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This work is dedicated to Professor Jacek Augustyniak

Differences in editing of mitochondrial *nad*3 transcripts from CMS and fertile carrots

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A high level of the nucleotide sequence conservation was found for mitochondrial *nad*3 gene of carrot. Three silent nucleotide substitutions differentiate *nad3* open reading frames from cytoplasmic male sterile and male fertile carrots. All these differences are preserved on the RNA level. Partial and silent editing also distinguished both carrots. Three of the C to U conversions were specific to the fertile line. In the two examined carrot lines editing did not affect the mode of alteration of encoded amino acids.

Exciting new findings have emerged as concerns plant mitochondria in relation to the manifestation of cytoplasmic male sterility (CMS) [1, 2]. Molecular studies on CMS have revealed the existence of a rearrangement modification in mitochondrial DNA which, in turn, leads to stamen sterility or pollen abortion in several plant species. An important feature of this rearrangement responsible for CMS was elucidated by the discovery of chimeric genes which are often cotranscribed with other standard mitochondrial genes. CMS provides a convenient means to produce hybrid seeds. Two CMS types are widely used for carrot hybrid seeds production: brown-anther and petaloid sterility [3]. Petaloid plants

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Abbreviations: AGPC, acid guanidinium thiocyanate-phenol-chloroform; AMV, avian myeloblastosis virus; CMS, cytoplasmic male sterility; CTAB, hexadecyltrimethylammonium bromide; RT-PCR, reverse transcriptase polymerase chain reaction.

lack stamens which depending on the nuclear background of the sterile cytoplasm have been transformed into petals, petal-like, bract-like or carpelloid structures [4]. The molecular aspects of CMS in carrots have been investigated in several laboratories [5–8]. Recently Szklarczyk *et al.* [8] reported specific organization and transcription of *atp*9 gene from petaloid carrot cytoplasm and also speculated that C to U conversion in the second glutamine triplet of the *atp*9 gene extension could play role in fertility restoration. Here, we attempted to test if the type of cytoplasm might influence RNA editing of another carrot mitochondrial gene. We chose *nad*3 gene for its high content of edited nucleotides. Wilson & Hanson [9] studied the *nad*3 transcripts population from petunia CMS lines and showed that the extent of editing at the three assayed positions varied depending on nuclear background of the sterile cytoplasm. However, Hanson *et al.* [10] have detected no correlation of transcripts editing with either sterility or fertility restoration. These results prompted us to analyse RNA editing in carrot *nad*3. Previously we examined the sequence and transcription of the *nad*3 and *rps*12 genes in four lupin species and found that the structure and organization of these genes were conserved [11]. The aim of this study was to analyse genomic structure and degree of RNA editing of the *nad*3 genes from CMS petaloid and fertile carrot cytoplasm.

MATERIALS AND METHODS

Plant material. Carrot inbred lines: 2163A (petaloid CMS) and 2163B (maintainer) were provided by Production and Breeding of Horticultural Plants Ltd. (PHRO, Krzeszowice, Poland).

Isolation of nucleic acids. Total cellular DNA for the polymerase chain reaction (PCR) was extracted from young leaves according to Gawal & Jarret [12] with an additional chloroform extraction step included. Prior to this step the aqueous phase collected from the first extraction was supplemented with 0.1 vol. of 10% CTAB, 0.7 M NaCl solution. Carrot mitochondrial DNA (mtDNA) was isolated from root tissue using the procedure of Steinborn *et al.* [13]. The ammonium acetate precipitation step was omitted. Plasmid DNA to be used as a sequencing template was isolated with Qiagen Miniprep Kit. PCR fragments to be used for subsequent cloning and sequencing were purified using Qiaex II Gel Extraction Kit (Qiagen). Total cellular RNA was extracted from young umbels using the modified AGPC method. After the first isopropanol precipitation described in the original procedure [14], the RNA pellet was re-hydrated and lithium chloride re-precipitated [15].

DNA cloning. The mtDNA library of petaloid line 2163A was constructed in Bluescript II KS phagemid vector (Stratagene) as described by Szklarczyk *et al.* [8]. The resulting library was screened with *nad*3 probe from 2163A line, labelled as described by Rurek *et al.* [11]. The insert size from two positive clones was determined using *Bam*HI digestion.

The PCR amplified *nad*3 genes were cloned into pGEM T-Easy vector (Promega) according to procedure of manufacturer's recommendations.

PCR amplification of DNA. The PCR reaction mixture contained in 50 μl the following components: 60 ng of total DNA, 0.45 μM of each primer, 10 mM Tris/HCl, pH 8.8, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% (v/v) Triton X-100, 200 μM of each dNTP, 0.03 units of PrimeZyme polymerase (Biometra) per microlitre of the reaction mixture. Five synthetic primers: P1, P2, P3, P4, P5 specific to the angiosperm consensus DNA fragment including the *nad*3 gene alone or *nad*3 with flanking sequences were used. PCR was carried out in a Biometra 050–500 cycler. For the above primers one step amplification was used: the initial denaturation for 95°C for 5 min was followed by 30 cycles of 45 s at 92°C, 30 s at 57°C and 2 min at 72°C. The PCR

was terminated by elongation cycle of 10 min of 72°C.

List of primers. Oligonucleotides used for PCR, sequencing and reverse transcription:

P1 – 5′ ATG TCG GAA TTT GCA CCT ATT TGT ACT 3′

P2 – 5′ TTA CTC CCG ATC CGA AGC ACC 3′

P3 – 5′ ATG CCG CTC CGC GAG CAA GG 3′

P4 – 5′ CTC CTC TAC CAT GAC GAA TCA A 3′

P5 – 5′ VAR DKW CAA GAG GCA TCT TCC AT 3′

DNA sequencing. This was performed by the dideoxy chain termination method [16] using either Perkin Elmer (ABI Prism BigDye Terminator Cycle Sequencing Kit) or Amersham-Pharmacia Biotech (Thermo Sequenase Fluorescent (*Cy5, 5′ end) Labelled Primer Cycle Sequencing Kit with 7-deaza-dGTP) and P1, P3 and P4 primers. Sequencing reactions were set up and cycled according to the manufacturer's recommendations. Reaction products were resolved and analysed using either the AbiPrism 377 DNA Sequencer or Pharmacia-Amersham ALF Sequencer.

Reverse transcription of RNA. The cDNA from six separate RT-PCR runs was synthesized from total RNA of carrot lines 2163A and 2163B and specific primer P5 by AMV reverse transcriptase (Promega) following the Promega protocol. For the reaction 4 μg of total RNA, 0.5 μg of P5 primer per μg of RNA and 15 units of AMV reverse transcriptase were used. The cDNA synthesis was terminated by phenol/chloroform extraction. The RT products were ethanol precipitated, dissolved in water and used for amplification.

RESULTS AND DISCUSSION

The *nad*3 gene from petaloid and fertile carrot cytoplasm

In order to isolate carrot *nad*3 gene, a genomic library of mt DNA from 2163A line was screened with PCR fragment amplified from petaloid carrot DNA using P1 and P2 primers. Two positive clones were identified with insert sizes of 4.5 and 17 kb. Restriction mapping and hybridization analysis (not shown) of these clones confirmed that they contained the *nad*3 gene. Sequence analysis of the 4.5 kb clone revealed a 357-nucleotide open reading frame (ORF) identified by similarity as the *nad*3 gene: the *nad*3 ORF showed more than 93% sequence identity with the respective sequences of dicotyledons and about 90% identity with other angiosperms (GenBank data). Downstream from the *nad*3 ORF, a coding sequence of *rps*12 gene is located. The spacer region between these ORFs has 48 nucleotides.

Once the sequence of *nad*3 gene from the petaloid line was determined it was possible to perform PCR amplification of this gene with specific primers using a genomic template from the same line. Both genomic (from the library) and amplified genomic sequences were identical proving that in petaloid carrot *nad*3 sequence does not exhibit heteroplasmy.

PCR amplification was also used to determine the sequence of *nad*3 gene from fertile carrot. Comparison of the sequence of *nad*3 genes from the two kinds of cytoplasm revealed three single-nucleotide substitutions within a DNA segment of *nad*3 ORFs and its flanking regions including 25 nucleotides at 5′ end, 48 nucleotides of the spacer and 18 nucleotides of the *rps*12 ORFs. Differentiating nucleotides are located in the following positions of the *nad*3 genes: 15th, 105th and 312th (EMBL accession numbers: AF285878, AF285879). In the case of the fertile line they contain A, T and T, while the sequence of petaloids has G, C and C, respectively. No sequence differences were observed when the same fragment of another clone from petaloid line was analysed. All substitutions appeared in the third codon position and therefore the deduced amino-acid sequences of NAD3 proteins remained identical.

The conserved arrangement of carrot *nad*3 and a part of *rps*12 sequences suggest their be-

longing to one transcription unit. To establish their cotranscription, an RT-PCR experiment was performed with two primers: P5 and P3. The P5 primer corresponds to the 3′ flanking region of the *rps*12 gene, while the P3 primer to 5′ flanking region of the *nad*3 gene. Both for fertile and CMS carrots PCR yielded a product of about 780 bp, strongly suggesting cotranscription of *nad*3 and *rps*12 (Fig. 1).

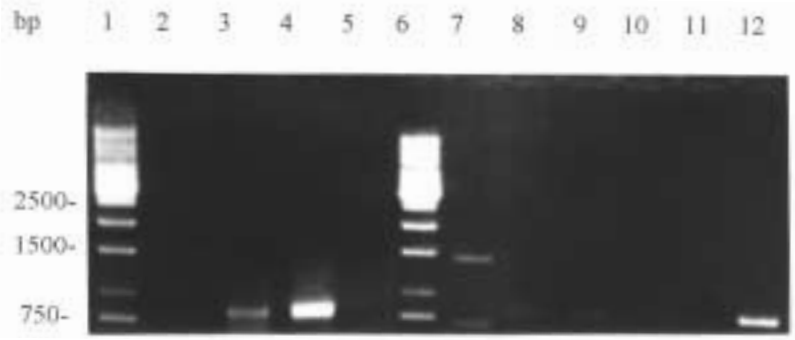

Figure 1. RT-PCR analysis of the cotranscription of *nad*3 and *rps*12 genes in CMS and fertile carrots.

Lanes 1, 6 and 7: molecular mass marker. Lane 3: the product of RT-PCR obtained by amplification with P3 and P5 primers in 2163B fertile line. Lane 4: the positive control of PCR (*nad*3-*rps*12 genes amplified with P3 and P5 primers). Lanes 8 and 9: the product of RT-PCR obtained by amplification with P3 and P5 primers in 2163A sterile line. Lane 12: the positive control of PCR. Lanes 5 and 11: negative control of amplification without RNA. Lanes 2 and 10: negative control of amplification without AMV reverse transcriptase.

The length of the product confirms that organization of the *nad*3-*rps*12 transcription unit in carrots follows the conserved pattern found in other plant species [9, 17].

Editing of the *nad*3 transcripts in petaloid and fertile carrots

To study the extent of RNA editing, the PCR derived cDNAs of *nad*3 transcripts of the two carrot lines studied were cloned and sequenced. Comparison of genomic and cDNA sequences revealed 19 editing sites – all of them of C to U type commonly found in plant mitochondria [18] (EMBL accession number: AJ300556). The nucleotide positions of these sites are the following: 5, 39, 43, 44, 61, 62, 89, 102, 208, 209, 215, 230, 231, 247, 266, 275, 317, 344 and 349. Another editing site was found 15 nucleotides upstream from *nad*3 ORF. The last C to U conversion was observed within the 5′ part of *rps*12 gene (not shown). Most of the editing sites are shared between the two analysed carrot lines. Positions 43, 89, and 102 are edited only in fertile carrot, and these positions are located in codons 15, 30 and 34 (Table 1). The fertile carrot also exhibits the presence of partial editing in codons 15, 30, 34 and 77 (Table 1). The partial editing of codon 15 concerns the first letter of the codon. The second letter of this codon is edited both in the sterile and fertile lines. In the case of codon 77, partial editing concerns the third letter. The presence of partial editing in the fertile line was not fully investigated, because only three clones of this line

Table 1. Differences in editing of some codons in the *nad*3 transcripts from sterile 2163A and fertile 2163B line of carrot

Codon number	Codon changes carrot line 2163A	Codon changes carrot, line 2163B
15	C**C**G-CUG (**P-L**)	**C**CG-UUG (**P-L**)*
30	UCC (S)	U**C**C-UUC (**S-F**)*[1]
34	ACC (T)	AC**C**-ACU (**T**)*[1]
77	U**CC**-UUU (**S-F**)	U**CC**-UUU (**S-F**)*

Edited nucleotides and the corresponding amino-acid changes are indicated by bold letters. A *star* (*) indicates the presence of partial editing of clones of the fertile line. [1] indicates the presence of unedited codons in part of transcripts of fertile line.

were analysed. No cases of partial editing were observed for three analysed clones of petaloid line 2163A, although we cannot exclude that they could have been detected if a larger number of cDNA molecules were checked. Lu and Hanson [19] distinguished three categories of transcripts with respect to the editing extent. Our results indicate that carrot *nad*3 mRNA might belong to the second category transcripts, which are heterogeneous in editing extent and in which partially edited transcripts are readily detectable. It is interesting to note that *nad*3 transcripts of petunia and *Oenothera* also belong to this category [19].

In both carrot lines editing of the *nad*3 transcripts results in replacement of four prolines by leucines and one proline by phenylalanine. Three serine codons are converted into phenylalanine triplets and in one case trypto-

Table 2. Comparison of amino-acid changes resulting from codon editing of carrot mitochondrial *nad*3 transcripts with *nad*3 transcripts of other higher plants

Codon number	D. carota	Lupinus sp.	Petunia sp.	H. annuus	Oenothera sp.	Magnolia sp.	B. napus	A. cepa	T. aestivum	S. bicolor	O. sativa	Z. mays	C. lacryma-jobi	P. sylvestris
2	S-L	S-L	S-L	S-L	S-L	S-L	S-L	S-L	T-L			S-L	S-L	
13	I								I					
15	P-L		P-L	P-L	P-L	P-L		P-L	P-L	P-L	P-L	P-L	P-L	S-L
21	P-L	P-L	P-L	P-L		P-L	L	P-L	P-L	P-L	P-L	P-L	P-L	
27		P-L		P-L		P-L	P-L	P-L	P-L	P-L	P-L	P-L	P-L	P-L
30	S-F													P-S
34	T			T										
49													S-F	
62													P-L	
64													P-S	
70	P-F	P-F	P-F	P-F	P-F	P-F	P-F	P-F	P-F	P-F		P-F	P-F	S-F
72	P-L	P-L	P-L	P-L	P-L	P-L		P-L	P-L	P-L		P-L	P-L	P-L
77	S-F	S-F	S-F	S-F		S-F		S-F	S-F	S-F		S-F	S-F	S-F
83	P-S	P-S	P-S	P-S	P-S	P-S	P-S	P-S	P-S	P-S		P-S	P-S	P-S
84		P-L	P-L		L-F	P-L	P-L	P-L	P-L	-L		P-L	P-L	
89	P-L	P-L	P-L	P-L	P-L	P-L		P-L				L		P-L
92	S-F	S-F	S-F	S-F	S-F	S-F		S-F	S-F			S-F	S-F	S-F
106	S-F	S-F	S-F	S-F	S-F	S-F		S-F	S-F	S-F			S-F	
115	S-L	S-L	S-L	S-L	S-L	S-L	S-L	S-L	S-L	S-L		S-L	S-L	S-L
117	R-W	R-W	R-W	R-W	R-W	R-W	R-W	R-W	R-W	R-W		R-W	R-W	R-W

Abbreviations: D. carota, *Daucus carota*; H. annuus, *Helianthus annuus*; B. napus, *Brassica napus*; A. cepa, *Allium cepa*; T. aestivum, *Triticum aestivum*; S. bicolor, *Sorghum bicolor*; O. sativa, *Oryza sativa*; Z. mays, *Zea mays*; C. lacryma-jobi, *Coix lacryma-jobi*; P. sylvestris, *Pinus sylvestris*. The data used in this Table are adapted from the following references: *Petunia* sp. [9], *H. annuus* [17], *Oenothera* sp. [21], *Magnolia* sp. [17], *A. cepa* [17], *B. napus* [22], *T. aestivum* [20], *S. bicolor* [23], *O. sativa* [24], *Z. mays* [25], *C. lacryma-jobi* [26], *P. sylvestris* [27].

phan replaces arginine (Table 2). In neither of the two examined carrot lines editing does affect the mode of alteration of the encoded amino acids (Tables 1 and 2).

The characteristic feature of the carrot transcripts is the editing resulting in silent codon alterations. We observed two codon changes corresponding to silent events. In the case of fertile carrot, they were found in codons 13 and 34, for the petaloid carrot only in codon 13 (Tables 1 and 2).

Data collected in Table 2 show that most of the edited codons are evolutionarily conserved in all angiosperms. It is interesting to note that two silent changes which appeared in codons 13 and 34 of carrot are very rare among plants. The peculiar feature of RNA editing of the *nad*3 transcripts, is the preediting of codon 84 in carrot, sunflower and wheat in spite of the overall panediting of this triplet in the rest of plants [17, 20].

Generally it seems that a part of differences in genomic sequences between all the investigated species are minimized by editing of their mRNAs.

Differential editing of the *nad*3 transcripts in fertile and CMS carrots raises the possibility that regulation of the extent of RNA editing could generate multiple forms of NAD3 gene products. In this study we have demonstrated that, in carrot mitochondria, the partially edited codon 30 could eventually lead to the change of one amino-acid residue in the protein sequence.

The presented data report for the first time that petaloid and fertile carrots differ in the nucleotide sequence and editing of RNA of *nad*3 gene.

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