Mitochondrial genomes organization in alloplasmic lines of sunflower (*Helianthus annuus*) with various types of cytoplasmic male sterility

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**Background.** Cytoplasmic male sterility (CMS) is a common phenotype in higher plants, which often is associated with rearrangements in mitochondrial DNA (mtDNA), and is widely used to produce hybrid seeds in a variety of valuable crop species. The CMS phenomenon investigations are also promote understanding of a fundamental issue of nuclear-cytoplasmic interactions in the ontogeny of higher plants. In the present study, we analyzed the structural changes in mitochondrial genomes of three alloplasmic lines of sunflower (*Helianthus annuus*). The investigation was focused on CMS line PET2, as there are very few reports about its mtDNA organization.

**Methods.** The NGS sequencing, *de novo* assembly, and annotation of sunflower mitochondrial genomes were performed. The comparative analysis of mtDNA of HA89 fertile line and two HA89 CMS lines (PET1, PET2) occurred.

**Results.** The mtDNA of the HA89 fertile line was almost identical to the HA412 line (NC_023337). The comparative analysis of HA89 fertile and CMS (PET1) analog mitochondrial genomes revealed 11852 bp inversion, 4732 bp insertion, 451 bp deletion and 18 variant sites. In mtDNA of HA89 (PET2) CMS line 77 kb translocation, 711 bp and 3780 bp deletions, as well as 1558 bp, 5050 bp, 14330 bp insertions were determined. There are also revealed 83 polymorphic sites sites in the PET2 mitochondrial genome, as compared with the fertile line

**Discussion.** Among the revealed rearrangements the 1558 bp insertion resulted in new open reading frames formation - *orf228* and *orf246*. The *orf228* and *orf246* could be the main reason for the development of PET2 CMS phenotype, whereas the role of other mtDNA reorganizations in CMS formation is negligible.
Mitochondrial genomes organization in alloplasmic lines of sunflower (Helianthus annuus) with various types of cytoplasmic male sterility

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Abstract

Background. Cytoplasmic male sterility (CMS) is a common phenotype in higher plants, that is often associated with rearrangements in mitochondrial DNA (mtDNA), and is widely used to produce hybrid seeds in a variety of valuable crop species. Investigation of the CMS phenomenon promotes understanding of fundamental issues of nuclear-cytoplasmic interactions in the ontogeny of higher plants. In the present study, we analyzed the structural changes in mitochondrial genomes of three alloplasmic lines of sunflower (Helianthus annuus). The investigation was focused on CMS line PET2, as there are very few reports about its mtDNA organization.

Methods. The NGS sequencing, de novo assembly, and annotation of sunflower mitochondrial genomes were performed. The comparative analysis of mtDNA of HA89 fertile line and two HA89 CMS lines (PET1, PET2) occurred.

Results. The mtDNA of the HA89 fertile line was almost identical to the HA412 line (NC_023337). The comparative analysis of HA89 fertile and CMS (PET1) analog mitochondrial genomes revealed 11852 bp inversion, 4732 bp insertion, 451 bp deletion and 18 variant sites. In the mtDNA of HA89 (PET2) CMS line we determined 27,5 kb and 106,5 kb translocations, 711...
bp and 3780 bp deletions, as well as, 5050 bp and 15885 bp insertions. There are also 83 polymorphic sites in the PET2 mitochondrial genome, as compared with the fertile line.

**Discussion.**

The observed mitochondrial reorganizations in PET1 resulted in only one new open reading frame formation (orfH522), and PET2 mtDNA rearrangements led to the elimination of orf777, duplication of atp6 gene and appearance of four new ORFs with transcription activity specific for the HA89 (PET2) CMS line – orf645, orf2565, orf228 and orf285. Orf228 and orf285 are the atp9 chimeric ORFs, containing transmembrane domains and possibly may impact on mitochondrial membrane potential. So orf228 and orf285 may be the cause for the appearance of the PET2 CMS phenotype, while the contribution of other mtDNA reorganizations in CMS formation is negligible.

**Introduction**

In plants, the phenomenon of cytoplasmic male sterility (CMS) stems from interaction between mitochondrial and nuclear genomes resulting in microsporogenesis disorders (Touzet & Meyer, 2014). All known natural CMSs, as well as most of the artificially obtained examples, are characterized by a special type of mitochondrial DNA (mtDNA) with numerous structural rearrangements as compared to the mtDNA of the fertile plants of the same species (Ivanov & Dymshits, 2007; Horn, Gupta & Colombo, 2014; Garayalde et al. 2015). The mitochondrial genomes of higher plants have comparatively large sizes with a multitude of noncoding and repetitive sequences that can result in the complex of sub-genomic structures (Chen & Liu, 2014). These features of plant mtDNA promote a large number of recombination events, leading to the appearance of new sequences and new open reading frames, that in turn often results in CMS development (Horn, Gupta & Colombo, 2014; Touzet & Meyer, 2014).

Natural CMS forms have been described in more than 150 species of flowering plants (Fujii & Toriyama, 2009). Most CMS sources in crops are obtained using interspecific hybridization. The first CMS in a sunflower was discovered by Leclercq (1969) in an interspecific hybrid between *Helianthus petiolaris* Nutt (PET1) and *Helianthus annuus*. Comparison of mitochondrial DNA organization of the fertile line and the male-sterile line carrying the PET1 cytoplasm revealed the presence of an 11-kb-inversion and 5-kb-insertion
These rearrangements of the mitochondrial genome produced a new open reading frame \( \text{orfH522} \) in the 3'-flanking region of the \( \text{atp1} \) gene encoding the alpha subunit of mitochondrial F1 ATPase. A new \( \text{orfH522} \) is co-transcribed with the \( \text{atp1} \) gene as a polycistronic mRNA (Moneger, Smart & Leaver, 1994). Using antibodies specific to the product of \( \text{orfH522} \) gene (16-kDa-protein), Horn et al. (1996) showed that this was the only difference between the mitochondrial translation products of fertile and CMS lines. The 16-kDa protein is synthesized in all tissues of a plant. It is embedded in the mitochondrial membranes and is believed to disrupt its integrity (Horn et al., 1996). Expression of \( \text{orfH522} \) in tapetum cells leads to premature apoptosis. Release of cytochrome C from the mitochondria, activates the proteolytic enzyme cascade, eventually leading to degradation of nuclear DNA and cell death (Balk & Leaver, 2001; Sabar et al., 2003). Interestingly, stable transgenic CMS tobacco lines carrying the \( \text{orfH522} \) gene were obtained (Nizampatnam et al., 2009). When dominant nuclear restorer gene (Rf) is present in the sunflower genome, fertility is restored due to the anther-specific lowering of the co-transcript of \( \text{orfH522} \) and the \( \text{atp1} \) gene (Moneger, Smart & Leaver, 1994; Horn, 2003). A possible mechanism leading to a reduction in the number of the chimeric \( \text{atp1-orfH522} \) transcripts by the restorer gene is polyadenylation of RNA matrices, causing accelerated degradation of RNA molecules by the ribonuclease (Gagliardi & Leaver, 1999).

The CMS-Rf system is widely used for the commercial production of F1 hybrid seeds for many important crops, including maize, sorghum, sunflower etc. (Liu et al., 2011, Bohra et al., 2016). Almost all commercial sunflower hybrids are currently based on a single source of CMS discovered by Leclercq (1969) and described above. Such genetic homogeneity of cultivated hybrids makes them extremely vulnerable to new virulent strains of the pathogens and can lead to negative phenomena, for example, epiphytotics development (Levings, 1990). For instance, leaf blight pandemic affected only one type maize hybrids (namely, Texas-type), while other types of CMS were less susceptible to this disease (Bruns, 2017). To create new CMS-Rf systems, prevent mtDNA unification, and reduce genetic vulnerability of sunflower hybrids to biotic and abiotic stresses, it is urgent to search for and introduce the new CMS sources into sunflower breeding. Although more than 70 cytoplasmic male sterility types have been identified in sunflower (Garayalde et al. 2015), they have not been sufficiently studied, resulting in limitation of their use in commercial hybrid breeding. Undoubtedly, research of the cytoplasmic
male sterility phenomenon is important for investigating the fundamental problem of nuclear-cytoplasmic interaction in the ontogeny of higher plants (Hanson & Bentolila, 2004). Previously, the comparison of mitochondrial genome organization between 28 CMS sources of sunflower, performed with Southern hybridization, demonstrated that some types of CMS (for example, ANN2, PET2, PEF1, etc.) have a different organization of the mtDNA from the PET1-like cytoplasms (Horn, 2002). Sequencing and comparing of whole mitochondrial genomes of various CMS sources will provide additional information about the molecular changes in their mtDNA, which in turn could help to suggest new mechanisms of the male sterility formation.

In the current study, we investigated structural changes in mitochondrial genomes of HA89-alloplasmic lines: fertile line and two analog lines with different types of cytoplasmic male sterility - PET1 and PET2. The results obtained for the PET2 CMS type formed the basis for further research.

Materials and methods

Plant material

Fertile line HA89 and isonuclear CMS lines - PET1 and PET2 of sunflower were obtained from the genetic collection of the N. I. Vavilov Institute of Plant Genetic Resources (VIR, Russia). The lines had the same nuclear genome (HA89), but they differed in chloroplast and mitochondrial genomes, inherited from their wild ancestors. The CMS sources were initially obtained by the interspecific hybridization of domesticated sunflower (Helianthus annuus) with H. petiolaris Nutt (Leclercq, 1969; Whelan, 1980).

Mitochondrial DNA extraction, genome library construction and NGS sequencing

We extracted the organelle fraction with a reduced amount of nuclear DNA from leaves of 14-day sunflower seedlings, following the protocol of Makarenko et al. (2016). For every line, we used the same quantity of leaf tissue from five plants. The DNA isolation was performed with PhytoSorb kit (Syntol, Russia), according to the manufacturer’s protocol. The NGS libraries preparations were made using 1 ng of DNA and Nextera XT DNA Library Prep Kit (Illumina, USA), following the sample preparation protocol by Illumina. For the qualitative control of libraries, Bioanalyzer 2100 (Agilent, USA) was used. The libraries quantitation was performed with the Qubit fluorimeter (Invitrogen, USA) and qPCR. Libraries for NGS sequencing were diluted up to the concentration of 8 pM. Libraries were sequenced on different sequencing...
platforms. Fertile line and PET1 NGS libraries were sequenced with NextSeq 500 sequencer using High Output v2 kit (Illumina, USA). A total number of 13,240,057 150-bp paired reads were generated for fertile line and 14,758,067 reads - for PET1 line. PET2 library was sequenced with HiSeq2000 and MiSeq platforms using TruSeq SBS Kit v3-HS and MiSeq Reagent Kit v2 500-cycles (Illumina, USA). A total number of 4,471,774 125-bp and 4,931,318 250-bp paired reads were generated for the PET2 line.

**Analysis of sequencing data**

Quality of reads was determined with Fast QC. Trimming of adapter-derived and low quality (Q-score below 25) reads was performed with Trimmomatic software (Bolger et al., 2014). Using the Bowtie2 tool v 2.3.3 (Langmead & Salzberg, 2012) sequencing reads were aligned to the reference sequence from NCBI databank (NC_023337.1). The Bowtie 2 alignments were done only for concordant paired reads (--no-mixed, --no-discordant options). Variant calling was made with samtools/bcftools software (Li, 2011) and manually revised using the IGV tool (Thorvaldsdottir, Robinson & Mesirov, 2013). De novo assembly was performed with SPAdes Genome Assembler v 3.10.1 (Nurk et al., 2013) with different K values equal to 75, 85, 95, 127, and read coverage cutoff value equal to 30.0 (--cov-cutoff option). The potential ORFs were identified using ORFfinder. The graphical genome map was generated using the OGDRAW tool (Lohse, Drechsel & Bock, 2007). Transmembrane domains were predicted using the TMHMM Server v.2.0 (available online: http://www.cbs.dtu.dk/services/TMHMM-2.0/).

**Validation of genome assembly. PCR and Sanger sequencing**

The contigs obtained in de novo assembly were aligned to the reference sunflower mitochondrial genome (NC_023337.1) using BLAST. Validation of discovered rearrangements was made by PCR analysis and Sanger sequencing. PCR reactions were performed with LongAmp Taq PCR Kit (New England BioLabs, USA) for reactions with expected amplicons more than 1.5 kb, and with Tersus Plus PCR kit (Evrogen, Russia) for other reactions, including Sanger sequencing. For 28-29 cycles of PCR, we used 0.4 uM of primers (Table 1) and 1 ng of extracted DNA. The direct sequencing of purified amplicons was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, USA) and ABI Prism 3130xl Genetic Analyser (Applied Biosystems, USA).
RNA extraction and qRT-PCR

Total RNA from the leaves of five samples of each line was extracted with guanidinium thiocyanate-phenol-chloroform reagent kit – ExtractRNA (Evrogen, Russia). RNA quality and concentration were measured using the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA) and the Qubit fluorimeter (Invitrogen, USA). 0.5 μg of total RNA was treated with DNase I (Thermo Fisher Scientific, USA) according to the manufacturer’s instruction. First-strand cDNA was synthesized using MMLV RT kit (Evrogen, Russia) and specific primers. The qPCR was performed with designed primers (Table 1) and PCR kit with EvaGreen dye (Syntol, Russia) on Rotor-Gene 6000 (Corbett Research, Australia).

Results and Discussion

Organization of fertile line and PET1 mtDNA

De novo assembly of the fertile line and PET1 mitochondrial genomes revealed between 9 and 12 large contigs (10-115 kb long), depending on K value. The optimum K value was 95. The obtained large contigs covered up to 95% of the reference genome. The remaining 5% of the mitochondrial genome correspond to repeats, therefore they were the breakpoints of contigs formation. Among numerous repeats in the mitochondrial genome, only one large (12,933 bp long) and six small (203-728 bp long) repeats played a crucial role in genome assembly and prevented single scaffold formation. Four regions (415-1192 bp long) with 99% chloroplast DNA identity also affected the mitochondrial genome assembly. Eventually, manual assembly based on predominant contigs obtained by SPAdes supplemented by analysis of reads alignment by Bowtie2 and validation of controversial regions performed by PCR analysis and Sanger sequencing, allowed summary sequencing of data in completed mitochondrial genomes. Circular mtDNA of HA89 and PET1 lines are presented in Figures 1 and 2.

The mtDNA comparative analysis of sunflower fertile lines HA412 (NCBI accession NC_023337.1) and HA89 revealed two single nucleotide thymidine insertions: in positions 35690-35691 and 129368-129369 of NC_023337. Two SNP in the noncoding part of 301 kb genome is a negligible difference. We did not amend the HA89 mitochondrion sequence in the NCBI GenBank and, in this paper, used the same positions of mtDNA for HA412 and HA89 lines for simplicity.
The PET1 mitochondrial genome had structural rearrangements as well as polymorphic sites compared to the fertile lines’ (HA412/HA89) mtDNA. Previously, using the restriction analysis and Sanger sequencing, large structural variations of mtDNA associated with the sterility of plants were detected in the PET1 CMS type of sunflower – a 11 kb inversion and a 5 kb insertion (Kohler et al., 1991). The results of the current study not only confirmed the presence of these reorganizations in the HA89 PET1 mitochondrial genome but also allowed detection of more precise genome changes: 11852 bp inversion, 4732 bp insertion, 451 bp deletion. The revealed insertion was 98% identical to the PET1 insertion that can be obtained from GenBank NCBI (accession Z23137.1). Comparing with the fertile line genome, we have identified 18 variants in HA89(PET1) mtDNA. Among the nucleotide variations, 8 were localized in SSR loci, 2 deletions (single and dinucleotide) and 7 SNP, including 1 transition and 6 transversions, were predominantly located in noncoding regions (Table 2). The exceptions were nonsynonymous mutations in orf777 (Asp251Glu), nad6 (Ser232Tyr), rpl16 (Lys32Gln). The HA89 (PET1) complete mitochondrial genome sequence has been deposited in the NCBI databank (accession MG735191).

Organization of PET2 mtDNA

We assembled the PET2 mitochondrial genome following the described procedure that was used for identification of HA89 fertile and PET1 complete mtDNA sequences. The complete mitochondrion of HA89(PET2) is 316586 bp (Figure 3) and, in comparison with the HA89 fertile line, contained large-scale reorganizations of the mtDNA structure as well as minor changes represented by variable sites. Among significant rearrangements, two translocations, two deletions and two insertions were determined.

Even in a single plant cell, the mitochondrial genome is represented by several DNA molecules with various structure (Sloan et al., 2012). The so-called “master chromosome” – the single mtDNA molecule is a rare type of mitochondrial genome organization (Gualberto et al., 2014). More often, a mitochondrion includes a set of sub-genomic forms (Yang et al., 2015). Because sub-genomes could form differing master chromosomes, the statement of translocations in plant mitochondrial genomes is equivocal. To compare complete mitochondrial genomes of HA89(PET2) and fertile HA89 lines, two translocations of approximately 27,5 kb and 106,5 kb (positions 37112-64614 bp and 194439-300945 bp in HA89 fertile line mtDNA) could be established. Using specific PCR primers (table 1) we could demonstrate that the mtDNA in
sunflower can also form a 154-kb sub-genomic circle molecule (positions 36393-190650). In the
sunflower genome there is a repeat region of 722 bp (36393-37114 = 189929-190650 positions
of the fertile line) with 100 % similarity, which makes the cyclization of sub-genome circular
molecule possible. In the case of HA89(PET2) this sub-genome circle molecule has the wrong
insertion point in the genome as compared with the fertile analog and thus results in
translocations appearance.

Deletion of 711 bp (35682-36393 positions in fertile line mtDNA) resulted in the absence
of orf777 in the HA89(PET2) mitochondrial genome. The orf777 codes for a putative protein
with unknown function, which shows no similarities with other mitochondrial proteins. So its
elimination can hardly be the molecular reason for CMS development. The other deletion of
3780 nucleotides (190659-194439 positions) affects only noncoding sequences. Notably, the
deletions are associated with sub-genome integration regions (36393-37114 and 189929-190650
positions). So there is a possibility, that deletions in these regions could impair master
chromosome assembly, which results in the translocations formations, as described above.

More significant mtDNA reorganizations are two revealed insertions – 5050 bp and
15885 bp. Among them, the 5050 bp insertion is most likely not involved in the origin of the
CMS phenotype. This insertion was found in the intergenic region atp6-cox2 (275230-275231
positions of fertile line mtDNA), and it does not lead to the formation of new open reading
frames (ORFs) directly in the place of insertion into the mitochondrial genome. Moreover, there
are no large (more than 300 nucleotides) ORFs within the sequence of 5050 bp insertion. The
exception is orf645 putatively translated into a polypeptide of 215 amino acids. Twenty-three
amino acids at the N-terminus of this 215 amino acid protein were similar to N-terminus of
ribosomal protein S3. We determined transcripts of orf645 in PET2 CMS line by qPCR, using
specific primers (Table 1). However, mRNA of orf645 was absent in the fertile line and PET1
CMS line. Most often the molecular reason for CMS phenotype development is the emergence of
chimeric ORFs with transmembrane domains, such as ATPase complex subunits, respiratory-
related proteins, etc. (Gillman, Bentolila & Hanson, 2007; Yang, Huai & Zhang, 2009).

Consequently, even if orf645 is translated in vivo, its role in male sterility development most
likely is negligible, as there are no similarities with respiratory/ATP synthesis-related proteins.

The 15885 bp insertion in the intergenic region nad4L-ccmFc is more complicated than
the 5050 bp insertion and includes different ORFs. First of all, it should be highlighted that most
of the insertion (9482 bp) has 100% similarity to another PET2 mtDNA region (126260-135741 positions). The repeating part of the insertion could be divided into two parts – 6097 bp (35686-41782 positions of PET2 mitochondrion) are common (99-100% identity) for Helianthus mitochondrion: 269147-275243, 273418-279514 and 126260-132356 positions of fertile line, PET1 and PET2 CMS lines, respectively. Such repeat predominantly consists of noncoding sequence, except \textit{atp6} gene. The other 3385 bp (41783-45167 positions of PET2 mitochondrion) have 100% similarity to the part of 5050 bp insertion (132357-135741 positions of PET2 mitochondrion). However, this part of the insertion does not contain coding sequence. The next part of the 15885 bp insertion counts 4849 nucleotides which are unique for the PET2 mitochondrion (45168-50016 positions). The is encoded in this part of the insertion and we determined \textit{orf2565}. The last 1554 bp of the 15886 bp insertion (50017-51570 positions of PET2 mitochondrion) have complex origin. 390 nucleotides of this insertion (50017-50406 positions of PET2 mitochondrion) are similar to 114179-114568 positions of fertile line mtDNA, the next 271 bp (50406-50676 positions) are unique, then 500 bp (50677-51177 positions) are complement to 114587-115087 positions of the HA89 fertile mitochondrion and the rest 393 nucleotides (50677-51570) are also unique. From a functional point this region presents duplication of \textit{atp9} gene combined with 271 bp insertion and deletion of 12 bp, which resulted in two new ORFs formation – \textit{orf228} and \textit{orf285} (Reddemann, Horn 2018). So the 15885 bp insertion, especially its last 1554 nucleotides, is the most important mitochondrial genome rearrangement, probably associated with PET2 CMS phenotype. It is also notable that 15885 bp insertion has the same region in mtDNA of PET2 as the other reorganizations - 711 bp and 3780 bp deletions and 106,5 kb translocation. Summarizing the functional changes data obtained, the 15885 bp insertion consists of four coding sequences: duplicated \textit{atp6} gene and three new ORFs - \textit{orf2565}, \textit{orf228} and \textit{orf285}. \textit{Atp6} chimeric ORFs or new ORFs co-transcribed with \textit{atp6} are the quite common causes of CMS development in different plant species (Kim, Kang & Kim, 2007; Jing et al., 2011; Tan et al., 2017). So we proposed that \textit{atp6} gene and its colocalized area are of particular interest as the candidate sequence for CMS phenotype development. The annotation of \textit{Helianthus annuus} mitochondrion (NC_023337.1) presents the \textit{atp6} gene coding mRNA which consists of 906 nucleotides. But the fact is that in the 5’ adjoining sequence (150 bp) to \textit{atp6} start codon there is the other one initiating codon (ATG). Transcription from this codon results in new mRNA
counting 1056 nucleotides. The elongated transcript could be translated in putative ATP6 protein with additional 50 amino acids, wherein 37 of 50 amino acids are identical with N-terminus of coxI. The similar extension of protein was discovered in another CMS type of sunflower - ANT1 (Spassova et al., 1994), but the described protein had additional 87 aa at the C-terminus of the ATP6 protein. So we assumed that extended transcript, which could be produced in \textit{atp6} duplicated region, may cause CMS phenotype. To verify the hypothesis, the expression levels of \textit{atp6} and 5' elongated \textit{atp6} transcripts were analyzed using qPCR with the same reverse primer but different forward primers (Table 1). The elongated \textit{atp6} transcript has been expressed at the same level as the \textit{atp6} gene (Gene ID: 18250997) both in PET1, PET2 CMS and fertile lines. So the \textit{atp6} transcript counting 1056 nucleotides is the normal one, and there is a mistake in its annotation in NC_023337.1. Moreover, the \textit{atp9} gene also (Gene ID: 18250970) has wrong transcript annotation – it counts only 261 bp instead of proper one with 300 bp. Using qPCR and specific primers (Table 1) we established the same expression level for 261 bp and 300 bp transcripts in all three studied lines. Notable that the relative expression level (ΔCt) of \textit{atp6} gene to \textit{atp1} gene in PET2 CMS line had no significant difference as compared with fertile and PET1 CMS analogs, despite \textit{atp6} duplication in PET2 mitochondrion. Thereby \textit{atp6} duplication in PET2 mitochondrion does not involve in CMS appearance.

All three revealed open reading frames (\textit{orf2565}, \textit{orf228} and \textit{orf285}) had demonstrated transcription activity in PET2 CMS line but not in the fertile or PET1 CMS analogs. The \textit{orf2565} translates to the putative polypeptide of 855 amino acids. Homology search in the NCBI database using BLAST pointed to similarity to DNA polymerase (type B). The impact of \textit{orf2565} on CMS phenotype development is quite doubtful, taking into account that the polypeptide has no transmembrane domains. The \textit{orf228} encodes a polypeptide of 76 aa, of which 75 are identical to C-terminus of ATPase subunit 9, as well as a new start codon (AUG) is formed due to the 271 bp insertion. Naturally, ATPase subunit 9 has two transmembrane domains – near N- and C-terminus, in \textit{orf228} there are also two predicted transmembrane domains (Reddemann, Horn 2018). However, it should be noted that the N-terminus transmembrane domain of \textit{orf228} encoded polypeptide lacks four amino acids as compared with \textit{atp9} ones. This difference, in turn, could affect polypeptide (\textit{orf228}) interaction with mitochondrial membrane and so results in mitochondrial membrane potential changes. The \textit{orf285} encodes a polypeptide of 95 aa, of which 18 are complement to N-terminus of ATPase
subunit 9. In total orf228 and orf285 polypeptides share 93 of 99 amino acids of the atp9 protein. The polypeptide encoded by orf285 also has the transmembrane domain which formed by predominantly 20-42 aa. Thus both ORFs (orf228, orf285) could be the main reason the development of PET2 CMS type

The comparative analysis of PET2 mitochondrion sequence with complete mtDNA of fertile line revealed 83 polymorphic sites – 14 SSR, 13 small indels (1-29 bp) and 56 SNP (Table 2). Among nucleotide variations only two (synonymous) SNP were in the coding sequence of genes rps3 and atp6. Additional analysis of distribution of variants can offer insight into functional properties and evolution of sunflower mtDNA (Triska et al 2017). Interestingly, PET1 and PET2 share ten polymorphic sites as compared with the fertile line. The obtained HA89(PET2) mitochondrial genome sequence has been deposited to the NCBI GenBank (accessions MG770607.1). It is important to note that the sets of primers used for identification of PET2 insertions may be used for designing molecular markers for this type of CMS in sunflower.

Conclusions

Comparative analysis of HA89 fertile and PET1 CMS analog mitochondrial genomes revealed 11852 bp inversion, 4732 bp insertion, 451 bp deletion and 18 variant sites. In the mtDNA of HA89 (PET2) CMS line we determined 27.5 kb and 106.5 kb translocations, 711 bp and 3780 bp deletions, as well as, 5050 bp, 15885 bp insertions and 83 polymorphic sites. From a functional point of view, there is the elimination of orf777, duplication of atp6 gene and appearance of four new ORFs with transcription activity specific for the HA89 (PET2) CMS line – orf645, orf2565, orf228 and orf285. We hypothesize that orf228 and orf285 could be the main reason for the development of PET2 CMS phenotype, while the contribution of other mtDNA reorganizations in CMS formation is negligible.

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Table 1 (on next page)

The primers sets used for HA89 (PET2) genome reorganizations validation and gene expression analysis.

All PCR reactions were held for HA89 fertile and CMS (PET2) lines. For simplicity primers were named according to their position in the HA89 fertile genome. The ‘F’ and ‘R’ letters denote the PCR strand orientation - forward (plus) and reverse (minus), respectively.
| The purpose                                                                 | Primer name | Primer sequence (5’-3’)              | Line         | The expected amplicon size (kbp) | The real amplicon size (kbp) |
|---------------------------------------------------------------------------|-------------|----------------------------------|--------------|---------------------------------|-------------------------------|
| Validation sub-genome structure                                          | 189837F     | CGTGAAGCCGGGATGGTATT              | Fertile      | -                               | 0.9                           |
|                                                                          | 37192R      | CAAGTGATCCCCCATCCAGG              | PET1         | -                               | 0.9                           |
|                                                                          | 189660F     | AGGAGTGAGATGGACGCTCT              | Fertile      | -                               | 1.8                           |
|                                                                          | 37954R      | AAGTGGTGCAACCCCCCTTGAA           | PET1         | -                               | 1.8                           |
|                                                                          |             |                                  | PET2         | -                               | 1.8                           |
|                                                                          |             |                                  |              |                                 |                               |
| 153,5 kb circle                                                          | orf645F     | GCCCTTCCACCTCTCGTTTGA             | Fertile      | -                               | -                             |
|                                                                          | orf645R     | TCCGAAAGCAGGCGCTAAAT             | PET2         | 0.162                           | 0.162                         |
| The analysis of orf645 expression                                        | orf2565F    | TCAATCCATGTGTTCTCGCT             | Fertile      | -                               | -                             |
|                                                                          | orf2565R    | CGGAAAGAAGACCCTCGGT              | PET2         | 0.147                           | 0.147                         |
|                                                                          |             |                                  |              |                                 |                               |
| The analysis of atp6 906/1056 bp transcripts                              | atp6F       | AGAACTGTAACTGACAACGC             | PET1         | 0.106                           | 0.106                         |
|                                                                          | atp6R       | ACCTGAGTCCGACTCTGACTC            | PET2         | 0.106                           | 0.106                         |
|                                                                          | atp6-1056F  | TCCCCATGCCTTTCTTGTTGCT           | PET1         | -                               | 0.28                          |
|                                                                          | atp6R       |                                  | PET2         | 0.28                           | 0.28                          |
|                                                                          |             |                                  |              |                                 |                               |
| The analysis of atp9 261/300 bp transcripts                               | atp9F       | CATTGGGGCAACGATGCA               | Fertile      | 0.107                           | 0.107                         |
|                                                                          | atp9R       | CCTCGATTCATTTCCGTGCT             | PET1         | 0.107                           | 0.107                         |
|                                                                          | atp9F       |                                  | PET2         | 0.107                           | 0.107                         |
|                                                                          | atp9.300R   | TGAAAAAGAAAAAGCGTGAGGAGA         | PET1         | 0.233                           | 0.233                         |
|                                                                          |             |                                  | PET2         | -                               | 0.233                         |
|                                                                          |             |                                  |              |                                 |                               |
| The analysis of atp1 expression                                           | atp1F       | CCCATGGCACACGCAAGATA             | Fertile      | 0.14                            | 0.14                          |
|                                                                          | atp1R       | CAGAAACGCTCAACTGTGGC             | PET2         | 0.14                            | 0.14                          |
|                                                                          |             |                                  |              |                                 |                               |
| The analysis of orf285 expression                                         | atp285F     | TCCCATCATGACCTACCCGT             | Fertile      | -                               | -                             |
|                                                                          | atp9.300R   |                                  | PET2         | 0.243                           | 0.243                         |
|                                                                          |             |                                  |              |                                 |                               |
| Sanger                                                                    | 274655F     |                                  | Fertile      | -                               | -                             |
| Resequencing the 5’ and 3’ ends of 5050 bp insertion                      | Pet2-seqF   | GAGGAACGAGACGACCA                | PET2         | 0.7                             | 0.7                           |
|                                                                          | Pet2-seqR   | AGGGAGGGAGGAAGTGAC               | Fertile      | -                               | -                             |
|                                                                          | 275503R     | TAACCCGCTGCAAGAGTGAGG            | PET2         | 0.7                             | 0.7                           |
|                                                                          | 35202F      | AGCTCTCCCCCATCGGTAGTT            | Fertile      | -                               | -                             |
| Detection the 5’                                                          |             |                                  |              |                                 |                               |
| PCR Forward | PCR Reverse | Genotype | Pet2-InsL | Pet2-InsR |
|-------------|-------------|----------|-----------|-----------|
| 271194R     | GGTCATCAGTTCGAGTGCA | PET2     | 2.5       | 2.5       |
| Pet2-insF   | AGGAAAAGACCCACACAGGCA | Fertile  | -         | -         |
| 194675R     | TAGCTCTTCGGAGCAGTCT | PET2     | 2.7       | 2.7       |
Table 2\textit{(on next page)}

Variation sites in mitochondrial DNA of HA89 CMS lines PET1 and PET2.

Nucleotide positions are specified according to fertile line mtDNA. IGR – an intergenic region. In case of indels, the deletions are indicated as “-“ and the inserted nucleotide are in bold.
| Position | Type  | Fertile | PET1 | PET2  | Localization       |
|----------|-------|---------|------|-------|--------------------|
| 3031     | SSR   | G5      | G6   |       | IGR nad2-ccmC      |
| 3107     | SSR   | T5      | T6   |       | IGR nad2-ccmC      |
| 3275-3276| INDEL | TA      | TTTA |       | IGR nad2-ccmC      |
| 3281-3281| INDEL | AT      | ATT  |       | IGR nad2-ccmC      |
| 4715     | SSR   | T8      | T9   |       | IGR nad2-ccmC      |
| 6207     | SSR   | A8      | A7   |       | IGR nad2-ccmC      |
| 6660     | SNP   | A       | G    |       | IGR nad2-ccmC      |
| 7404     | SSR   | G10     | G9   |       | IGR nad2-ccmC      |
| 7919     | INDEL | A       | -    |       | IGR nad2-ccmC      |
| 9796     | SNP   | T       | C    |       | IGR nad2-ccmC      |
| 10467    | SNP   | A       | C    |       | IGR nad2-ccmC      |
| 10924    | SNP   | A       | C    |       | IGR nad2-ccmC      |
| 12314    | SNP   | T       | C    |       | IGR nad2-ccmC      |
| 19594    | SNP   | G       | A    |       | IGR ccmC-atp4      |
| 23917    | SNP   | G       | T    |       | IGR ccmC-atp4      |
| 31803    | SNP   | A       | C    |       | IGR nad4L-orf259   |
| 34099    | SNP   | A       | C    |       | IGR nad4L-orf259   |
| 34135-34136| INDEL | AT   | ATGT |       | IGR nad4L-orf259   |
| 34162    | SNP   | T       | C    |       | IGR nad4L-orf259   |
| 35031    | SNP   | C       | A    |       | IGR nad4L-orf259   |
| 35114    | SNP   | C       | A    |       | IGR nad4L-orf259   |
| 35478    | SNP   | T       | C    |       | IGR nad4L-orf259   |
| 35511    | SNP   | G       | A    |       | IGR nad4L-orf259   |
| 35596    | SNP   | G       | C    |       | IGR nad4L-orf259   |
| 36360    | SNP   | T       | G    | -     | orf259 Asp251Glu   |
| 42295    | SNP   | C       | A    |       | IGR coxIII-rpl15   |
| 46039    | INDEL | A       | -    |       | IGR rpl5-nad4      |
| 49272    | SSR   | C11     | C9   | C10   | IGR nad4-ccmB      |
| 50856    | SNP   | C       | A    |       | IGR nad4-ccmB      |
| 51678    | SSR   | G10     | G9   | G9    | IGR nad4-ccmB      |
| 62360    | SNP   | T       | G    |       | IGR nad4-ccmB      |
| 62403    | SNP   | G       | A    |       | IGR nad4-ccmB      |
| 63433-63434| INDEL | TC   | TCC  |       | IGR nad4-ccmB      |
| 71497-71498| INDEL | GT   | GGGGCT |       | IGR rpl10-nad1     |
75332  SNP  A  C  C  IGR rpl10-nad1
91105  SNP  G  T  C  IGR rpl10-nad1
91106  SNP  A  C  IGR rpl10-nad1
105474  SSR  T35  T25  IGR nad1-coxl
108200  SNP  T  G  IGR coxl-rps11
115915  SNP  T  G  IGR atp9-rps4
116777  SNP  G  T  G  IGR atp9-rps4
119331  SNP  G  A  IGR atp9-rps4
121108-121109  INDEL  CC  CTTC  IGR atp9-rps4
122990  SNP  A  C  rps4 (synonymous)
133546  SNP  T  A  IGR rnr26-rnr5
133547-133548  INDEL  AT  AGG  IGR rnr26-rnr5
133548  SNP  T  G  IGR rnr26-rnr5
133549  SNP  A  C  IGR rnr26-rnr5
156213  SNP  C  A  IGR rps13-nad6
156621-156622  INDEL  CC  CCTAC  IGR rps13-nad6
157459  SNP  T  G  IGR rps13-nad6
169028  SNP  G  T  nad6 (Ser232Tyr)
170185  SSR  T14  T12  IGR nad6-ymf16
174932-174933  INDEL  AC  ACTCGACTGAA AGGAAAGGTA CGAAGTGGC  IGR nad6-ymf16
175179  SNP  G  T  IGR nad6-ymf16
178406  SSR  T9  T8  ymf16 intron
184739  SSR  A10  A11  IGR ymf16-cob
188363  SSR  T11  T10  cob intron
189980  SNP  G  T  cob intron
195008  SNP  G  T  cob intron
195015  SNP  C  A  cob intron
200174  SNP  G  A  ccmfC intron
200515  SNP  G  A  ccmfC intron
202672  SNP  T  C  IGR orf873-atp1
204990  SNP  C  A  IGR atp1-ccmFn
204846-204847  INDEL  AA  ATA  IGR atp1-ccmFn
207965  SSR  G10  G12  IGR atp1-ccmFn
| Start Position | Type   | 1st Base | 2nd Base | Description           |
|----------------|--------|----------|----------|-----------------------|
| 209335-36      | INDEL  | AA       | -        | IGR $\text{atp1-ccmFn}$ |
| 209458         | SNP    | G        | A        | IGR $\text{atp1-ccmFn}$ |
| 212638         | SSR    | C9       | C12      | IGR $\text{atp1-ccmFn}$ |
| 215916         | SNP    | C        | T        | IGR $\text{ccmFn-nad7}$ |
| 223917         | SNP    | A        | C        | IGR $\text{nad7-rps3}$ |
| 223925-223926  | INDEL  | GA       | GAA      | IGR $\text{nad7-rps3}$ |
| 226977-226978  | INDEL  | AC       | ACGTTGTTTTC | rpl16 (Lys32Gln) |
| 230112         | SNP    | A        | C        | IGR rpl16-matR         |
| 232826         | SNP    | G        | T        | IGR rpl16-matR         |
| 239880         | SNP    | G        | A        | IGR rpl16-matR         |
| 239988         | SNP    | A        | C        | IGR rpl16-matR         |
| 241035         | SNP    | G        | A        | IGR rpl16-matR         |
| 241475         | SNP    | A        | C        | IGR rpl16-matR         |
| 246053         | SNP    | C        | T        | IGR rpl16-matR         |
| 248266         | SSR    | A14      | A10      | A9                     | IGR rpl16-matR |
| 249347         | SSR    | T8       | T9       | IGR rpl16-matR         |
| 249361         | SNP    | C        | A        | IGR rpl16-matR         |
| 260901         | SNP    | G        | T        | IGR nad9-atp6          |
| 262080         | SNP    | G        | A        | IGR nad9-atp6          |
| 269062         | SNP    | G        | C        | IGR nad9-atp6          |
| 269134         | SNP    | A        | C        | IGR nad9-atp6          |
| 270676         | SNP    | G        | T        | IGR atp6-coxII         |
| 273344         | SNP    | C        | A        | IGR atp6-coxII         |
| 276834         | SNP    | T        | G        | IGR atp6-coxII         |
Figure 1

Graphical mitochondrial genome maps of HA89 fertile line.
Figure 2

Graphical mitochondrial genome map of HA89(PET1) line
**Figure 3**

Graphical mitochondrial genome map of HA89(PET2) line