Available online at www.sciencedirect.com

ScienceDirect

Rice Science, 2019, 26(5): 282−289

Research Paper

Differential RNA Editing of Mitochondrial Genes in WA-Cytoplasmic Based Male Sterile Line Pusa 6A, and Its Maintainer and Restorer Lines

Umakanta NGANGKHAM[1, 2], Swarup Kumar PARIDA[1, 3], Ashok Kumar SINGH[4], Trilochan MOHAPATRA[1, 5]

([1]*National Research Centre on Plant Biotechnology, New Delhi 110012, India;* [2]*Indian Council of Agricultural Research-Research Complex for North East Hill Region, Indian Council of Agricultural Research, Umiam 793103, India;* [3]*National Institute of Plant Genome Research, New Delhi 110067, India;* [4]*Division of Genetics, Indian Agricultural Research Institute, New Delhi 110012, India;* [5]*Indian Council of Agricultural Research, New Delhi 110001, India*)

Abstract: RNA editing changes the nucleotides at the transcript level of mitochondrial genes which results in synthesis of functional proteins. This study was designed to find the editing sites which could be implicated in male fertility restoration and to develop editing based markers for differentiation of cytoplasmic male sterility and maintainer lines from each other. DNA and RNA from young panicles were isolated from three-line system of hybrid rice PRH10, wild abortive (WA) cytoplasm based male sterile (A line Pusa 6A), maintainer (B line Pusa 6B) and restorer (R line PRR78) lines. Pusa 6A and PRR78 having the same WA cytoplasm are allo-nuclear and iso-cytpolasmic lines. The genomic and cDNA amplicons for eight mitochondrial genes (*18SrRNA*, *atp6*, *atp9*, *cobII*, *coxI*, *coxIII*, *nadI* and *rps3*) were sequenced and compared. Differences in genomic and cDNA sequences were considered as editing. Two hundred and thirty editing sites having base substitution or insertion/deletion were identified with the highest in *18SrRNA* (5.74%) and the lowest in *coxI* (0.60%). The highest editing sites were observed in fertile maintainer Pusa 6B followed by PRR78 and Pusa 6A, of which random five editing sites in five different rice mitochondrial transcripts namely *atp9*, *cobII*, *coxIII*, *rps3* and *18SrRNA* were chosen and validated through cleaved amplified polymorphism sequence (CAPS) analysis and found to be partially edited in four genes. The identical editing sites of different mitochondrial genes from maintainer and restorer lines might reflect their possible contribution to fertility restoration of sterile WA cytoplasm.

Key words: cleaved amplified polymorphism sequence; cytoplasmic male sterility; hybrid rice; RNA editing; mitochondrial gene; wild abortive; nucleotide

RNA editing is an important posttranscriptional modification process that increases the diversities of transcriptomes and proteomes in eukaryotic organisms (Wu et al, 2017). In the mitochondria of flowering plants, the primary transcripts undergo 'editing' that involves predominantly C to U substitutions (Covello and Gray, 1989). Besides substitutions, insertions and/or deletions of other nucleotides have also been reported (Hanson et al, 1996; Smith et al, 1997; Stuart et al, 1997). The consequences of RNA editing are either modification of the coded information for some amino acids or generation of new initiation and/or termination codons of the proteins. The first report of RNA editing was described in 1986 which involved addition or deletion of uridine residues in the RNAs of kinetoplastid, the trypanosomal mitochondria (Benne et al, 1986). Since then, it has been reported as a frequent phenomenon of both monocotyledonous and

Received: 19 July 2018; **Accepted:** 10 October 2018

Corresponding authors: Trilochan MOHAPATRA (tmnrcpb@gmail.com); Umakanta NGANGKHAM (ukbiotech@gmail.com)

Peer review under responsibility of China National Rice Research Institute

http://dx.doi.org/10.1016/j.rsci.2019.08.002

dicotyledonous higher plant species (Hiesel et al, 1989; Gray et al, 1992), abundantly in mitochondria as compared to chloroplast (Chateigner-Boutin et al, 2008). The first evidence of RNA editing in the plant mitochondria comes from comparison of the protein sequence of subunit 9 (*atp9*) of wheat mitochondrial ATP synthase (ATPase) and its corresponding gene sequence (Graves et al, 1990). Specific RNA editing data for plant mitochondrial *coxII* mRNA are able to illustrate the basic features of RNA editing in plant mitochondria (Cattaneo, 1991). Higher degree of conservation editing sites in different plant species has indicated sharing of editing sites by both monocots and dicots. However, species specific RNA editing patterns have also been reported in several plant mitochondrial genes (Covello and Gray, 1990).

Cytoplasmic male sterility (CMS), a maternally inherited trait, is widely observed in more than 150 plant species (Liu et al, 2007), where the male organ is sterile/non-functional but having functional female organ leading to compulsory cross-pollination. In many crops, the fertility of male sterile line is restored by fertility restorer (*Rf*) gene(s), which are encoded by nuclear genome. Such systems have been exploited in many crops for commercial production of hybrid seeds and to understand the nuclear-cytoplasmic organelles interaction. The best studied case of a nucleo-cytoplasmic incompatibility leading to the expression of CMS phenotype is the CMS-T of maize. In this case, mitochondrial dysfunction and male-sterility are associated with mitochondrial DNA rearrangements creating a new ORF (*T-urf13*), which is transcribed and translated into a chimeric polypeptide (Dewey et al, 1986). Other examples are the chimeric genes *pcf-S* of petunia (Young and Hanson, 1987), and *orfB* and *orf224* of the 'Polima' CMS in rapeseed (Handa et al, 1995). The transcripts of all these chimeric genes, with the exception of *T-urf13*, were found to be edited. Correlation of RNA editing in the plant mitochondrial gene with CMS has also been observed for *atp9* in transgenic tobacco (Hernould et al, 1993). Transgenic tobacco plants showed male sterility when the unedited *atp9* (non-functional) was introduced, while all the transgenic plants carrying the edited (functional) form of *atp9* were found to be fertile. Cell type specific RNA editing in *atp6* mitochondrial gene is involved in CMS in *Sorghum bicolour* (Howad and Kempken, 1997). The complete editing of an *atp6* gene that might restore the fertility of BT-type CMS has also been reported in rice (Iwabuchi et al, 1993). Das et al (2010) and Chakraborty et al (2015) have reported that the sterility of wild abortive (WA) cytoplasm is due to competition between translated products of edited and unedited *orfB* transcripts, which leads to impaired biogenesis of ATPase complex in rice mitochondria. Most of the mitochondrial DNA rearrangements in CMS identified in plants are associated with the genes encoding subunits of ATP synthase (Ji et al, 2014).

The present study was carried out to gain insight into RNA editing taking place in eight mitochondrial genes from WA-cytoplasm which involves in vital mitochondrial processes and are widely used in rice hybrid breeding, and to examine possible relationship of any editing sites with expression of CMS.

MATERIALS AND METHODS

Rice materials

Rice materials included three lines of hybrid rice (*Oryza sativa* L.), Pusa Rice Hybrid 10 (PRH10) seed production viz. CMS line Pusa 6A, maintainer line Pusa 6B and restorer line Pusa Rice Restorer 78 (PRR78). These lines were grown in the research field of the Indian Agricultural Research Institute, New Delhi, India, following standard agronomic practices.

Isolation of total genomic DNA and cellular RNA

Total cellular DNA was isolated from young panicles of Pusa 6A, Pusa 6B and PRR78 following Doyle and Doyle (1990). DNA was purified, quantified and diluted to 50 ng/μL for PCR reaction. Total cellular RNA was isolated from young panicles using SV Total RNA Isolation System (www.promega.com) following the manufacturer's protocol.

Primer designing, PCR and RT-PCR amplification

The primer sequences of eight rice mitochondrial genes namely *atp9*, *atp6*, *coxI*, *coxIII*, *cobII*, *18SrRNA*, *rps3* and *nadI* were taken from Ngangkham et al (2010) (Table 1). The PCR constituents and cycling conditions with varying annealing temperature were carried out according to Ngangkham et al (2010). For reverse transcript PCR (RT-PCR), RNA was converted to cDNA using ImProm-II™ Reverse Transcription System (www.promega.com) following the manufacturer's protocol and cDNAs were directly used for gene specific PCR amplification. The amplicons were purified using E.Z.N.A. Gel Extraction Kit

Table 1. Eight rice mitochondrial genes and their biological function used in this study.

Gene	Biological function	Forward primer (5′–3′)	Reverse primer (5′–3′)	Amplicon size (bp)
18SrRNA	Ribosomal subunit	GTGTTGCTGAGACATGCGCC	ATATGGCGCAAGACGATTCC	749
atp6	Subunit of ATPase	ATGGGTTTGAATCAGAGAGA	ATTCAATTATGAAATTACTC	999
atp9	Subunit of ATPase	GGTGTGGTGTTCAGTCTACC	GGGCCTCGTATCTCTATTTG	406
cobII	Apocytochrome B	AAGGAACCAACGATTCTCTC	CGGTCGAAAACTTGAACTAC	1 001
coxI	Cytochrome C oxidase subunit	TATTACCAGCCATTCTGGAG	CTACGAAGAAACGACGAATC	1 008
coxIII	Cytochrome C oxidase subunit	GGAGAGGGCATGATAAAGAC	AAATAGTGGAGGGTGCTTG	810
nadI	Subunit of NADH dehydrogenase	ATACACCAGGGCAACTAATG	AGGGAGTAGGGTGAGTAAGC	790
rps3	Ribosomal subunit	AAACCCTCTCTAAGGTGGAG	AGATAGTCACCCATCACACG	1 002

(www.Omegabiotek.com).

Cloning and sequencing

Purified PCR and RT-PCR products were cloned into pGEM-T Easy Vector System (www.promega.com), following the manufacturer's instruction. Recombinant white colonies were selected and used for plasmid isolation using AxyPrep Easy-96 plasmid DNA Kit (Axygen Biosciences, USA). Purified recombinant plasmids were sequenced with universal primers in both forward and reverse directions using a capillary-based Automated DNA Sequencer (MegaBACE 4000, Amersham Biosciences, USA). The vector sequences from the sequencing data were cleaned using VecScreen of NCBI online tools (http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html).

Detection of RNA editing sites through multiple sequence alignment and cleaved amplified polymorphism sequence (CAPS) analysis

For discovering of RNA editing sites, the high quality sequences obtained from genomic and cDNA of each rice mitochondrial gene were aligned and compared using BioEdit Sequence Alignment Editor (http://www.mbio.ncsu.edu/bioedit/bioedit.html). RNA editing site is considered only when a specific nucleotide base alteration was detected in the cDNA sequences, as compared to the corresponding genomic sequences of a rice line. Frequency of RNA editing for each rice mitochondrial genes was also estimated. To validate the presence of editing sites identified through sequencing allignment, SNP2CAPS software tool (Thiel et al, 2004) was used for identification of appropriate restriction enzymes whose target sites were altered or introduced due to editing. The restriction enzymes predicted by SNP2CAPS were used to validate the SNP in the enzyme target sites in the mitochondrial genes. PCR amplicon of each gene was quantified on 1% agarose gel along with different known quantity of uncut lambda DNA as standard for comparison. Digestion of amplified products was carried out according to the manufacturer's protocol and the digested products were resolved on 1.5% agarose gel using 0.5× TBE buffer. Fragment size of the digested products was determined using 100 bp DNA ladder plus (Fermentas, Lithuania) and further compared with that of the undigested control to infer editing site.

RESULTS

Editing in Pusa 6A, Pusa 6B and PRR78

Comparison of the cDNA sequence of eight rice mitochondrial genes for sterile (Pusa 6A), maintainer (Pusa 6B) and restorer (PRR78) lines individually, with their corresponding genomic DNA sequences, led to identification of 230 nucleotides being edited, which consisted of substitution and insertion/deletion of nucleotides, from a total of 6 765 nucleotides, with an overall editing frequency of 3.39% (Table 2). Maximum edited nucleotides (52 sites) were observed in *atp6*, followed by *18SrRNA* (41), *cobII* (36), *nadI* (35), *rps3* (29), *coxIII* (23), *atp9* (8) and *coxI* (6). The frequency of RNA editing ranged from 0.60% (*coxI*) to 5.74% (*18SrRNA*) among the eight mitochondrial genes studied. Overall, there were 196 substitutions (74.24%), 37 insertions (14.02%) and 31 deletions (11.74%) in the three rice lines (Table 2).

In the CMS line Pusa 6A, there were 42 nucleotides edited (Table 2), which was the lowest among the three lines studied. Of these, 1 was deletion, 13 were insertions and 28 were substitutions. The maximum of 11 edited nucleotides were found in *coxIII* transcripts (1.36%), of which 3 were insertions, 1 was deletion and 7 were substitutions. This was followed by 10 edited nucleotides in *rps3* transcript, 9 in *cobII*, 8 in *atp6*, 2 in *18SrRNA* and 1 each in *nadI* and *atp9*, while in *coxI*, there was no editing.

Table 2. Statistics of eight mitochondrial genes in Pusa 6A, Pusa 6B and PRR78.

Gene	Pusa 6A					Pusa 6B					PRR78					TPPE	TF (%)
	NS	NI	ND	NT	F (%)	NS	NI	ND	NT	F (%)	NS	NI	ND	NT	F (%)		
18SrRNA	2	–	–	2	0.27	2	–	1	3	0.40	29	7	–	36	4.80	41	5.47
atp6	6	2	–	8	0.80	31	3	2	36	3.60	11	1	–	12	1.20	52	5.21
atp9	-	1	–	1	0.24	2	1	5	8	1.97	–	–	1	1	0.24	8	1.97
cobII	7	2	–	9	0.90	20	3	1	24	2.40	12	2	–	14	1.40	36	3.60
coxI	–	–	–	–	0.00	6	–	–	6	0.60	–	–	–	–	0.00	6	0.60
coxIII	7	3	1	11	1.36	10	3	2	15	1.85	9	1	1	11	1.36	23	2.84
nadI	–	1	–	1	0.12	2	–	–	2	0.25	19	2	11	32	4.05	35	4.43
rps3	6	4	–	10	1.00	13	–	6	19	1.89	2	1	–	3	0.30	29	3.19
Total	28	13	1	42	0.62	86	10	17	113	1.67	82	14	13	109	1.61	230	3.39

NS, Number of substitution sites; NI, Number of insertion sites; ND, Number of deletion sites; NT, Number of total sites edited; F, Frequency; TPPE, Total physical position edited; TF, Total editing frequency; '–', None.

In the case of PRR78, there were 109 edited bases which included 82 substitutions, 14 insertions and 13 deletions. The maximum edited nucleotides were observed in *18SrRNA* (36), followed by *nadI* (32), *cobII* (14), *atp6* (12), *coxIII* (11), *rps3* (3) and *atp9* (1) with no editing in *coxI* transcript. When compared to iso-cytoplasmic Pusa 6A line, there were 67 additional edited nucleotides in four genes viz. *18SrRNA*, *atp6*, *cobII* and *nadI* in PRR78 (Table 2).

In contrast to the above two parental lines carrying WA cytoplasm that has male sterilization effect, the frequency of editing was much higher in the maintainer line, Pusa 6B carrying normal fertile cytoplasm. There were a total of 113 edited nucleotides distributed across all the eight genes studied. Of these, 86 were base substitutions, 10 were insertions and 17 were deletions. The majority of these edited bases were different from those in the other two rice lines. The maximum editing took place in *atp6* transcript (36), followed by *cobII* (24), *rps3* (19), *coxIII* (15), *atp9* (8), *coxI* (6), *18SrRNA* (3) and *nadI* (2). As compared to isonuclear CMS line Pusa 6A, Pusa 6B had 71 new edited sites distributed across the eight mitochondrial genes.

CAPS analysis of editing sites

The RNA editing sites detected in the transcripts of eight rice mitochondrial genes were validated through CAPS analysis using appropriate restriction enzymes as predicted by SNP2CAPS. The editing sites in eight rice mitochondrial genes were predicted to have target sites for 18 different tetra-cutter restriction enzymes. Of these, five different rice mitochondrial transcripts (*atp9*, *coxIII*, *cobII*, *rps3* and *18SrRNA*) were chosen and examined through CAPS analysis.

In case of *atp9*, a 406 bp amplicon was obtained from both mitochondrial DNA and cDNA in all the three lines. When digested with *Dpn*I, the amplicons from genomic DNA were not affected in any of the three lines, while the cDNA got digested only in Pusa 6B giving a fragment of 390 bp, but not in the other two lines. This validated the T/G substitution in the *Dpn*I site GATC in Pusa 6B (Fig. 1-A).

PCR amplification using primers specific to *coxIII* gene gave 810 bp fragments for the mitochondrial DNA templates of Pusa 6A, Pusa 6B and PRR78, which weren't digested by *Hpy*CH4IV. The same 810 bp RT-PCR product for *coxIII* could not be digested in maintainer line Pusa 6B. In contrast, cDNAs from Pusa 6A and PRR78 were digested into fragments of 350 bp and 300 bp, and even lesser size (Fig. 1-B), revealing post transcriptional creation of two or more ACGT target sites for the enzyme *Hpy*CH4IV including a C/A substitution in the sequenced region. This result again validated editing in *coxIII* transcript in Pusa 6A and PRR78 but not in Pusa 6B.

In case of *cobII* gene, a 1 001 bp fragment was amplified in all the three lines when mitochondrial DNA was used as a template, which could not be digested by *Tsp*509I. The 1 001 bp fragments from cDNA of Pusa 6A and PRR78 were digested in where there was no digestion for cDNA of Pusa 6B (Fig. 1-C). In this digestion, cDNA amplicon from Pusa 6A and PRR78 gave a multiple fragments including four distinct and detectable fragments with different sizes of 701, 551, 450 and 300 bp. The combination of two digested fragment pairs such as 700 with 300 bp and 550 with 450 bp might be constituted the pre-digested amplicon. This result indicated the occurrence of two sites edited in sterile cytoplasms which wasn't edited completely and cDNAs of Pusa 6A and PRR78 consisted of both the partial edited transcripts.

Gene specific primers for *rps3* gene gave a PCR amplicon of size 1 002 bp in all the three lines, which was cleaved by enzyme *Bfa*I only in Pusa 6B to give

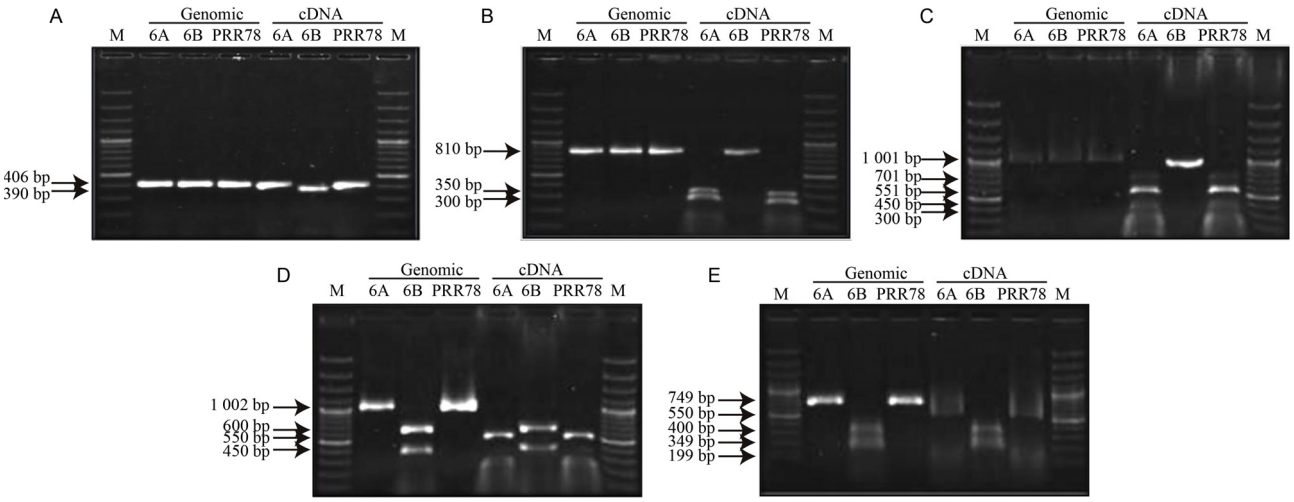

Fig. 1. Validation of RNA editing in mitochondrial genes by cleaved amplified polymorphism sequence analysis.

M, 100 bp DNA ladder plus; 6A, CMS line Pusa 6A; 6B, Maintainer line Pusa 6B; PRR78, Restorer line PRR78.

The recognition sites of *Dpn*I (GATC) for *atp9* (A), *Hpy*CH4IV (ACGT) for *coxIII* (B), *Tsp*509I (AATT) for *cobII* (C), *Bfa*I (CTAG) for *rps3* (D) and *Alu*I (AGCT) for *18SrRNA* (E) were affected by SNPs T/G, C/A, T/A, G/C and A/T as predicted by SNP2CAPS, respectively. The digested PCR fragments were separated in 1.2% agarose gel.

two fragments (Fig. 1-D), whereas, the amplicons in Pusa 6A and PRR78 remained uncut. This revealed a target site for the enzyme existing in the mtDNA only in Pusa 6B. When the RT-PCR products of 1 002 bp were digested using the same enzyme, digestion pattern in Pusa 6B was identical to that observed for PCR product suggesting that no further change had occurred post transcriptionally. However, in case of Pusa 6A and PRR78, the RT-PCR products for the *rps3* got digested to produce multiple fragments in contrast to PCR product. This indicated that multiple editing led to creation of target site CTAG for the enzyme *Bfa*I.

Similar to the *rps3* gene patterns, the amplicon of 749 bp obtained from the *18SrRNA* gene was digested by *Alu*I in Pusa 6B giving two fragments of 400 bp and 349 bp but not in Pusa 6A and PRR78, revealing thereby presence of one target site AGCT for the enzyme in Pusa 6B only. In RT-PCR using RNA templates, a 749 bp amplicon was obtained in all the three lines. This fragment got digested to give multiple fragments in Pusa 6A and PRR78 unlike to the PCR products, while the restriction fragment size remained identical to that from mtDNA template in Pusa 6B (Fig. 1-E). This result also revealed that editing took place at multiple sites in the *18SrRNA* transcript of Pusa 6A and PRR78.

DISCUSSION

RNA editing, a post transcriptional process, and CMS are two important phenomena associated with higher plant mitochondria where inadequate or defective RNA editing in specific mitochondrial genes causes male sterility (Hu et al, 2013). RNA editing is reported to regulate expression of several CMS-related mitochondrial genes (Howad and Kempken, 1997). Presence of edited and unedited forms of mRNA may give rise to biologically active proteins having different physical and/or functional properties (Gray and Covello, 1993). In some cases, RNA editing is an absolute requirement, which restores critical amino acid residue(s), in absence of which non-functional proteins might get synthesized as in case of wheat *cox2* mRNA (Covello and Gray, 1989).

Effect of isonuclear-alloplasmic interaction on RNA editing

Alloplasmic lines are created by exchanging the cytoplasm of one species line with another through substitution backcrossing while maintaining the nuclear genome, which leads to disruption of the interactions between the nucleus and cytoplasm that have co-evolved (Kianian and Kianian, 2014). It provides an opportunity to understand the interactions between the cytoplasmic organelles and the nucleus. The fertile maintainer line Pusa 6B with totally different mitochondrial genome from the sterile line Pusa 6A having WA sterile cytoplasm had identified 113 editing sites in eight mitochondrial genes, which is expected according to the hypothesis that decreased RNA editing frequency contributes to male sterility (Howad and Kempken, 1997; Yu et al, 2013). Presence

of additional 71 (63.71%) edited nucleotides in Pusa 6B as compared to Pusa 6A covering all the eight mitochondrial genes might indicate occurrences of higher and different mode of post-transcriptional editing in the mitochondrial genome of Pusa 6B, since it has male fertile cytoplasm compared to male sterile cytoplasm Pusa 6A (Table 2). While compared to Pusa 6A, the maximum additional edited nucleotides in Pusa 6B were observed in *atp6*, followed by *cobII* with the minimum in *nadI* and *18SrRNA* although nuclear background were identical, and therefore the same trans-factors for editing process. Since most of the maximum additional edited nucleotides were observed in genes involved in ATP synthesis, this shows the important role of editing in vital mitochondrial processes. These results also showed that the cytoplasm may play a vital role in the editing process. Similar results are also reported by Hu et al (2013), in which five isonuclear-alloplasmic male sterile lines, when compared to their corresponding CMS lines of rice, display different editing efficiencies at the conserved sites of *atp6*, *atp9* and *cox2* genes.

Effect of allonuclear-isoplasmic interaction on RNA editing

The male sterile line Pusa 6A and the restorer line PRR78 are allonuclear-isoplasmic parental lines since both having the same WA based sterile cytoplasm and thus the same mitochondrial genome constitution. Unlike in Pusa 6A line, there were 67 additional edited nucleotides in four genes viz. *18SrRNA*, *atp6*, *cobII* and *nadI* genes in PRR78. However, there was a decrease in number of total sites edited for *rps3*. Out of the total 12 edited nucleotides in the remaining three genes viz. *atp9*, *coxI* and *coxIII*, six editing sites had similar editing patterns in both iso-cytoplasmic Pusa 6A and PRR78 lines, which couldn't be correlated to male sterility. However, a total of 35 editing sites in six genes (*18SrRNA*, *atp6*, *codII*, *coxIII*, *rps3* and *nadI*) observed in Pusa 6A but not in PRR78, indicating that RNA editing isn't specific to the restorer lines. However, 67 new editing sites in PRR78 compared to its iso-cytoplasmic Pusa 6A, pointed to difference in nuclear-cytoplasmic interaction, which can be ascribed to the presence of fertility restorer (*Rf*) gene in PRR78.

Varying degree of editing in different mitochondrial transcripts in different lines

Comparison of the transcript sequence of eight rice mitochondrial genes in the parental lines of PRH10, viz. sterile (Pusa 6A), maintainer (Pusa 6B) and restorer (PRR78) lines revealed a total of 3.39% edited nucleotide, ranging from 0.60% to 5.74%, which consisted of substitutions and insertions/ deletions. The frequency of RNA editing ranged from 0.60% (*coxI*) to 5.47% (*18SrRNA*) in the eight mitochondrial genes studied, which are agreeable to those reported earlier (Giege and Brennicke, 1999; Picardi et al, 2010), and similar in-consistency of editing frequency across these eight genes as a function of gene length, which is also reported in earlier studies in *Arabidopsis*, *Brassica* and *Beta* (Mower and Palmer, 2006). Abundance of base substitution (74.2%) type of post transcriptional editing at multiple positions in transcripts of the rice mitochondria is consistent with the earlier observations in several monocotyledonous and dicotyledonous plant mitochondrial genes (Schuster et al, 1990). This result has suggested the existence of apparent coding anomalies in rice mitochondria. When we compared the RNA editing sites between Pusa 6B and PRR78, there were 21 editing sites in four genes namely, *cobII* (11), *coxIII* (6), *atp6* (3) and *rps3* (1), which were identical in both the fertile lines but absent in sterile line Pusa 6A, for which further analysis is required to validate this editing sites with the male sterility mechanism. Since, alteration of RNA editing in CMS lines compared to other normal cytoplasm is not always necessarily the mechanism of CMS in plants (Horn et al, 2014). A relevant example in rice is that the presence of *WA352* gene, whose protein interacts with the nuclear encoded mitochondrial protein COXII, triggers premature tapetal programmed cell death and consequent pollen abortion (Luo et al, 2013) and hence male sterility in the WA CMS system, which is in contradict to Das et al (2010) who had earlier showed that an unedited *orfB* transcript as molecular cause for this CMS system in rice and co-segregated with the male sterility. RNA editing is also influenced by many factors, such as tissue type, organ type, developmental stage, nucleo-cytoplasmic interactions and nuclear background (Grosskopf and Mulligan, 1996; Zehrmann et al, 2008) as well as by environmental stress (Liew et al, 2017). Such kinds of phenomena are also reported by Hu et al (2013), which indicated that nucleo-cytoplasmic interactions affect the RNA editing of *cox2*, *atp6* and *atp9* transcripts. Therefore, further analysis and characterization of these potential RNA editing sites in candidate mitochondrial genes is required to identify the mitochondrial gene responsible for male sterility.

Partial/incomplete editing in mitochondrial transcripts

Presence of multiple digested fragments of RT-PCR products of five mitochondrial genes namely *atp9*, *coxIII*, *rps3*, *18SrRNA* and *cobII* during the CAPS analysis, showed multiple sites of RNA editing. However, there was discrepancy between the number of editing sites revealed by sequencing and that based on CAPS analysis. For instance, in case of *cobII* gene, one edited sites corresponding to restriction enzyme target site (AATT) for restriction enzyme, *Tsp*509I based on sequence analysis was predicted. However, digestion of the same amplicon using the same restriction enzyme *Tsp*509I leads to four fragments and mixed of smaller fragments as compared to two digested fragments expected. This might be due to occurrence of incomplete/partial editing of gene transcripts and sequencing only one cloned RT-PCR product that was sequenced which do not represent the whole populations of differentially edited transcripts. An editing site of a particular gene is considered as partially/incompletely edited when the site is not edited in all the transcript pool. It is well known that partially/incompletely edited transcripts do exist in plant mitochondria (Schuster et al, 1990; Mower and Palmer, 2006; Zehrmann et al, 2008), which were represented in the RT-PCR products that were digested. While sequence comparison of genomic and multiple cDNA clones of *Nadh3* gene in *Oenothera* mitochondrion, Schuster et al (1990) found none of the cDNA clones were completely edited for all potential editing sites, suggesting that such completely edited transcripts might be rare as compared to abundant of mixture transcripts edited partially. Abundance of partial editing process is further shown by sequence comparison of *Vitis venefera* mitochondrion through RNAseq which detects 76% of editing sites as partial editing, of which 28% is found to be tissue specific (Picardi et al, 2010). They further suggested that partial editing was derived from incomplete editing transcripts as well as partial edited from mixed tissue sample. Therefore, the discrepancy of editing sites in these studies might be due to failure of potential partial editing site detection because of restriction of single cDNA clone sequencing thereby precluding multiple partially edited transcripts from the transcript pool and assessment of tissue specificity of editing sites.

REFERENCES

Benne R, van den Burg J, Brakenhoff J P, Sloof P, van Boom J H, Tromp M C. 1986. Major transcript of the frameshifted *coxII* gene from trypanosome mitochondria contains four nucleotides that are not encoded in the DNA. *Cell*, **46**(6): 819–826.

Cattaneo R. 1991. Different types of messenger RNA editing. *Annu Rev Genet*, **25**: 71–88.

Chakraborty A, Mitra J, Bhattacharyya J, Pradhan S, Sikdar N, Das S, Chakraborty S, Kumar S, Lakhanpaul S, Sen S K. 2015. Transgenic expression of an unedited mitochondrial *orfB* gene product from wild abortive (WA) cytoplasm of rice (*Oryza sativa* L.) generates male sterility in fertile rice lines. *Planta*, **241**: 1463–1479.

Chateigner-Boutin A L, Ramos-Vega M, Guevara-Garcia A, Andres C, de la Luz Gutierrez-Nava M, Cantero A, Delannoy E, Jiménez L F, Lurin C, Small I, León P. 2008. CLB19, a pentatricopeptide repeat protein required for editing of *rpoA* and *clpP* chloroplast transcripts. *Plant J*, **56**(4): 590–602.

Covello P S, Gray M W. 1989. RNA editing in plant mitochondria. *Nature*, **341**: 662–666.

Covello P S, Gray M W. 1990. Differences in editing at homologous sites in messenger RNAs from angiosperm mitochondria. *Nucl Acids Res*, **18**(17): 5189–5196.

Das S, Sen S, Chakraborty A, Chakraborti P, Maiti M K, Basu A, Basu D, Sen S K. 2010. An unedited 1.1 kb mitochondrial *orfB* gene transcript in the wild abortive cytoplasmic male sterility WA-CMS system of *Oryza sativa* L. subsp. *indica. BMC Plant Biol*, **10**: 39.

Dewey R E, Levings C S, Timothy D H. 1986. Novel recombinations in the maize mitochondrial genome produce a unique transcriptional unit in the *Texas* male sterile cytoplasm. *Cell*, **44**(3): 439–449.

Doyle J J, Doyle J L. 1990. Isolation of plant DNA from fresh tissue. *Focus*, **12**: 13–15.

Giege P, Brennicke A. 1999. RNA editing in *Arabidopsis* mitochondria effects 441 C to U changes in ORFs. *Proc Natl Acad Sci USA*, **96**: 15324–15329.

Graves P V, Bégu D, Velours J, Neau E, Belloc F, Litvak S, Araya A. 1990. Direct protein sequencing of wheat mitochondrial ATP-synthase subunit 9 confirms RNA editing in plants. *J Mol Biol*, **214**(1): 1–6.

Gray M W, Hanic-Joyce P J, Covello P S. 1992. Transcription, processing and editing in plant mitochondria. *Annu Rev Plant Physiol Plant Mol Biol*, **43**: 145–175.

Gray M W, Covello P S. 1993. RNA editing in plant mitochondria and chloroplasts. *FASEB J*, **7**: 64–71.

Grosskopf D, Mulligan R M. 1996. Developmental and tissue specificity of RNA editing in mitochondria of suspension cultured maize cells and seedlings. *Curr Genet*, **29**(6): 556–563.

Handa H, Gualberto J M, Grienenberger J M. 1995. Characterization of the mitochondrial *orfB* gene and its derivative, *orf224*, a chimeric open reading frame specific to one mitochondrial genome the *Polima* CMS in rapeseed *Brassica napus* L. *Curr Genet*, **28**(6): 546–552.

Hanson M R, Sutton C A, Lu B W. 1996. Plant organelle gene expression: Altered by RNA editing. *Trends Plant Sci*, **1**(2): 57–64.

Hernould M, Suharsono S, Litvak S, Araya A, Mouras A. 1993. Male sterility induction in transgenic tobacco plants with an unedited *atp9* mitochondrial gene from wheat. *Proc Natl Acad Sci USA*, **90**(6): 2370–2374.

Hiesel R, Wissinger B, Schuster W, Brennicke A. 1989. RNA editing in plant mitochondria. *Science*, **246**: 1632–1634.

Horn R, Gupta K J, Colombo N. 2014. Mitochondrion role in molecular basis of cytoplasmic male sterility. *Mitochondrion*, **19**: 198–205.

Howad W, Kempken F. 1997. Cell type-specific loss of *atp6* RNA editing in cytoplasmic male sterile *Sorghum bicolor. Proc Natl Acad Sci USA*, **94**: 11090–11095.

Hu J H, Yi R, Zhang H Y, Ding Y. 2013. Nucleo-cytoplasmic interactions affect RNA editing of *cox2*, *atp6*, and *atp9* in alloplasmic male sterile rice (*Oryza sativa* L.) lines. *Mitochondrion*, **13**(2): 87–95.

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**(4): 1437–1446.

Ji J J, Huang W, Li D W, Yin Y X, Chai W G, Gong Z H. 2014. A CMS-related gene, *Ψatp6-2*, causes increased ATP hydrolysis activity of the mitochondrial F1Fo-ATP synthase and induces male sterility in pepper (*Capsicum annuum* L). *Plant Mol Biol Rep*, **32**(4): 888–899.

Kianian P M A, Kianian S F. 2014. Mitochondrial dynamics and the cell cycle. *Front Plant Sci*, **5**: 222.

Liew Y J, Li Y, Baumgarten S, Voolstra C R, Aranda M. 2017. Condition-specific RNA editing in the coral symbiont *Symbiodinium microadriaticum*. *PLoS Genet*, **13**(2): e1006619.

Liu Z L, Xu H, Guo J X, Liu Y G. 2007. Structural and expression variations of the mitochondrial genome conferring the WA type of CMS in rice. *J Integr Plant Biol*, **49**(6): 908–914.

Luo D P, Xu H, Liu Z L, Guo J X, Li H Y, Chen L T, Fang C, Zhang Q Y, Bai M, Yao N, Wu H, Wu H, Ji C H, Zheng H Q, Chen Y L, Ye S, Li X Y, Zhao X C, Li R Q, Liu Y G. 2013. A detrimental mitochondrial-nuclear interaction causes cytoplasmic male sterility in rice. *Nat Genet*, **45**(5): 573–577.

Mower J P, Palmer J D. 2006. Patterns of partial RNA editing in mitochondrial genes of *Beta vulgaris*. *Mol Genet Genomics*, **276**(3): 285–293.

Ngangkham U, Parida S K, De S, Kumar A R, Singh A K, Singh N K, Mohapatra T. 2010. Genic markers for wild abortive WA cytoplasm based male sterility and its fertility restoration in rice. *Mol Breeding*, **26**: 275–292.

Picardi E, Horner D S, Chiara M, Schiavon R, Valle G, Pesole G. 2010. Large-scale detection and analysis of RNA editing in grape mtDNA by RNA deep-sequencing. *Nucleic Acids Res*, **38**(14): 4755–4767.

Schuster W, Hiessel R, Wissiinger B, Brennicke A. 1990. RNA editing in the cytochrome b locus of the higher plant *Oenothera berteriana* includes a U to C transition. *Mol Cell Biol*, **10**(5): 2428–2431.

Smith H C, Gott J M, Hanson M R. 1997. A guide to RNA editing. *RNA*, **3**(10): 1105–1123.

Stuart K, Allen T E, Heideman S, Seiwert S D. 1997. RNA editing of kinetoplast protozoa. *Microl Mol Biol Rev*, **61**(1): 105–120.

Thiel T, Kota R, Grosse I, Stein N, Graner A. 2004. SNP2CAPS: A SNP and INDEL analysis tool for CAPS marker development. *Nucleic Acids Res*, **32**(1): 5.

Wu B, Chen H M, Shao J J, Zhang H, Wu K, Liu C. 2017. Identification of symmetrical RNA editing events in the mitochondria of *Salvia miltiorrhiza* by strand specific RNA sequencing. *Sci Rep*, **7**: 42250.

Young E G, Hanson M R. 1987. A fused mitochondrial gene associated with cytoplasmic male sterility is developmentally regulated. *Cell*, **50**(1): 41–49.

Yu J H, Zhao Y X, Qin Y T, Yue B, Zheng Y L, Xiao H L. 2013. Discovery of microRNAs associated with the S type cytoplasmic male sterility in maize. *J Integr Agric*, **12**(2): 229–238.

Zehrmann A, van der Merwe J A, Veritskiy D, Brennicke A, Takenaka M. 2008. Seven large variations in the extent of RNA editing in plant mitochondria between three ecotypes of *Arabidopsis. Mitochondrion*, **8**(4): 319–327.

(Managing Editor: WANG Caihong)