Transcript analyses for mitochondrial sterile type rearrangement in sunflower

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Abstract

Transcript analyses were performed to investigate the expression of mitochondrial orfH873 and orf H522 genes present in the rearranged region of PET1. Total RNAs used in cDNA synthesis were isolated from fertile and sterile developing inflorescences of isogenic couple SW501 and fertile restored hybrid Sambred 254 at R_1 stage. Using orfH522 sequence as specific primer no transcripts were detected in the fertile line but the male sterile and restored hybrid genotypes investigated showed the same transcript pattern. Results reported here provide the first evidence that other orf from mitochondrial rearranged area - orfH873 is expressed in fertile, sterile and restored hybrid inflorescence buds of sunflower.

Keywords: cDNA, complimentary DNA, cytoplasmic male sterility, *Helianthus annus, orfH 873*.

Introduction

Sunflower belongs to the Composite family, with a terminal inflorescence composed of ray (sterile) flowers in the periphery and numerous hermaphrodite (fertile) flowers arranged in arcs which converge toward the centre of the capitulum. Due to these morphological characteristics, the hybrid breeding which requires an efficient and complete control of pollination is a tedious operation.

Cytoplasmic male sterility (CMS) is conveniently used in plant breeding for the production of a range of F_1 hybrid crops because it avoids manual emasculation of plants. CMS is a maternally inherited trait that has been described in more than 150 plant species and is characterized by the inability to produce functional pollen, although vegetative development and female fertility are usually unaffected [9, 16]. More than 60 CMS sources have been described in the genus *Helianthus*. These CMS types either occurred spontaneously or were found as a result of inter- and intraspecific crosses as well as due to mutagenesis [18].

The best-characterized system of cytoplasmic male sterility in sunfower is the so called PET1 cytoplasm, which has been obtained by an interspecific cross between *Helianthus petiolaris Nutt.* and *Helianthus annuus L.* [12] and that is used worldwide in commercial hybrid breeding to date [4].

It has been found that CMS is associated with aberrant recombination events in the mitochondrial (mt) genome resulting in the generation of chimeric ORFs, which are expressed as novel polypeptides [16]. In sunflower, the PET1−CMS protein is associated with the expression of a novel mitochondrial gene, *orfH522*, located downstream of the *atpA* gene (encoding the *α* subunit of the mitochondrial ATP synthase). *orfH522* is co-transcribed with the *atpA* gene and is expressed as a mitochondrial polypeptide of ~16 kDa in all tissues [6, 7, 10, 11]. The male-fertile phenotype can be restored by the introduction, in a cross, of nuclear *Rf* genes, which lead to a specific reduction of *atpA−orfH522* co-transcript and ORFH522 protein levels in male florets [2, 8,13, 20]. This demonstrates that interactions between nuclear and mt genomes are involved in the regulation of male fertility and flower development.

Significant progress has been made in identifying the molecular mechanisms leading to cytoplasmic male sterility. However, very little is known about other *orfs* from mitochondrial sterile type rearrangement in sunflower. Comparing the mitochondrial DNA organization of fertile and male- sterile lines, two other open reading frames *orfH708 and orfH873* with unknown function are present in the rearranged area with a difference for the CMS cytoplasm (PET1), where the *orfH708* sequence is disrupted by the 5 kb insertion. Due to the insertion, the *orfH522* is created in the 3'-flanking region of the *atpA* gene [10, 11, 19].

The presence of *orfH873* have been demonstrated in all 28 investigated CMS sources and the fertile cytoplasm in contrast with *orfH708* that did not show hybridization signal at more than one-third of sunflower sterile cytoplasms [5]. Moreover, southern hybridizations obtained with *orfH708* were highly polymorphic. No transcripts of *orfH873* and *orfH873* have been detected in etiolated seedlings of PET1 and the fertile cytoplasm [10].

The findings that *orfH873* was detected in fertile and sterile lines and was not disrupted like *orfH708* as a result of mtDNA rearrangements in sterile cytoplasm sustain the possibility that this open reading frame might be transcribed in inflorescence tissues at the beginning of reproductive stage development. The potential transcription of these *orf* is suggested and the fact, that most mitochondrial genes show enhanced expression during microsporogenesis in maize [13] rapeseed [3] and, more specifically, in microsporocyte and developing tapetal tissue in sunflower anthers [20].

Thus, our approach to unravel the possible expression of mitochondrial *orfH873,* an other gene present in plant mtDNA rearrangement associated to male sterile phenotype in sunflower is the transcript analyses by RT-PCR using CMS system at early reproductive stage.

Materials and methods

Plant materials. Sunflower (*Helianthus annuus L.*) seeds were kindly provided by Center for Scientific Research "Magroselect" (Soroca, R. Moldova). We used fertile SW501 and male sterile SW501CMS lines that are near-isonuclear. The fertile (maintainer) line carried the *H. annuus L.* cytoplasm and the sterile line contained the *H. petiolaris* cytoplasm. The fertile restored hybrid Sambred 254 resulted from a cross between the sterile line SW501 CMS and a restorer line RW637 Rf carrying the dominant nuclear restorer genes.

Sunflower plants were cultivated in pots under standard greenhouse conditions during the spring. The inflorescence tissues were analyzed in early stage of flower development R_1 according to [17]**.** The R_1 stage refers to the time when the inflorescence begins to enlarge and is visible as a bud with the bracts closed forming a star-like structure.

RNA isolation. To examine expression patterns, the total RNAs were extracted from developing inflorescence buds using Trizol reagent (GIBCO BRL) according to manufacture's protocol. Yield and purity was monitored by denaturing 1, 44% agarose gel electrophoresis [15] and by UV absorbance (A_{260}/A_{280}) using Genesis 10UV/VIS spectrophotometer (Fisher). A yield ranging 2-3 µg/µl RNA extract from sunflower tissues has obtained, the ratio $\lambda_{260/280}$ was between 2,0 and 2,1.

RT-PCR reactions. Total RNA (2µg) was treated with RQ1 RNase-Free DNase - 1u/µg RNA (Promega) at 37°C for 30 min. according to manufacturer's instructions. The treated RNA was used for reverse transcription in a reaction which contained: 1 x amplification buffer, 250 µM of each dNTP, 0,25 µg rimers oligo dT_{12-18} and 2,5 µg random hexanucleotides, 1 units/µl RNase inhibitor, 2,5 mM $MgCl_2$ in a total volume of 20 µl in the presence of 4 units/µl of M-MLV reverse transcriptase (Promega). The mixture was incubated for 60 minutes at 37°C. The reaction was stopped by an incubation of 5 minutes at 100°C [15].

The reaction mixture used for PCR amplification contained 2µl of reverse transcription reaction, 20 pmol of the sense and antisense primers, 200 µM of each dNTP, 2,5 mM $MgCl_2$, 10 µl of the 10x PCR buffer supplied with the Tag DNA polymerase (Qiagen) which was used at a concentration of 2,5 U per reaction, in a total volume of 100 µl. Primers designed for RT-PCR were the following oligonucleotides (5`-3`): *A* (AGTGAATCATTGGCT ACGTTTAGAG) and *B* (AAAGTACCTTCCATCAGATCCTTCT) for *orfH873*; *C* (GGC GCACTCTCTTTTTCTGT) and *D* (CTTGAATGGCAGTGGTGATG) for *orfH522* gene.

The PCR reaction was performed in PTC 100 thermocycler (MJ Research) programmed: 2 min. at 95°C; followed by 40 cycles: 30 s at 95°C, 1 min at 60°C, and 2 min and 30 s at 72°C. The amplified PCR products were separated and detected using agarose gels (1%) electrophoresis and ethidium bromide staining according to standard protocols [15]. The molecular size of the amplified products was evaluated using 1kb and 100 bp DNA ladder for comparison. RT-PCR was performed three times using samples collected from three distinct biological replicates. Database searches and primer design was performed using Genbank/EMBL and Primer3 program.

Results and discussion

To accomplish the goals, the primers for *orfH522* as positive control for CMS rearrangement and *orfH873* that is localized in the 3` flanking region of *atpA* in fertile line and in the 3`-flanking region of the cob gene in sterile mtDNA were designed based on the coding sequence of sunflower CMS Baso (EMBL/GenBank accession X55963) and sunflower Baso (EMBL/GenBank accession X53537) respectively (Fig. 1). As these *orfs* share homology in the 5`-coding region with *orfB* [10] it was important to exclude these regions (57 pb for *orfH522* and 30 pb for *orfH873*) from sequence to avoid obtaining undesired products.

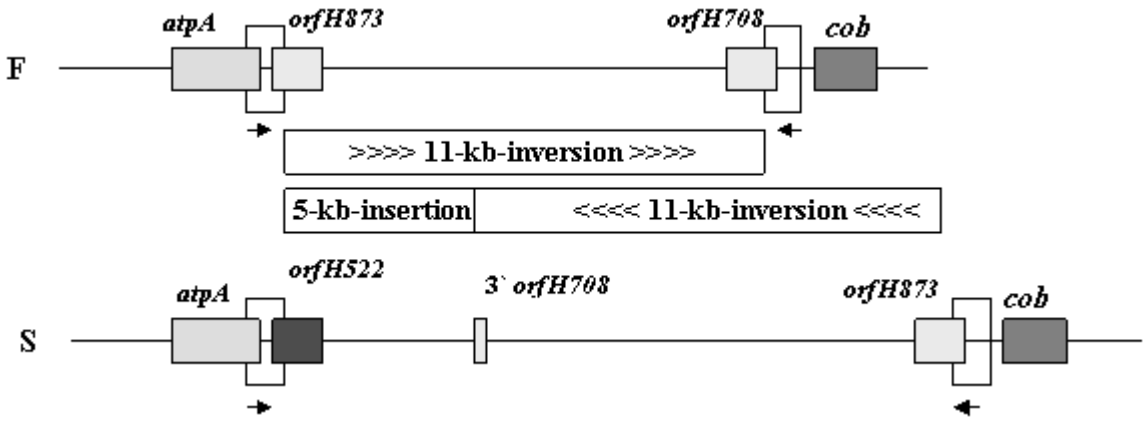

Figure 1. Organization of the mitochondrial DNA fanked by the *atpA*- and the *cob*-gene of fertile and male-sterile (PET1) lines. Boxes with arrows give the orientation of the inverted repeat of 261 bp according to Kohler et al., 1991 [5].

In order to confirm the transcription of male sterility associated gene, we performed a first RT-PCR using oligonucleotides for *orfH522* sequence. Two discrete PCR products with very close sizes were amplified from cDNAs prepared from sterile and restored hybrid plants as shown in figure 2, lanes S and H. The first fragment correspond to that with expected size 321 bp and the second additional had the size ~ 400 bp. Sequencing results and Blast search analyses have shown 96-99 % homology of this additional PCR product to the same *orfH522* [1]. It contained the sequence of the reverse PCR primers followed by the gene sequence up to ATG codon and 41 nucleotides more, which are present in 265-bp inverted repeated sequence found in the CMS sunflower [11].

As expected, no PCR product was obtained from the line SW501 with the original fertile cytoplasm (control) using these primers (Fig. 2 line F).

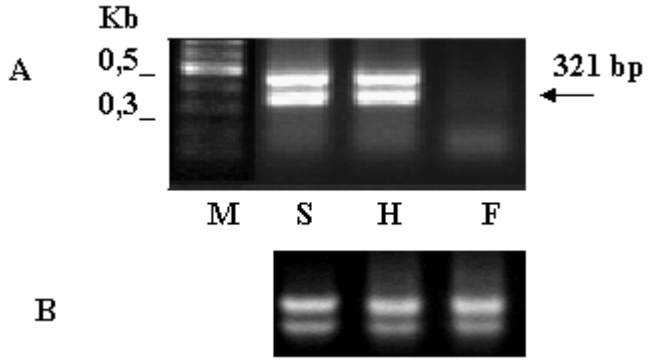

Figure 2. RT-PCR amplification products from sunflower samples using *orfH522* sequence primer. (A) RT-PCR was performed on the total RNA isolated from R_1 - inflorescence buds of fertile SW501 (F), sterile SW501CMS (S) and restored hybrid Sambred 254 (H). Lane M contain 100 pb ladder (Takara) and the sizes of the respective bands. (B) The bottom panel is the corresponding ethidium bromide-stained samples to visualize RNA quantity and quality.

Transcripts analyses of *orfH873* revealed identical patterns for male fertile, sterile and restored hybrid plants, that is a fragment with expected size - 410 bp (Figure 3, line F, S, H).

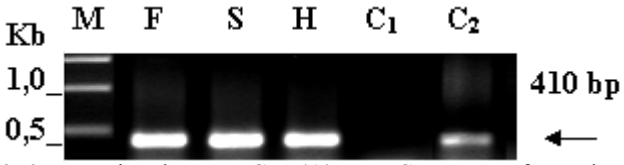

Figure 3. Detection of *orfH873* transcripts by RT-PCR. (A) RT-PCR was performed on the total RNA isolated from developing inflorescence buds of fertile SW501 (F), sterile SW501CMS (S) and restored hybrid Sambred 254 (H). C_1- sample with M-MLV reverse transcriptase omitted, C_2 - sample without DNase treatment. Lane M contain 1kb ladder (Biorad) and the sizes of the respective bands.

The RT-PCR analysis with gene-specific primers for CMS rearrangement revealed *orfH873* gene expression in sunflower R_1-stage inflorescence of both isonuclear lines and restored hybrid. This finding in accordance with published data related no transcripts of *orf873* detected in etiolated seedlings of PET1 and the fertile cytoplasm [10] suggest that *orfH873* gene expression is developmentally regulated and may play a role in flower development. As no differences were observed between fertile and sterile plants seems that *orfH873* is not involved in CMS mechanism. Therefore, a more detailed analysis of this gene expression pattern in different tissues and developmental stages is necessary to identify its role in higher plants during development.

Our data show similar abundant PCR product of *orfH522* gene in inflorescence apices of PET1-CMS sunflower and restored hybrid at R_1 stage of reproductive development. This indicates the existence of enhanced expression of CMS associated gene as early as flower meristems develop. The lack of differences observed in transcript (321 bp) amount between CMS and restored hybrid samples is due to fact that only very early stages of anther development were studied and at inflorescence level without to distinguish specific tissue. It was shown that the *atpA-orfH522* transcripts are preferentially destabilized in a tissue-specific manner upon restoration of fertility apparent at al stages of floret development, but more pronounced in male florets at the leptotene and tetrad/microspore stages [2, 14].

In conclusion, results reported here provide the first evidence that another *orf* from mitochondrial rearranged area - *orfH873* is expressed in fertile, sterile and restored hybrid inflorescence at early stages of sunflower reproductive development. These are preliminary results and may lead to new investigations to gain further understanding in this field.

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