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Research paper

Mapping of 5′ and 3′-ends of sunflower mitochondrial *nad6* mRNAs reveals a very complex transcription pattern which includes primary transcripts lacking 5′-UTR

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ABSTRACT

Nad6 orf, encoded in the sunflower mitochondrial genome in single copy contains a non-conserved 3′-extension.
The transcription of the *nad6* locus generates a highly complex pattern. Three main transcripts of 1240, 960 and 870 nt have been characterized by different approaches. The two smaller ones are apparently the most abundant of the steady-state RNA population and are generated as primary transcription products as well as by processing of the 1240 nt transcript. Their 5′-UTRs are absent or very short.
Whereas the 3′-ends of the 960 nt transcripts contain a TGA codon the shorter one terminates at positions excluding the stop codon.
The fate of transcripts to promote the synthesis of NAD6 sunflower protein seems, thus, to rely on the occurrence of mechanisms yet to be identified.
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1. Introduction

Plant mitochondrial genomes show an extraordinary complexity in terms of organization and structure. Although up to 30 times larger than their metazoan and fungal counterparts, their informational content (50–60 genes) is only 3–4 times larger [1].

In human mitochondrial DNA, genes are organized into two transcription units which are transcribed from a single bidirectional promoter located in the non-coding regulatory region [2].

In higher plant mitochondria mono-, as well as polycistronic transcription units might vary in number and structure. In either case the transcription initiation might be driven by single or multiple promoters [3,4].

In addition to their organization, plant mitochondrial genes show a surprising complexity in terms of transcription and RNA processing. RNA editing (C-to-U and less frequently U-to-C conversion), *cis-* or *trans*-splicing of one or several introns, base modifications, 3′-end polyadenylation and maturation of 5′- and 3′-termini, are examples of reactions which reflect the complex RNA processing in plant mitochondria [3,5].

The *nad6* gene can be taken as an example of the complexity in structure and expression of plant mitochondrial genes. The *nad6* open reading frames (*orfs*) investigated so far range from 618 bp (e.g. *Arabidopsis thaliana* [6,7], *Brassica campestris* [8], *Dacus carota* (EBI a.n. AY007818), *Oryza sativa* [9,10], *Lupinus luteus* [11], *Triticum aestivum* [12,13]) to 1128 bp in *Zea luxurians* (EBI a.n. DQ645537). Interestingly significant length and primary structure differences among all the known plant *nad6 orfs* have been found only after the first 600 nucleotides (nt). Despite the emerging genomic differences, data concerning transcription are limited to the group of the shortest ones.

In rice [10], wheat [13], and maize [14] the mitochondrial *nad6 orf* is co-transcribed with other genes whereas in *B. campestris* [8], lupin [11], *Arabidopsis* and cauliflower [7] the *nad6* seems to be expressed as a single monocistronic mRNA. In addition, the *Arabidopsis* and cauliflower *nad6* mRNAs have been shown to undergo processing upstream of the translation stop codon [7].

Here we report results concerning both the characterization of the *nad6 orf* present in the mitochondrial genome of Gloriasol sunflower variety and the processing and maturation of its mRNAs. The gene is present in single copy [8] and has been located downstream of the cluster *trnS–trnF–trnP*.

Abbreviations: cRT, circularized reverse transcription; mtDNA, mitochondrial DNA; *nad6*, gene coding for subunit six of NADH dehydrogenase; *orf*, open reading frame; TAP, tobacco acid pyrophosphatase.

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When compared with the orthologous genes identified in the mitochondrial genomes of other higher plants, the sunflower *nad6 orf* displays a novel 3′-extension of about 310 bp.

Transcription of *nad6 orf*, analysed by RT-PCR, cRT-PCR and RNA capping experiments, showed the existence of three main transcripts and the absence of alternative stop codons generated by RNA editing.

2. Materials and methods

2.1. Isolation of mitochondria and mitochondrial RNA

Mitochondria were isolated from 6-day-old etiolated shoots of sunflower (*Helianthus annuus*, cv. Gloriasol). Gloriasol is a sunflower fertile commercial hybrid line, based on the male sterile line CMS89, that carries the *Helianthus petiolaris* cytoplasm [15]. Gloriasol seeds were provided by ISEA (present affiliation: Agroservice spa, http://www.agroservicespa.it/, Italy).

After differential centrifugation, mitochondria were further purified through 0.6–1.8 M discontinuous sucrose gradients [16]. Purified mitochondria were lysed in a buffer containing 100 mM Tris–HCl pH 8.0, 10 mM EDTA and 0.4% SDS (w/v) and the RNA was recovered by phenol–chloroform extractions, ethanol precipitation and DNase free-RNase (Promega) treatments.

2.2. cRT-PCR, RT-PCR and editing analysis

Circular RT-PCR was used to determine 5′- and 3′-extremities of the mitochondrial *nad6* mRNAs. Prior to circularization by T4 RNA ligase total mtRNA was treated with tobacco acid pyrophosphatase (TAP) (Invitrogen) to generate primary transcripts with a 5′-monophosphate extremity. Up to 25–50 μg of TAP treated mtRNA were ligated by 80 U of T4 RNA ligase (New England Biolabs) in a final volume of 50 μl containing the supplied buffer. The circularized RNA was recovered after phenol/chloroform extractions and ethanol precipitation, using 1/10 of 1 M sodium acetate pH 4.6. Reverse transcription reaction was carried out using Superscript™ III (Invitrogen), following manufacturer's instructions, and the reverse primer Rcir (primers used in the present work are listed in Section 2.8). PCR reaction was performed using the couple of primers Rcir/Fcir, 10% of the single strand cDNA synthesis reaction and both Invitrogen Taq DNA polymerase (0.4 U) and Stratagene native *pfu* DNA polymerase (0.4 U) under the following conditions: 94 °C for 3 min; 30 cycles at 92 °C for 30 s, 65 °C for 30 s and 72 °C for 45 s.

RT-PCR was also used to verify co-transcription of both *nad6* and *trnP* genes. The primer Rev1 was used during the single-strand cDNA synthesis as well as during PCR with the For1 primer. Amplification conditions were: 94 °C for 3 min; 35 cycles at 92 °C for 30 s, 60 °C for 30 s and 72 °C for 40 s.

Editing studies were carried out by further RT-PCRs. To prime the first-strand cDNA synthesis Rev2, Rev3 and Rev4 primers (see Section 2.8) were used while the three double strand cDNAs were generated by PCR using the three couples of primers: For2/Rev2, For3/Rev3 and For4/Rev4 (see Section 2.8). The amplification conditions were: 94 °C for 3 min; 30 cycles at 92 °C for 30 s, 60 °C for 30 s and 72 °C for 45 s.

Cloning of amplification products was performed using a TopoTA vector (Invitrogen). *Escherichia coli* cells (TOP10 F′ strain, Invitrogen) were used for transformation. Sequencing of cDNA clones was performed using an ABI 377 automatic DNA sequencer (Applied Biosystems).

2.3. "In vitro" capping

Twenty-five μg of total mitochondrial RNA was labelled in a total volume of 50 μl containing 1× capping buffer (Ambion), 120 units of RNase inhibitor (Invitrogen), 1 mM S-Adenosyl Methionine (Ambion), 140 μCi [α-^{32}P]GTP (800 Ci/mmol) and 30 units of Guanylyltransferase (Ambion) at 37 °C for 1 h. After the reaction, RNA was recovered by a gel-filtration through a Sephadex G-50 spin column.

2.4. On filter RNase protection experiments

In order to define the region/s containing putative transcription initiation sites the genomic region located between the *trnP* gene and the *nad6* gene was subdivided into three partially overlapping segments defined as a, b and c. The three segments, respectively 300, 450 and 650 bp long, were amplified by PCR reaction using the three couples of primers For1/a-Rev, b-For/b-Rev, c-For/c-Rev under the following conditions: 94 °C for 5 min; 35 cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, in presence of Taq DNA polymerase (0.4 U) and Stratagene native *pfu* DNA polymerase (0.4 U).

The amplified DNA segments a, b and c were transferred onto Hybond N$^+$ membranes (see Section 2.6) and hybridized with the ^{32}P-cap-labelled RNAs. Hybridizations were carried out at 65 °C in 1 M phosphate buffer pH 7.2, 7% (w/v) SDS. The filter was washed at 65 °C for 10 min in 2× SSC and at 65 °C for 10 min in 0.5× SSC. In order to remove unhybridized cap-labelled RNA, hybridized filters were incubated in the following buffer: 10 mM Tris–HCl pH 7.8, 1 mM EDTA, 300 mM NaCl at 37 °C for 1 h, 100 μg RNase A and 200 units of RNase T1 (Roche).

2.5. In solution RNase protection experiments

In order to identify the position of transcription initiation sites a RNA probe complementary to the b DNA fragment was synthesized as follows: DNA segment b was amplified by PCR (as above) using the couple of primers T7b-For2/b-Rev2 (see Section 2.8) and the 14.8 kbp PstI restriction fragment as a template. The amplified DNA segment b was purified from a 1% ultrapure agarose gel (Invitrogen) and used as a template to transcribe *in vitro* the complementary riboprobe according to the RiboMax™ Large RNA Production System-T7 protocol (Promega).

The complementary 450 nt long riboprobe was used to hybridize in solution total ^{32}P-cap-labelled RNA of sunflower mitochondria following the Ribonuclease Protection Assay (RPA) III™ protocol (Ambion). Protected RNA fragments were resolved onto a 6% polyacrylamide gel (Acryl/Bis-acryl 19:1), 7 M Urea, 1× TBE.

Thirty-two-P-labelled DNA marker fragments were obtained by polymerase chain reactions using the three couples of primers M1-For/b-Rev, M2-For/b-Rev, M3-For/b-Rev (see Section 2.8) and the 14.8 kbp PstI restriction fragment as a template under the following amplification conditions: 94 °C for 5 min; 30 cycles at 94 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min in a buffer containing 10 mM Tris–HCl pH 8.8, 50 mM KCl, 100 mM $MgCl_2$, 10 mM dTTP, 10 mM dGTP, 10 mM dCTP, 0.5 mM dATP, 100 μCi [α-^{32}P] ATP (800 Ci/mmol), Taq DNA polymerase (0.4 U) and Stratagene native *pfu* DNA polymerase (0.4 U). Single radioactive fragments were recovered by phenol–chloroform extractions, ethanol precipitations and then purified from a 1% ultrapure agarose gel (Invitrogen).

2.6. Southern blotting

One μg of each PCR-generated DNA fragment (a, b and c, Fig. 4A,B) were resolved on 0.8% ultrapure agarose gel (Invitrogen) and transferred onto Hybond N$^+$ membrane (Amersham-Pharmacia) by a 0.5 M NaOH solution. Hybridizations on Southern blots were performed as in Section 2.4.

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