Comparative analysis of mitochondrial genomes of maize CMS-S subtypes provides new insights into male sterility stability

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Abstract

Background: Cytoplasmic male sterility (CMS) is a trait of economic importance in the production of hybrid seeds. In CMS-S maize, spontaneous revertants appear frequently in field-grown female populations where only male-sterile plants were expected. Loss of sterility conferring regions or other rearrangements in the mitochondrial genome were reported to associate with these reversions. However, the relationship about mitochondrial function and sterility stability is largely unknown.

Results: Here we reported different spontaneous reversion rates between two CMS-S subtypes, one with high rate of reversion to fertility (CMS-Sa) and another with more stable sterility (CMS-Sb). Through next-generation sequencing, we assembled and compared mitochondrial genomes of two CMS-S subtypes. Phylogenetic analysis revealed strong similarity between two mitochondrial genomes. The sterility associated regions, S plasmids, and terminal inverted repeats (TIRs) were intact in both. Two subtypes maintained high level of sterility gene orf355 transcription in anther tissue. Majority of the functional genes are identical in both amino acid and nucleotide sequence in both subtypes, except for that of *NADH dehydrogenase subunit 1* (*nad1*). In the mitochondrial genome of CMS-Sb, 3.3-kilobase sequence was lost in the 17-kb repeat region, leading to the elimination of one copy of *nad1* exon1. Consequently, there are two copies of *nad1* exon1 in CMS-Sa, but only one copy in CMS-Sb. During pollen development, obvious induction of *nad1* transcription and mitochondrial biogenesis were observed in anther of CMS-Sa, but not in anther of CMS-Sb. We suggested that the impaired mitochondrial function in anther of CMS-Sb is associated with the enhanced sterility stability.

Conclusions: Comprehensive analysis revealed the sequence diversity in copy number of mitochondrial encoding gene *nad1* in two subtypes of CMS-S maize. This copy number shift affects the gene transcription and organelle biogenesis of anther tissue, and is associated with different spontaneous sterility reversion. Our study suggests the mechanism that the robustness of mitochondrial function underlying the sterility stability of CMS-S maize.

Background

Cytoplasmic male sterility (CMS) is the maternally inherited inability of plants to produce viable pollen, whereas plant growth and female fertility is normal [1, 2]. There are three major groups of CMS in maize, CMS-C, CMS-T, and CMS-S, which is defined according to the sterilizing factors in mitochondria and the corresponding main restorers in nucleus. Maize CMS is an economically valuable trait used for the production of hybrid seeds. CMS-T system was first used in three-line system technology to produce hybrid maize, which accounted for about 85% of U.S. hybrid seed until the 1970 epidemic of southern corn leaf blight [3, 4]. Since only one single major restorer (*Rf3*) is required for pollen fertility in the S-system, breeder switched to CMS-S to produce hybrid maize.

CMS-S maize have a clear elucidated mitochondria-nucleus interaction system, where the sterilizing factor orf355 initiates microspore degeneration while a single main restorer Rf3 cleaves orf355

transcripts to restore fertility. Mechanically, CMS-S mitochondria have two linear plasmids called S1 and S2, which have exactly the same terminal inverted repeats (TIRs) as that in CMS-associated region. These plasmids actively recombine with the circular genome to linearizing the mitochondrial DNA (mtDNA) [5–8]. The sterilizing factor orf355 locates just at the near end of the linearized mtDNA and expresses at bi-cellular stage of microspores development, finally leading to the gametophytic sterility of maize [9, 10]. In the presence of Rf3, the 1.6-kb transcripts containing orf355 are cleaved via posttranscriptional modification, leading to the reduction of transcripts level and fertility restoration [11].

However, CMS-S maize is a relative unstable system where revertants are reported to arise in real time through genetic mutation [12]. Although the reversions usually take place in a single floret or a small sector in the tassel, this would potentially reduce the purity of hybrid seeds and hinder the utilization of CMS-S. Spontaneous fertility reversions usually take place in absence of main restorer Rf3. For example, nuclear-encoded restorer Rf9 does not cause the cleavage of 1.6-kb transcripts but decreases the abundance of the linearized transcription mtDNA template [8]. In addition, loss of the free S1 and S2 plasmids of mitochondria blocks the rearrangement with the sterility-associated region, causes fertility reversion in the CMS-S maize [9]. In some case, mtDNA rearrangement disrupts the sterility associated region such that the sterilizing factor either was lost or cannot be expressed. For example, a 7.3-kb inversion in mtDNA separates the TIR sequences from the CMS-associated region in some special revertants. As a result, orf355 is no longer transcribed from mtDNA linear ends but co-transcribed with cox2. Although orf355 transcripts can still be detected in the plant, they are not highly expressed and no longer initiated the sterility of the plant [13]. Furthermore, the *restorer-of-fertility lethal 1* (*rfl1*) mutant disrupts mitochondrial gene expression and the accumulation of α-subunit of the ATP synthase (ATPA), which link the functional plasticity of mitochondria with the spontaneous reversion in CMS-S [14].

Maize mitochondrial genome is about 500kb in size, containing a suite of relatively conserved protein-coding genes within different cytotypes [15]. However, the relative placement of the genes and the intergenic spacer regions within the mitochondrial genome vary extensively among different subgroups of maize [16]. A major reason for the highly variable structural organization of the maize mtDNA is the abundance of recombination-active repeated sequences [17]. The shuffling of mtDNA sequences by recombination plays a role in evolution, changing gene organizations and creating gene chimeras [16]. All the three types of CMS in maize, as well as CMSs reported in other species to date, are caused by chimeric ORFs that generated from mtDNA rearrangement. Mitochondrial rearrangements associated with the loss of portions of essential genes cause poor growth or lethality in maize [18, 19]. On the other hand, rearrangements affecting only noncoding regions of mtDNA are usually neutral, but sometimes cause different kind of phenotype, such as fertility reversion in CMS-S maize. Maize mitochondrial genomes have a special type of DNA sequence that acquired from plastid, which accounts for 4.4% of CMS-S mitochondrial genome [20]. The plastid-derived DNA sequences change rapidly in content and location among five maize mitochondrial genomes [15]. However, the relationship about plastid-derived DNA sequence and recombination is not clear.

On the other hand, mitochondria is a dynamic organelle that modulate its function and biogenesis in respond to fluctuating energy demand triggered by developmental signals and environmental stimuli [21]. This key property is referred as mitochondrial robustness, and has important implication for understanding why severe mutations of mitochondrial can be compatible with life or only manifest in specific tissue. Study in animal suggests that the tissue-specific control coefficients of different respiratory chain complexes counteract the influence of mitochondrial, probably vice versa [22]. Plant male sterility might be the outcome of inability of mitochondria to meet the increasing energy demand of microspore development [2], but the relationship between mitochondrial robustness and sterility determination is largely unknown. Here we described a special mitochondrial genome of CMS-S subtype that retained an intact orf355/orf77 region but lost the second copy of *nad1*- exon1. This Stoichiometric change reduced the mitochondrial biogenesis and genes transcription in anther tissue, which render low spontaneous reversion rate of the sterile plant.

Materials And Methods

Plant Material and growth condition

The subtype CMS-Sa and CMS-Sb were collected independently from different breeders, and sterility of both of them can be counteracted by the Rf3 restorer. Jing72464 is the female parent of elite hybrid line in China, which was developed by the Maize Research Institute, Beijing Academy of Agricultural and Forestry Science. Both subtypes were introduced into Jing72464 to develop the sterile inbred. By crossing two cytoplasm donors with the fertile Jing72464 (NB), the sterile F_1 was obtained, which were consecutively backcrossed with Jing72464 for more than ten generation. Molecular markers were applied to select the Jing72464 nuclear background in each backcross generation, which made the sterile and partially rescued families as near isogenic lines. The plant materials were planted in the Hainan maize propagation base in Yacheng, Hainan (HN-YC, 18.3°N, 109.5°E) and Beijing farms (40.1° N, 116.4° E).

Revertants screen

Fertility survey was conducted in Beijing farms (40.1° N, 116.4° E) in summer of 2021. During flowering time, we searched every plant for exerted anther every day. The plants with even only one exerted anther in any sector of the tassels were designated as “spontaneous revertants” in this study. Practical observation indicated that majority of these plant cannot acutely revert to fertility, although most of the exerted anthers exhibited small portion of I_2-KI stainable pollen. The starch granule in these pollens implied the potential of germination, and represented the partial recovery of mitochondrial function in the supposed sterile pollen. In addition, the exerted anthers in CMS-Sa usually appeared several days after natural pollination, which cannot meet the functional silk to complete fertilization.

Pollen viability test

Pollen viability was checked using the Iodine-potassium Iodide staining method. The pre-exerted anthers were cut with tweezers in 50 µl of I_2-KI solution on the top of glass slide, after that large debris was

carefully removed. The stained pollens without cover glass were directly observed under a light microscope at 20× magnification. At least 100 pollen grains per visual field were counted randomly under the light microscope. The successful seed set was used as the basis for tests of pollen fertility. Pollen from the exerted anther were carefully collected and crossed onto ears of CMS-Sb Jing72464 inbred plants to confirm the pollen fertility. In some cases, anthers were cut with tweezers and the pollen was applied directly to filaments. Progeny families from the above tests were planted and checked the fertility to see whether the restoration of pollen fertility can transmit from pollen.

Library construction and sequencing

Mitochondrial DNA was extract according to previous study [23]. Ten-days-old etiolated seedling were homogenized in the homogenization buffers (0.4 M mannitol, 10 mM TES pH 7.5, 5 mM EGTA, 0.05% cysteine, 0.2% BSA) in blender, then filtered with four layers cheesecloth and one layer Miracloth. Crude mitochondria were obtained from differential centrifugation (1000g, 5 min; 2000g, 10min; 10000g, 20min). The crude mitochodnrial pellets were suspended in 2 ml homogenization buffer, then purified using the sucrose step gradients centrifugation. Sucrose step gradients were made with sterile 60%, 47%, 35%, and 20% sucrose solutions (in 10 mM tricine, 1 mM EGTA, pH 7.5) in ultraclear tubes prior to starting the preparation. The resuspended mitochondria were carefully layered onto the sucrose gradients and centrifuge at 30,000 rpm in a SW41 rotor for 60 minutes at 4°C. The mitochondria were condesed at the interface between 35% and 47% layer. Collect the mitochondria and dilute with a total of 3 volumes of homogenization buffer. Purified mitochondria were obtained by centrifuging the supernatant at 10,000g for 30 minutes at 4°C. The DNeasy plant mini kit (QIAGEN, Hilden, Germany) was used to extract DNA from the mitochondrial fraction.

The libraries were constructed with TruSeq Nano DNA LT Sample Prepararion Kit (Illumina, San Diego, CA, USA). Briefly, the mitochondrial DNA was sheared into fragments with length ~ 350 bp using S220 Focusedultrasonicators (Covaris, USA). Adapters were ligated onto the 3’ end of the sheared fragments. After PCR amplification and purification, the final libraries were sequenced on the Illumina sequencing platform HiSeq X Ten platform (Illumina Inc., San Diego, CA, USA) and 150 bp paired-end reads were generated. The sequenced data was de novo assembled using ABySS with different k-mer values (k = 31 to 127, step by 2 each time) to choose the best k-mer value of assembly. To verify the quality and accuracy of our assemblies, the original reads were mapped to the corresponding mitogenomes using BWA, and then Pilon was used to polish the final assembly. Mitochondrial protein-coding genes were predicted using the GeSeq, and then we manually revised the start/stop codons and the exon-intron boundaries of genes. We investigated the GC content with a shell script and visualized the circular physical map of all mitogenomes using OGDRAW. The Synteny and SNP analysis of different mitogenomes were analyzed by MUMmer.

Quantification of mitochondrial DNA levels

Mitochondrial DNA in maize seedlings were quantified as previously described study with slight modifications [24]. Total DNA was extracted from a pool of five primary root tips of maize seedlings, or

from the anthers that peeled form the pre-emerged tassels. A total of 40 ng of DNA was assessed by real-time PCR using a Light Cycler 480 (Roche). The reaction was performed in a final volume of 20 µl with SYBR green master mix (Roche) and 1.25 mM of the forward and reverse primers. Mitochondrial DNA levels in total DNA were measured by primer pairs C33-C34 and C35-C36, then normalized to the internal standard nucleus-encoded single-copy fragment C39-C40 as described previously[24]. The average of at least three technical repeats was used for each biological data point. Primer sequences are shown in Additional file 1: Table S1.

RNA Extraction and Expression Analysis

Whole seedlings with the first expanded true leaf were sampled from each subtypes materils. Anther tissue were carfully collected from the florets of pre-emerge tassels. Total RNA was isolated using the Plant RNA Purification Kit (Thermo Fisher Scientific, K0801, MA, US). Full-length cDNA was generated from 2 µg total RNA per sample using random primer (cDNA synthesis kit, Thermo Fisher Scientific, K1622). The resulting cDNA were made a 1:10 dilution for the following quantitative RT-PCR (qRT-PCR). qRT-PCR was conducted using TB Green® Premix Ex Taq™ II Real-Time PCR System (Takara, Japan, RR820A) in a typical 20 µL PCR mixture that included 10 µL of premix, 3 µL of template cDNA, and 0.4 µM of each PCR primer. Cycling conditions were 95°C for 2 min, followed by 40 cycles at 95°C for 20 s, 56°C for 1min, and 72°C for 30 s, and samples were run on the LightCycler 480 Real-Time PCR System (Roche, Basel, Switzerland). The melt curve data were utilized in every experiment to verify that the appropriate PCR product was being amplified. The mitochondria-ecoded gene *rrn18* was used as the internal control to monitor sample uniformity of initial RNA input and reverse transcription efficiency. The $2^{-\Delta\Delta Ct}$ method was used to calculate the expression level of mitochondrial genes. The relevant primer sequences are in Additional file 1: Table S1.

Circular RT-PCR

Circular RT-PCR analysis was conducted to determine the primary and processed transcripts of *nad1* and *nad2* in the 17-kb repeat region. The extracted RNAs were treated with RNA 5' polyphosphatase (NEB, USA, #M0356) prior to self-ligation. Reverse transcription of the circular cDNA were carried out using gene-specific primer. Two round PCR were conducted to amplified the sequence falking the 5’ and 3’ extremities. The product of second round PCR were seperated by agarose gel electrophoresis, and the bands of expected size were sequenced by cloning into vector. Detailed procedures were carried out according to previous work[25] and the manufacturer’s instructions. The primers used for reverse transcription and cRT-PCR amplifications are listed in Additional file 1: Table S1.

Results

Different spontaneous reversion rate in two subtypes of CMS-S maize

In the practice of hybrid breeding programs, two subtypes of CMS-S (designated as CMS-Sa and CMS-Sb) were used as the cytoplasm donors to generate sterile female parents of the elite hybrids maize (see method). In the analysis of 319 plants derived from subtype CMS-Sa, 188 of them had visible anther exerted from the florets. These anthers were smaller in size and usually hanged on the basal region of the tassels sectors after natural pollination (Fig. 1a-d). The spontaneous reversion rate in CMS-Sb was significantly lower than that of CMS-Sa, only 4 of the 270 progenies shown visible anther at the same stage. The anthers in subtype Sb were severely shrunken, implying no viable pollen in the locules (Fig. 1a-d). Pollen development was checked via I-KI staining method. Among the 178 analyzed revertants in CMS-Sa, majority (170/178) of them had variable proportion of stainable pollen (Fig. 1e). In contrast, none of the 4 revertants in subtype Sb had stainable pollen. To test the pollen viability of CMS-Sa, revertants were crossed as pollen parents onto ears of pollen-sterile testers. A total of 23 families were produced with considerable seed set, indicating the good viability of the normal pollen (Fig. 1f). Notably, natural pollination of these plants already finished at the time when the anther exerted from the floret, implying that the fertility reversion is partial and in rare cases these leaked pollens had a chance to meet the unfertilized silk to complete fertilization. Because the experimental materials were near isogenic lines, variation in fertility reversion is likely due to non-nuclear difference. To test that, 15 of the above 23 families with good seed set were grown for examination of pollen fertility. As expect, none of these checked plants were fertile or partial fertile, indicating no nuclear restorer allele transmit from the pollen to the next generation.

Mitochondrial genomes of two CMS-S subtypes

Because mitochondrial genome structure, genes organization, and linearization all affect sterility determination, mitochondrial genomes of the two CMS-S subtypes were sequenced from highly purified mitochondrial DNA. Previous studies demonstrated that majority of CMS-S genome is present in linear form [6], we depicted the assemblies of subtypes genomes in circular form for better illustration. The two newly sequenced CMS-S subtypes genomes shown high similarity with each other, and with the reference genome [15], but exhibited variable size with 557,050-bp in CMS-Sa and 553,762-bp in CMS-Sb (Fig. 2 and Additional File 2: Fig. S1). Variation of large repeats (> 0.5-kb) account for majority of the size differences between the mitochondrial genomes of Subtype CMS-Sa and CMS-Sb. There are 22 predicted large repeats in the mitochondrial genomes of CMS-S maize [15], the two newly sequenced mitochondrial genomes shared the same size and copy number of all repeats except the 17-kb repeat region termed R17. The first copy of R17 were identical among CMS-Sa, CMS-Sb, and the reference, whereas the second copy of R17 eliminated 3.3-kb sequence in the internal region from the main mitochondrial genomes of CMS-Sb (Additional File 3: Fig. S2 and Additional File 4: Fig. S3). Majority of the functional genes are identical in both amino acid and nucleotide sequence in both two subtypes except *apocytochrome b* (*cob*) and *NADH dehydrogenase subunit 1*. The *cob* gene of CMS-Sa has a T to G substitution at position + 312, predicting an amino acid change from phenylalanine to leucine. There are two copies of *nad1* exon1 in CMS-Sa, but only one copy of *nad1* exon1 in CMS-Sb that due the 3.3-kb sequence.

Nucleotide substitutions mainly occurred in the plastid-derived sequence in newly sequenced CMS-S subtypes

There are 117 and 104 nucleotide substitutions in CMS-Sa and CMS-Sb in alignment with the reference genome, respectively. The nucleotide substitution frequency in CMS-Sa is higher than that of CMS-Sb. For CMS-Sa, the value is 2.1 substitutions/10,000 bp, and for CMS-Sb it is 1.88 (Additional file 5: Table S2). The frequency of indels in plant mitochondrial genomes is thought to be lower than the frequency of substitutions [15]. Consistently, the two new sequenced mitochondrial genomes have low ratio of indels to substitutions: each has 0.17 and 0.15 indels/substitution relative to reference, respectively. More than half of the indels have a length of five nucleotides, and length variation in SSR account for 41% of the total indels (Additional file 6: Table S3 and Additional file 7: Table S4).

CMS-Sa and CMS-Sb shared 92 identical nucleotide substitutions in comparison with the reference genome. These substitution sites were not evenly distributed across the genome, but were clustered to several hot spot regions (Fig. 3a). Notably, majority of these hot spots were sequences derived from plastid (mitochondrial plastid DNAs: *mtpt*). For example, a 3658-bp *mtpt* sequence is present in the mitochondrial genome of both subtypes, representing 22 substitutions in comparison with the reference. However, the corresponding 3681-bp homolog sequences in plastid were almost identical among CMS-Sa, CMS-Sb, and the reference plastid genome (NC_001666.2). To eliminate the possible interference of plastid genome during sequencing and assembly, we amplified these *mtpt* sequences from mitochondrial genome and plastid genome using suites of specific primers. Results indicated that *mtpt* sequences in the newly sequenced CMS-Sa and MS-Sb genomes were different from their ancestral homolog sequences in plastid. Thus, nucleotide substitutions actually taken placed in the mitochondrial genomes of CMS-Sa and CMS-Sb, and were more likely to occur in plastid-originated sequence. The mitochondrial substitutions rate in *mtpt* sequence was about 42.3 and 42.8 substitutions/10,000-bp in Sa and Sb, respectively, which were significantly higher than the rate in non-*mtpt* sequence (Fig. 3B). Majority of the clustered substitution sites surrounded the functional plastid-originated *trn* gene coordinates.

Transcript level of orf355 in two CMS-S subtypes

We next focus on the sterility associated region between two CMS-S subtypes. In CMS-S mitochondrial genome, there are two copies of 4.2-kb repeat (R4.2) containing orf355-orf77 sequences. The second copy of R4.2 that downstream of cox2 recombined with S plasmid to yield linearized CMS-S genomes, where the sterilizing 1.6-kb RNA transcribed from [8]. Previous studies demonstrated that expression of *orf355-orf77* is associated with the male sterility of maize CMS-S. Sequencing of the coding region revealed that the two subtypes shared the identical DNA sequence in R4.2 (Fig. 2). Most spontaneous reversions in CMS-S maize are associated with the integrity of free S plasmid and TIR sequence of main mitochondrial genome. However, the newly sequenced subtype CMS-Sa and CMS-Sb shared identical intact S1, S2 and TIR sequence with the reference genome. We next checked the transcript level of *orf355* and *orf77* in the leaf and anther of the two accessions in normalization against a mitochondrial rRNA gene, *rrn18*. A quantitative RT-PCR experiment demonstrated that the *orf355* transcript levels significantly

increased in anther of both subtypes compared with those of leaves. In addition, transcript abundance of orf355 was even slightly lower in CMS-Sb than that of CMS-Sa (Fig. 4). Thus, other beside the sterilizing factor may also influence the stability of sterility in CMS-S maize.

Similar post-transcriptional process in R17 region was observed in CMS-Sa and CMS-Sb.

The 3.3-kb deletion in the second copy of R17 accounted for the major differences between two CMS-S subtypes. R17 contains several functional genes, including five tRNA genes, the first exon of *NADH dehydrogenase subunit* (*nad1*), and the fourth and fifth exon of *nad*2. Notably, a 411-bp *mtpt* sequence located within the intergenic region between *nad*2-exon5 and *nad*1-exon1. The observed deletion in CMS-Sb taken place between the *mtpt* sequence and *trnP*, leading to the elimination of *nad*1-exon1 (Fig. 5). In maize mitochondrial genome, *nad*1 and *nad*2 genes were separated by group II introns (Fig. 6a). Mature functional RNA is generated through posttranscriptional modification of the corresponding precursors [20]. Previous study using NB mitochondrial maize illustrated that *nad*1-exon1 is co-transcribed with the upstream tRNAs to generate the polycistronic precursor RNAs [25]. Following endonucleolytic cleavages at specific sites adjacent to tRNAs, mature RNA of *nad*1-exon1 were released. R17 is present in all cytotype of maize but CMS-T [15], which implies that CMS-S probably have identical transcripts to those of NB maize. We performed Circular RT-PCR (cRT-PCR) to explore the post-transcriptional process of gene in R17. Result revealed that *nad*1-exon1 were processed downstream of *trn*P to generate the 5′ terminus and upstream of *mtpt* sequence to generate the 3′ terminus (Fig. 6b). Due to intact of the first copy of R17, no difference was observed in the fragment length and processing site of cRT-PCR products between two subtypes (Fig. 6b and Additional file 8: Fig. S4a). Similarly, cRT-PCR analysis only identified transcripts that flanking nad2-exon3, exon4, and exon5 adjacent to the first copy of R17. No nad2 transcripts that specific to the second copy of R17 were identified (Fig. 6c-d and Additional file 8: Fig. S4b). Our results identified no obvious differences in post-transcriptional process between CMS-Sa and CMS-Sb.

The sterility-stable subtype CMS-Sb displayed reduced mitochondrial function in anther as compared CMS-Sa

Mitochondrial function increases in anther to meet the large energy demand required for pollen development. We quantified the DNA amount ratio between the mitochondrial genomes and the nuclear genome to estimate average mitochondrial copy numbers per cell in different tissues. Quantitative real-time PCR (qRT-PCR) results suggested more mitochondrial genome copies per cell on average in the anther tissues than that of seedlings (Fig. 7a). However, mitochondrial copy number in anthers of CMS-Sb was lower than that of CMS-Sa, implying the impairment of mitochondrial biogenesis (Fig. 7a). We found correlation between mitochondrial biogenesis and genes transcription levels during pollen development. Transcript levels of most mitochondrial encoding genes significantly increased in anthers of CMS-Sa, but were not so much inducted in anthers of CMS-Sb (Fig. 7d). For example, transcripts levels of *nad1* and *nad2* were more than four times higher in anther of CMS-Sa than those of seedlings, whereas no obvious differences between anther and seedling tissues were seen in CMS-Sb (Fig. 7b, c).

These results suggested that lower mitochondrial function in CMS-S maize during pollen development enhances the stability of sterility.

Discussion

The CMS-S maize sterilizing gene orf355 is cytotoxic to the plant cell, prokaryotic expression of which has been previously shown to be lethal to host cells. Transcription of orf355 increases specifically in anther tissue, leading to the deterioration of mitochondrial function[10]. On the other hand, mitochondria is a dynamic organelle that modulate its function and biogenesis in respond to fluctuating energy demand triggered by developmental signals and environmental stimuli [21]. Plant microspore genesis is a high-energy-demanding process, but ATP required for pollen development must be supplied mainly by mitochondria, which due to the non-photosynthetic feature[26]. The mitochondrial biogenesis increased in reproductive cells with a 20 to 40 fold induction in copy number in some anther cells in maize [27]. And the expression levels of several mitochondrial genes increase in maize microspores accompany with the amplification of mitochondrion number [26]. We can speculated that the tissue-specific control of mitochondrial function counteract the influence of CMS-S gene orf355. The finally degeneration of microspores might be the outcome of inability of mitochondria to counteract the deleterious effect of orf355.

Robustness is a key property of mitochondria, and has important implication for understanding why severe mutations of mitochondrial can be compatible with life or only manifest in specific tissue[21]. Studies in animals proposed working model to explain such phenomenon[22]. Generally, metabolic expression of the defect in oxidative phosphorylation (OXPHOS) complexes presents a biochemical threshold. The threshold value varies according to the tissue and specific defects. Thus, in a given tissue with high biochemical threshold, even a low quantity of normal mitochondrial can suffice to maintain a normal level of oxidative phosphorylation. Conversely, if in another tissue with low biochemical threshold, this minute decrease in OXPHOS induce mitochondria collapse[22]. This hypothesis can well explain the spontaneous sterility reversion in CMS-S maize. In sterile pollen, the mitochondrial function inhibited by orf355 did not reach the threshold required for normal pollen development. However, mitochondrial function varies among tissues and even among different cells of the same tissue. Thus, anthers with higher mitochondrial function that reach the threshold for normal pollen development can reverse to fertility. Spontaneous sterility reversion in maize CMS-S population probably reflects the fluctuation of mitochondrial function during pollen development.

All reported maize mitochondrial genomes contain a basic suite of functional genes encoding component of macromolecular complexes, whereas the copy number of individual genes or exons varies among cytotypes [15, 20]. Variation in copy number of these essential OXPHOS genes would affect the transcription. We observed higher mitochondrial genes transcription in case of two copies of R17, but lower in one copy. A slightly alteration of the gene transcription would affect translation and efficient assembly of the complexes, ultimately affect the functional robustness of respiratory chain. Loss one copy of nad1 exon1 in CMS-Sb mitochondria might be sufficient to meet the energy requirements for

normal vegetative growth, however, potentially reduced the biochemical capacity in anthers during pollen development. Thus, this defect manifested only in specific situation such as pollen development where large amount of energy is required, however, was conform to the requirement of stable sterile female plants in hybrid seed production system (Fig. 8).

Another notable feature of the two newly sequenced mitochondrial genomes is the nucleotide substitution bias in plastid-derived sequence. One important feature of angiosperm mitochondrial genomes is the acquirement of foreign DNA from diverse sources, especially by intracellular gene transfer from the plastid [28–30]. Although most of the foreign plastid DNA resulted in non-functional sequences, growing body of evidence implied that the plastid-derived sequence play important role during plant development. In this study, we found higher nucleotides mutation rates in *mtpt* sequences than non-*mtpt* region. In addition, a 3.3-kb sequence is likely eliminated from the main genome with the help of the adjacent 411-bp *mtpt* sequence. An explanation for this result is that the *mtpt* region is recombinationally active and the transfer of plastid DNA into the mitochondrion is a relatively common event [20]. In this scenario, mtpt sequences recombined with homologous DNA from the plastid. In rare case replication of mtpt sequences may fail to utilize the recombinational repair machinery and periodically introduce mutations into the mitochondrial genome. In summary, our comparative analyses uncover the relationship between mitochondrial genes copy number shift and function plasticity, which provides new insights into male sterility stability of maize CMS-S.

Declarations

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Availability of data and materials:

The data that support the findings of this study have been deposited into CNGB Sequence Archive (CNSA)[31] of China National GeneBank DataBase (CNGBdb) with accession number CNP0002875.

Authors' contributions:

SX, WS and JZ conceived the study. SX and TN performed the experiment. AS and YZ assembled, annotated, and analyzed the genome. SX drafted the manuscript. JX and RZ provided input to the manuscript. SX and WS revised the manuscript. All authors read and approved the final manuscript.

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The authors declared that they have no competing interests.

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Figures

Figure 1

Different spontaneous reversion rate in two subtypes of CMS-S maize. (a) Pictures of tassel sectors on cytoplasm N, CMS-Sa, and CMS-Sb.

Scale bar, 1 cm. The lower left panel show full image of each tassel. Scale bar, 10 cm. The lower right panel show representative pollen grains that

stained with I-Ki solution in each subtypes. (b) Spontaneous reversion rate of CMS-S subtype Sa and Sb, plant with exerted anther in floret was

considered as spontaneous reverted individuals. (c) Image of anthers in CMS-Sa and CMS-Sb. Scale bar, 1 cm. (d) Anther size calculated using

Image J. Data are mean ± s.e.m. (n = 5). (e) Fertile pollen ratios, calculated as the ratio of darkly stained pollens versus the lightly stained pollens

in each plantlets. (f) Tests of pollen viability, as illustrated by seed set in ears pollinated using pollen from each subtypes.

Figure 2

Graphical genome maps display major difference between CMS-Sa and CMS-Sb. Both genomes have identical sequence of

4.2-kb repeat regions that contains the sterilizing gene orf355. The second copy of 17-kb repeat of CMS-Sb lost 3.3-kb sequence,

leading to the elimination of one copy of first exon of nad1. Yellow rectangles represent coding region of nad1. Repeat regions

sequences were labeled in brown color.

Figure 3

Nucleotide substitutions mainly occurred in the plastid-derived sequence in new sequenced CMS-S subtypes. (a) Mutation

bias in plastid-derived DNA sequences (mtpt) of CMS-S mitochondrial genome. Orange dots in upper panel represent nucleotides

mutation in each subtypes that compared with referee. Mtpt sequences are labeled in green color. Lower panel shows individual

nucleotide mutations in the representative 3658-bp mtpt sequence and chloroplast homolog among NB, CMS-Sa, and CMS-Sb. Mt,

mitochondrial ; Pt, plastid. (b) Mutation rate in mtpt and non-mtpt sequence of CMS-Sa and CMS-Sb, calculated as the number of

substitution or indel per 10,000 nucleotide.

Figure 4

Transcript level of sterilizing gene in CMS-Sa and

CMS-Sb. Transcript level of orf355 and orf77 in leaf and

anther in NB, CMS-Sa, and CMS-Sb. Data are mean ±

s.e.m. (n = 3). Different letters denote significant

differences determined by Tukey’s tests, $P < 0.05$.

Fig. 5

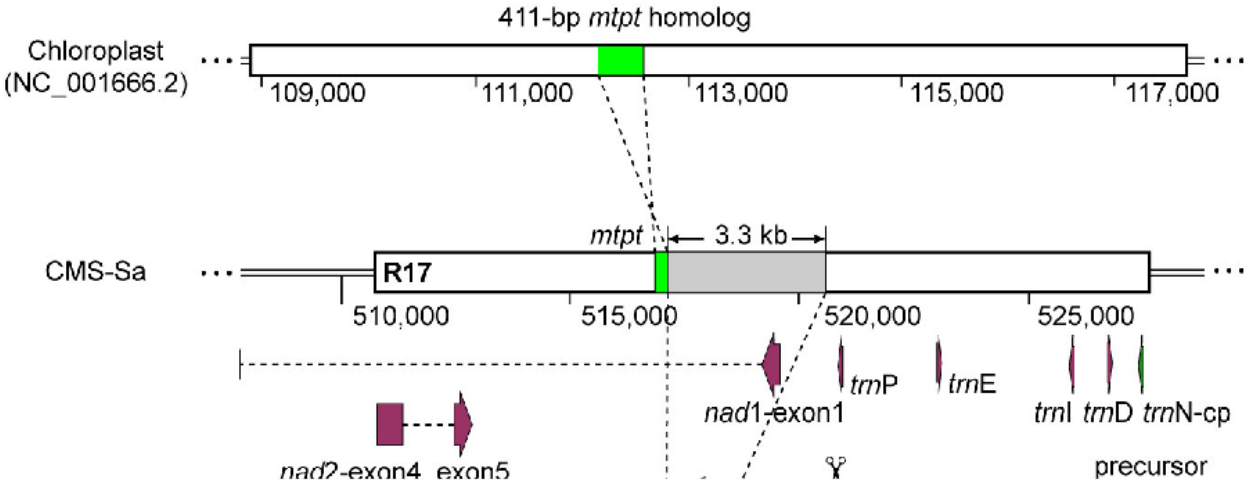

Figure 5

Schematic map of the second copy of 17-kb repeat in CMS-Sa and CMS-Sb. Coding genes are indicated by boxes of purple color. The mtpt sequence and its homolog in chloroplast genome are labeled in green color. The eliminated 3.3-kb sequence in CMS-Sb is labeled in gray color. The putative nad1 transcripts were cleaved at the sites adjacent to trnP to generate the 5′ end of precursor. 3′ end of nad1 precursor were tested at the sites adjacent to mtpt sequence.

Figure 6

Similar post-transcriptional process in R17 region was observed in CMS-Sa and CMS-Sb. (a) Diagrammatic representation

of nad1 and nad2 RNA transcripts that generated from post-transcriptionally processing of the precursor. (b and c) The cropped

gels of cRT-PCR products of nad1-transcripts 1 (B) and nad2-transcripts 2 (C). “+” indicated RNA samples treated with RNA 5'-

polyphosphatase. (d) Circular RT-PCR (cRT-PCR) in CMS-S revealed that nad1-exon1 were processed downstream of trnP to

generate the 5 terminus and upstream of mtpt sequence to generate the 3 terminus. No obvious difference was observed in the

fragment length and processing site of cRT-PCR products between subtype CMS-Sa and CMS-Sb, which due to the presence of

intact first copy of R17 is both genomes. cRT-PCR analysis revealed similar precursor of nad2-exon3, exon4, and exon5 that

flanking border of the first copy of R17. Consistent with previous work of Zhang et al, no specific precursor that flanking nad2-

exon4 and exon5 in the second copy of R17 were identified, which probably due to low abundance of this precursor .

Figure 7

The sterility-stable subtype CMS-Sb displayed reduced mitochondrial function in anther as

compared CMS-Sa. (a) Mitochondria biogenesis in leaf and anther of fertile, CMS-Sa, and CMS-Sb,

as illustrated by relative abundance of mitochondrial DNA versus nuclear DNA. Mitochondrial DNA

levels were measured by two independent primer pairs of mitochondria, then normalized to the

internal standard nucleus-encoded single-copy fragment. Data are mean ± s.e.m. (n = 6). (b and c)

Transcripts abundances of representative mitochondria encoding genes nad1 (B) and nad2 (C) of

Complex I. Abundances shown are relative to the level of CMS-sb (set to 1). Data are mean ±

s.e.m. (n = 3). (d) Relative transcripts levels of mitochondria encoding genes in leaf and anther

tissues. Transcripts levels were normalized to the minimum means among four groups (set to 1).

Data are mean ± s.e.m. (n = 3). The relative positions of genes as indicated by horizontal lines in

the mitochondrial schematic map of CMS-S.

Figure 8

A working model of spontaneous fertility reversion in CMS-S maize. Plant microspore genesis is a high-energydemanding

process, and the mitochondrial activity increases in anther to meet the energy required for pollen

development. Transcription of orf355 increases specifically in anther tissue, which inhibits mitochondrial function and

makes that below the threshold required for normal pollen development. However, mitochondrial activity varies among

tissues and even among different cells of the same tissue. In CMS-Sa, anthers with higher mitochondrial activity reach

the threshold for normal pollen development, can reverse to fertility. In CMS-Sb, Loss one copy of nad1 exon1

potentially reduces the mitochondrial robustness in anther tissue. Thus, this defect may be sufficient to meet the

energy requirements for normal vegetative growth, but manifests only in specific situation such as fertility reversion.

The blue arrow indicated the increase of mitochondrial activity from vegetative to reproductive developmental stage.

The dotted line indicated the threshold required for normal pollen development. The spring cartoon showed the

difference in mitochondria robustness of CMS-Sa and CMS-Sb.

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