTGATCCAAACAACCTTAG	TCGTACAGTCCTGAATTTATGAG	343	42
CGS31F/R	TCCACGTATGCAGAAAAAAAATG	GTGGACAAATCTCATGCGTTCA	249	48
CGS32F/R	CCACTATGAAAACAATCCCAAAT	ATGGGTTGATATTCCGCCTA	280	45
CGS33F/R	AATGGAGGTCGTGGGAACAAATC	ACGGGCGGGGGGCATAG	410	52
CGS34F/R	GCTTCAATCCTGTCGACCTCTAT	AATGCTGAACAGGAATCGAACA	421	48
CGS35F/R	GCCGCCGATGTAGAGAGGGTTC	CGGTGGGGACGGGGTTCTC	146	55
CGS36F/R	CGCCGCTGAGGTCATTGCTG	GCCGCTGCGACGACGACAT	184	55
CGS37F/R	CGCCCATAAAAACGAATCTCCAC	CGTTGGTGCACATGTTGAGTTTG	143	52
CGS38F/R	GGCGCTGGAGTTCAACGT	AAACGTTCGATCGCTTCAATAT	252	47
CGS39F/R	CCTAAATTCAATCCCCAAAAC	CCGGTTCCTCGCTCTGT	180	44
CGS40F/R	CCGGGACAGGTAGGCAGGATT	TGCGTGGTTGGGTTTTGCTAGT	286	52
CGS41F/R	CGTTTACTGTGGGGATTAGAA	CAGCCACATATGAAACGTAGA	338	42
CGS42F/R	CGCTTGCCGCCGAATCTT	GCCGGAGCTTACTGTTTGGACG	440	52
CGS43F/R	ATCGATCGGTCCCTCCTTGAAGA	CGCGAATCCCCTTGCCG	201	55
CGS44F/R	AGCCCAAAACCCAAAACAAAC	TTTGAGAGGCTGTCCATCCATAA	366	48
CGS45F/R	GCGCCAATATGCATGTTAAGAGA	CCTCCTTCTCCTCCCCTTTGTG	286	50
CGS46F/R	TCGCCATCAAACATTAATTCG	CGTAGTGGCACCGATTAAATT	196	45

Similarly, the scanning on chromosome 10 detected 84 loci out of 202 clones. Genomic DNA sequences of these loci were retrieved from the database and screened for the mitochondrial targeting signal peptide sequence. Out of 132 loci on chromosome 1, 58 (43.94%) were found to possess the mitochondrial targeting signal sequence and their probability value ranged between 0.045 and 0.992. For chromosome 10, 27/84 loci (32.14%) were detected for the possession of signal sequence and their probability value ranged between 0.052 and 0.983. The size of the signal sequence was ranged from 11 to155 amino acid. There was no linkage between the size of the signal peptide sequence and its probability value (Table 2).

STS marker development and validation

To assess the sequence polymorphism at the signal peptide sequence of genes selected (Table 2), genomic DNA sequences were retrieved along with their flanking sequences and used for the primer designing using Gene Tool software program. A set of 59 primer pairs were synthesized from Sigma- Bangalore (Table 3).

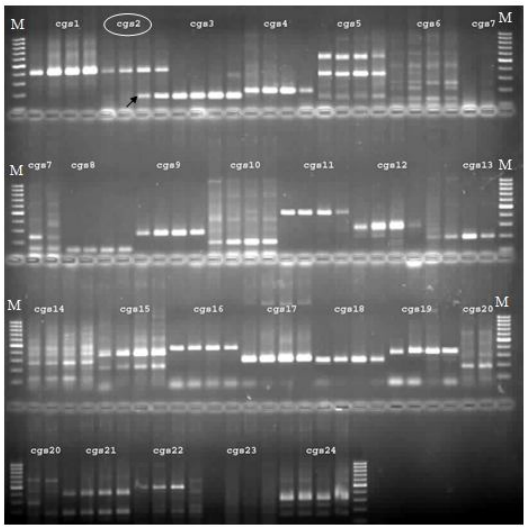

Figure 1a. PCR amplification and identification of polymorphic CGS-STS primers (CGS 1 to CGS24) on 2% agarose gel using cms (lane 1: IR58025A and lane 2: Pusa 6A) and restorer lines (lane 3: KMR3 and Lane 4: PRR78). Arrow mark indicates the amplification of 150bp polymorphic product of CGS 2 primer in restorer lines and absent in sterile lines. M: 100bp DNA ladder

PCR amplification analysis of these primers using two sterile lines and two restorer lines revealed two markers namely CGS2 and CGS36 exhibited polymorphism (Figure 1a and 1b) with the product sizes of 150bp and 350bp respectively. Screening those polymorphic markers among 30 cms, 34 maintainer and 54 restorer lines indicated the marker CGS36 was able to distinguish the restore lines with the maximum efficiency of 87.5%. Further screening of CGS36 among 500 samples of two F_2 populations and analysis of the marker for the TASSEL based marker-trait association study indicated CGS36 was highly significant at p value <0.001 and its phenotypic variance was calculated as 71.74% (Figure 2).

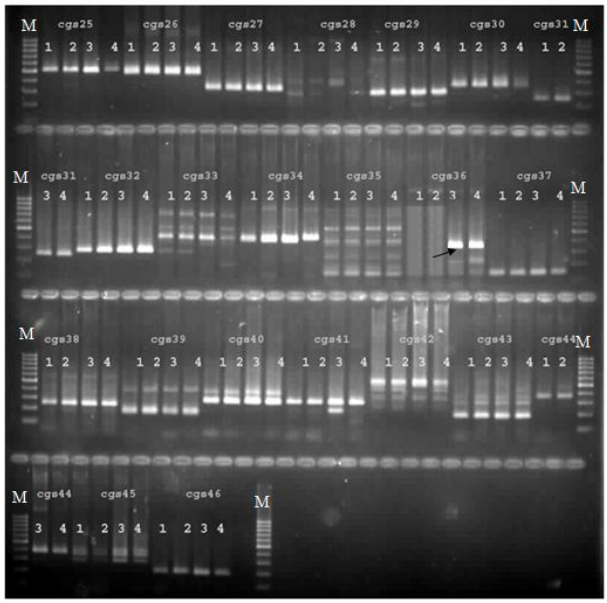

Figure 1b. PCR amplification and identification of polymorphic CGS-STS primers (CGS25-CGS46) on 2% agarose gel using cms (lane 1:IR58025A and lane 2: Pusa 6A) and restorer lines (lane 3: KMR3 and Lane 4: PRR78). Arrow mark indicates the amplification of polymorphic products of CGS 36 (350bp) primer in restorer lines and absent in sterile lines. M: 100bp DNA ladder

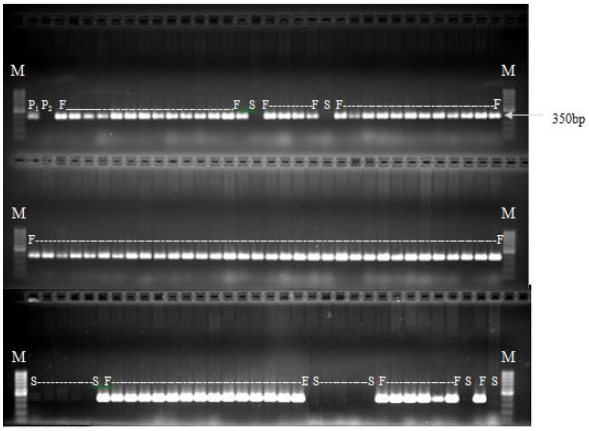

Figure 2: Validation of CGS 36F/R–STS primer among F_2 population of IR58025AxKMR3. P_1: cms line - IR58025A; P_2: restorer line - KMR3; S: sterile F_2 genotype; F: fertile F_2 genotype; Arrow mark indicates the presence of 350bp polymorphic product present among fertile genotypes and absent in sterile genotypes; M: 100 bp DNA ladder

DISCUSSION

Fertility restorer alleles (*Rf*s) are always tightly evolved with CMS trait during plant evolution. CMS trait is often associated with unusual open reading frames (ORFs) in mitochondrial genome (Schnable and Wise, 1998, Chase and Babay-Laughnan, 2004; Hanson and Bentolila, 2004). For the past 30 years, several strategies were implemented to detect the cms loci in mitochondrial genome. Following the methods on (i) identifying differentially expressed genes via screening of the mitochondrial cDNA library (ii) comparing the RFLP patterns of the mtDNA between the male sterile plant and maintainer line using known mitochondrial genes as probes to characterize the mutated mitochondrial genome and (iii) comparing the polymerase chain reaction (PCR) patterns of the mtDNA between the mutant plant and the fertile plant based on the entire mitochondrial genomic information, variations in mitochondrial genes associated with the cms trait were identified (Kadowaki *et al*., 1990; Iwabuchi *et al*., 1993). To date, at least nine mitochondrial genes for respiratory chain complexes have been discovered that cause cms in various plant species including *nad3*, *nad5* and *nad7* of complex I, *cox1* and *cox2* of complex IV, *atp1*, *atp6*, *atp8* and *atp9* of complex V (Akagi *et al*., 1994; Song and Hedgecoth, 1994; Brown, 1999; Vedel *et al*., 1999; Heazlewood *et al*., 2003, Hanson and Bentolila, 2004).

Accumulation of these altered gene products specifically in the microspores adversely affect the microspore development and cause pollen abortion, although the constitutive expression in other tissues observed (Wang *et al*., 2006b). Gene expression studies revealed nuclear restorer genes are targeted to mitochondria and interact with the cms genes in a gene to-gene fashion, modulate them at transcriptional or post- transcriptional level (Budar and Pelletier, 2001) and suppress the cytotoxic effect of aberrant ORFs (Wang *et al*., 2006b). It indicated cloning of *Rf* gene(s) is a key step to dissect the molecular mechanism of CMS and to clarify how interaction between the nuclear and cytoplasmic genes leads to pollen abortion and fertility restoration. Understanding the molecular basis of CMS-*Rf* interaction is critical for continued improvements in hybrid rice breeding. Characterization of *Rf* gene loci in several crop species demonstrated the *Rf* alleles contained pentatricopeptide (PPR) motif (Brown *et al*., 2003; Kazama and Toriyama, 2003; Koizuka *et al*., 2003). PPR proteins constitute a large family with 400 members in Arabidopsis and rice that are thought to be RNA binding proteins involved in posttranscriptional processes in mitochondria and chloroplasts (Bentolila *et al*., 2002) and only little data available on the functions of individual proteins in this family (Lurin *et al*., 2004). In a non restoring genotype (*rfrf*), homologous gene contained a deletion in the promoter region and was expressed in roots but not in floral buds (Li *et al*., 1998; Singh *et al*., 1996; Tang *et al*., 1998; Bentolila *et al*., 2002). Cloning and characterization of *Rf* gene in petunia indicated the gene product of *Rf-PPR592* led to a decrease in the gene product of the aberrant mitochondrial ORF pcf composed of portions of the coding region of *ATP synthase* subunit 9 and *cytochrome oxidase* subunit 2 fused to an ORF of unknown origin and concluded that *Rf*-PPR592 is likely involved in mediating a reduction in mRNA accumulation (Bentolila *et al*., 2002). Maize *Rf*2 gene encode for *aldehyde dehydrogenase* enzyme, which acts in conjunction with the *Rf*1 gene to restore fertility of T-cms (Deway *et al*., 1987, Kennel *et al*., 1987; Wise *et al*., 1996). The mechanism of *Rf* genes in maize was found to be similar to that of the petunia *Rf* gene. In rice, at present, four rice *Rf* genes namely *Rf*1 for BT cms, *Rf*2 for Ld–cms, *Rf*5 for HL cms and *Rf*17 for CW-cms were cloned and their molecular mechanisms elucidated (Toriyama *et al*., 2010). Amongst, *Rf*-1 and *Rf*5-HL cms encodes PPR protein (Kazama and Toriyama, 2003; Akagi *et al*., 2004; Komori *et al*., 2004, Hu *et al*., 2012), *Rf*2 encode glycine rich protein, *Rf*17 for CW cms encodes an unknown protein containing a part of the acyl-carrier protein synthase-like domain. So far, *Rf* genes for WA-cms is not cloned due to inconsistency in number of genes controlling for WA-cms. Polymorphic molecular marker (CGS36) identified in this study was derived from the signal peptide sequence region of putative protein aldo-keto reductase/oxidoreductase gene (loci LOC-Og10s 37330) at Chromosome 10 in rice, where *Rf* gene cluster region (s) were reported earlier could distinguish restorers and non restorers of WA cytoplasm. Analyses of these mitochondrial targeted oxidoreductase enzymes and PPR gene clusters are needed for a complete understanding of the evolution and molecular basis of WA- cms/R*f* system in rice.

Acknowledgement

This work was supported by Barwale Foundation-Research and Training Center, Hyderabad, India. We thank Mr. Dinesh C. Joshi, Executive Director, Barwale Foundation for his support and encouragement.

REFERENCES

Akagi H, Sakamoto M, Shinjyo C, Shimada H, Fujimura T (1994). A unique sequence located downstream from the rice mitochondrial apt6 may cause male sterility. Curr. Genet. 25: 52–58.

Bentolila S, Alfonso AA, Hanson MR (2002). A pentatricopeptide repeat-containing gene restores fertility to cytoplasmic male-sterile plants. Proc. Natl. Acad. Sci. 99: 10887-10892.

Bharaj TS, Virmani SS, Khush GS (1995). Chromosomal location of fertility restoring genes for wild abortive cytoplasmic male sterility using primary trisomics in rice. Euphytica 83: 169-173.

Bradbury PJ, Zhang Z, Kroon DE, Casstevens TM, Ramdoss Y, *et al.* (2007). TASSEL: software for association mapping of complex traits in diverse samples.Bioinformatics 23: 2633–2635.

Brown GG (1999). Unique aspects of cytoplasmic male sterility and fertility restoration in *Brassica napus*. J. Hered. 90: 351-356.

Brown GG, Formanova N, Jin H, Wargachuk R, Dendy C, Patil P, Laforest M, Zhang J, cheng WY and Landry BS (2003). The radish *Rfo* restorer gene of Ogura cytoplasmic male sterility encodes a protein with multiple pentatricopepetide repeats. Plant J. 35: 262-272.

Budar F, Pelletier G (2001). Male sterility in plants, occurrence, determinism, significance and use. C R Acad. Sci. 324: 543–550.

Chase CD, Babay-Laughnan S (2004). Cytoplasmic male sterility and fertility restoration by nuclear genes. In: Daniell H, Chase C (ed) Molecular Biology and Biotechnology of Plant Organelles: 593-622.

Dellaporta SL, Wood J, Hicks JB (1983). A plant DNA mini preparation: version II. Plant Mol. Biol. Rep. 1:19–21.

Dewey RE, Timothy DH, Levings CS (1987). A mitochondrial protein associated with cytoplasmic male sterility in the T cytoplasm of maize. Proc. Natl. Acad. Sci. 84: 5374–5378.

Fu HW, Xue QZ (2004). Analysis of restoring genes of three type of cytoplasmic male sterility in rice. Mol. Plant Breed. 2: 336–341.

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.

Hanson MR, Bentolila S (2004). Interactions of mitochondrial and nuclear genes that affect male gametophyte development. Plant Cell 16: 154-169.

He GH, Wang WM, Liu GQ, Hou L, Xiao YH, Tang M *et al*., (2002) Mapping of two fertility restoring gene for WA cytoplasmic male sterility in Minghui63 using SSR markers. Acta Genet. Sin. 29: 798-802.

Heazlewood JL, Whelan J, Millar AH (2003). The products of the mitochondrial orf25 and orfB genes are F_0 components in the plant F1Fo ATP *synthase*. FEBS Lett. 540: 201-205.

Hu J, Wang K, Huang W, Liu G, Gao Y, Wang J, Huang Q, Ji Y, Qin X, Wan L, Zhu R, Li S, Yang D, Zhu Y (2012). The Rice Pentatricopeptide Repeat Protein RF5 Restores Fertility in Hong-Lian Cytoplasmic Male-Sterile Lines via a Complex with the Glycine-Rich Protein GRP162. Plant Cell. 24(1): 109–122

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.

Kadowaki K, Suzaki T Kazama S (1990). A chimeric gene containing the 5′ portion of atp6 is associated with cytoplasmic male-sterility of rice. Mol. Gen. Genet. 224: 10-16.

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.

Kennel JC, Wise RP, and Pring DR (1987). Influence of nuclear background on transcription of a maize mitochondrial region associated with Texas male sterile cytoplasm. Mol. Gen. Genet. 210: 399-406.

Koizuka N, Imai I, Fujimoto H, Hayakawa T, Kimura Y, Kohno-Murase J, Sasaki T, Kawasaki S, Imamura J (2003). Genetic characterization of a pentatricopeptide repeat protein gene, orf687, that restores fertility in the cytoplasmic male sterile kosena radish. Plant J. 34: 407-415.

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.

Kotera E, Tasaka M, Shikanai T (2005). A pentatricopeptide repeat protein is essential for RNA editing in chloroplasts. Nature 433: 326-330.

Li XQ, Jean M, Landry BS, Brown GG (1998). Restorer genes for different forms of Brassica cytoplasmic male sterility map to a single nuclear locus that modifies transcripts of several mitochondrial genes. Proc. Natl. Acad. Sci. 95: 10032-10037.

Li YC, Yuan LP (1986). Genetic analysis of fertility restoration in male sterile lines of rice. In: IRRI (ed) Rice Genetics. Proc. Intl. Rice Genet. Symp. IRRI, Manila, Phillipines: 617-632.

Li ZB, Zhu YG (1986). Rice male sterile cytoplasm and fertility restoration. In: Smith WH, Bostian LR, Cervantes E (ed) Hybrid Rice. Proc Int Symp, IRRI, Manila, Phillipines: 85-102.

Lurin, C, Andres C, Aubourg S, Bellaoui M, Bitton F, Bruyere C, Caboche M, Debast C, Gualberto J, Hoffmann B (2004). Genome-wide analysis of Arabidopsis pentatricopeptide repeat proteins reveals their essential role in organelle biogenesis. Plant Cell 16: 2089-2103.

McCouch SR, Teytelman L, Xu Y, Lobos KB, Clare K, Walton M, Fu B, Maghirang R, Li Z, Zing Y, Zhang Q, Kono I, Yano M, Fjellstrom R, DeClerck G, Schneider D, Cartinhour S, Ware D, Stein L (2002). Development and mapping of 2240 new SSR markers for rice (Oryza sativa L.). DNA Res. 9: 199-207.

Nakamura T, Schuster G, Sugiura M, Sugita M (2004) Chloroplast RNAbinding and pentatricopeptide repeat proteins. Biochem. Soc. Trans. 32: 571-574.

Schmitz-Linneweber C, Williams-Carrier R, Barkan A (2005). RNA immunoprecipitation and micro array analysis show a chloroplast Pentatricopeptide repeat protein to be associated with the 5' region of mRNAs whose translation it activates. Plant Cell 17: 2791-2804.

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

Shen Y, Cai Q, Gao M, Wang X (1996). Isolation and genetic characterization of fertility restoring revertant induced from cytoplasmic male sterile rice. Euphytica 90: 17-23.

Singh M, Hamel N, Menassa R, Li XQ, Young B, Jean M, Landry BS, Brown GG (1996). Nuclear genes associated with a single Brassica CMS restorer locus influence transcripts of three different mitochondrial gene regions. Genetics 143(1):505–516.

Small I, Peeters N (2000). The PPR motif a TPR-related motif prevalent in plant organellar proteins. Trends Biochem. Sci. 25(2): 46-47.

Song J, Hedgcoth C (1994). A chimeric gene (*orf256*) is expressed as protein only in cytoplasmic male-sterile lines of wheat. Plant Mol. Biol. 26: 535-539.

Tan XL, Vanavichit A, Amornsilpa S, Trangoonrung S (1998). Genetic analysis of rice CMS-WA fertility restoration based on QTL mapping. Theor. Appl. Genet. 96: 994-999.

Tang HV, Chang R, Pring DR (1998). Co-segregation of single genes associated with fertility restoration and transcript processing of sorghum mitochondrial orf107 and urf209. Genetics 150: 383-391.

Teng LS, Shen ZT (1994). Inheritance of fertility restoration for cytoplasmic male sterility in rice. Rice Genet. Newslett. 11: 95-97.

Toriyama K, Fujii S, Itabashi E and Kazama T (2010). Molecular comparison of fertility restorer genes RF1, RF2, and RF17 for cytoplasmic male sterility in rice. In: Molecular Biology and "Omics" Technologies 28th International Rice Research Conference, 8-12 November 2010, Hanoi, Vietnam

Vedel F, Lalanne E, Sabar M, Chetrit P, De Paepe R (1999). The mitochondrial respiratory chain and *ATP synthase* complexes: composition, structure and mutational studies. Plant Physiol. Biochem. 37: 629–643.

Virmani SS (1996). Hybrid rice. Adv. Agron. 57: 378-462.

Virmani SS, Kumar I (2004). Development and use of hybrid rice technology to increase rice productivity in the tropics. IRRN 29:10-19.

Virmani SS, Raj KG, Casal C, Dalmacio RD, Aurin PA (1986). Current knowledge of and outlook on cytoplasmic-genetic male sterility and fertility restoration in rice. In: IRRI (ed) Rice Genetics. Proc. Intl. Rice Genet. Symp. IRRI, Manila, Phillipines: 633-648.

Wang ZH, Zou YJ, Li XY, Zhang QY, Chen LT, Wu H *et al*., (2006b). 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.

Wise RP, Dill CL, Schnable PS (1996). Interaction of mutator induced mutations of the nuclear fertility restorer and T-urf13 of T-cytoplasm maize mitochondria. Genetics 143: 1383–1394.

Yuan LP (2002). Hybrid Rice. China Agricultural Press, Beijing.

Yui R, Iketami S, Mikami T, Kubo T (2003). Antisense inhibition of mitochondrial *pyruvate dehydrogenaseE1* alpha subunit in anther tapetum causes male sterility. Plant J. 34: 57-66.

Zhang G, Bharaj TS, Ln Y, Vinnani S, Huang N (1997). Mapping of the *Rf*3 nuclear fertility restoring gene for WA cytoplasmic, male sterility in rice using RAPD and RFLP markers. Theor. Appl. Genet. 94: 27-33.

Zhu L, Lu C, Li P, Shen L, Xu Y, He P, Chen Y (1996). Using doubled haploid populations of rice for quantitative trait locus mapping. In: Khush GS (ed) Rice Genetics III. Proc. Third Intl. Rice Genet. Symp. IRRI, Manila, Philippines: 31-635.

Zhuang JY, Zheng KL (2000). Mapping major and minor QTL for rice CMS-WA fertility restoration. Rice Genet. Newslett. 17: 56-59.

****