Theor Appl Genet (2000) 100:263–270

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Organisation and expression of mitochondrial *atp9* genes from CMS and fertile carrots

Received: 29 March 1999 / Accepted: 30 April 1999

Abstract The F_0-F_1 ATPase subunit 9 gene of carrot mitochondria has been isolated from both petaloid male-sterile (*atp9-1*) and normal fertile cytoplasms (*atp9-3*). The position occupied in *atp9-3* by the TAA stop codon is, in the case of *atp9-1* replaced by the CAA triplet coding for glutamine, which makes this latter ORF 13 amino acid residues longer than that of the *atp9-3*. The 3' end of *atp9-3* gene is flanked by a direct repeat of 42 bp. The sequence of the repeat unit is also present at the 3' end of *atp9-1* but without reiteration. A truncated and presumably inactive version of *atp9* (*atp9-2*) was found to be present in cytoplasms regardless of the fertility phenotype which they condition. The *atp9-1* gene from petaloid cytoplasm appeared to be co-transcribed with the gene coding for 5S rRNA, and nuclear background influenced the accumulation of the respective transcript. The results are discussed with respect to a potential role of *atp9-1* in generating the petaloid form of CMS.

Key words Cytoplasmic male sterility · F_0-F_1 ATPase subunit 9 · Mitochondrial DNA · Carrot ·*Daucus carota*

Introduction

Cytoplasmic male sterility (CMS) is a maternally inherited inability of a plant to produce functional pollen. Sterility-inducing cytoplasms have been identified in over 150 plant species (Mackenzie et al. 1994). The action of specific nuclear genes can suppress the cytoplasmic dysfunction and restore fertility. For several plant species CMS-associated DNA sequences have been identified (Schnable and Wise 1998). They are located in the mitochondrial genome and often contain chimeric open reading frames (ORFs). Recently, the use of map-based cloning has led to the first successful isolation of the restorer gene *rf2* which is required for fertility restoration in T-cytoplasm maize (Cui et al. 1996).

Two CMS types are widely used for carrot hybrid seed production: brown-anther and petaloid sterility (Stein and Nothnagel 1995). Brown-anther sterility has been observed in many open-pollinated varieties and is characterised by deformed anthers which are a consequence of tapetum degeneration (Michalik 1971). Petaloid CMS was found in wild carrot (Thompson 1961; McCollum 1966) and subsequently introduced into cultivated germplasm (Eisa and Wallace 1969). Petaloid plants lack stamens which are transformed into petals or petal-like, bract-like or carpelloid structures depending on the nuclear background of the sterile cytoplasm (Erickson et al. 1982; Kitagawa et al. 1994). Cytoplasms of a few wild *Daucus* species were proven to maintain sterility upon their introduction into cultivated forms (Nothnagel et al. 1997). Genetic experiments indicate that several major nuclear genes control CMS in carrots (for review see Börner et al. 1995). Recently, a project has been undertaken to map restorer genes of the petaloid CMS system (Westphal and Wricke 1997).

Several reports have been dedicated to molecular aspects of cytoplasmic male sterility in carrot (Tanno-Suenaga and Immamura 1991; Scheike et al. 1992; Ronfort et al. 1995; Wright et al. 1996; Bowes and Wolyn 1998). Nevertheless, CMS-associated sequences have not been identified in this species. Szklarczyk (1997) reported that the *atp9* gene of the petaloid form exhibits Southern and transcription profiles which are different to those obtained for fertile and brown anther cytoplasms (Szklarczyk 1997). The purpose of the investigation reported here was to examine in more detail the

Communicated by R. Hagemann

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organisation and transcription of the carrot *atp9* gene from petaloid and fertile cytoplasms.[1]

Materials and methods

Plant material

The following carrot petaloid CMS lines (designation A) and their respective mantainers (B) were provided by Production and Breeding of Horticultural Plants Ltd. (PHRO), Krzeszowice, Poland: 2158A and 2158B, 2163A and 2163B, 2874A and 2874B, 9370A and 9370B, A88A and A88B, 52274FeA and 52274FeB. Line 1028A, exhibiting the brown-anther type of sterility, and *Daucus carota carota* plants were also provided by PHRO, Krzeszowice.

Other investigated accessions were bred and grown at the Institut für Züchtungsmethodik bei Gemüse in Quedlinburg, Germany. Those included: 908 and 1039 (brown-anther CMS lines), 932 (petaloid CMS line), 'Marktgärtner' (open-pollinated cultivar), *Daucus carota gummifer*, *D. halophilus*, hybrid *D. halophilus* × *D. c. sativus*, *D. c. commutatus*, hybrid *D. c. commutatus* x *D. c. sativus* and *D. c. maritimus*.

Isolation of nucleic acids

Total cellular DNA for the polymerase chain reaction (PCR) was extracted from young leaves according to Gawal and Jarret (1991) with an additional chloroform extraction step included. Prior to this step the aqueous phase collected from the first extraction was supplemented with a 1/10 volume of 10% CTAB, 0.7 *M* NaCl solution.

Carrot mitochondrial (mt) DNA was isolated from root tissue using the procedure of Steinborn et al. (1992). The ammonium acetate precipitation step was omitted.

Plasmid DNA to be used as a sequencing template was isolated with Qiagen Plasmid Midi Kit; otherwise, plasmid minipreparations were utilised (Le Gouill et al. 1994). PCR fragments for labelling and sequencing were purified using the High Pure PCR Product Purification Kit purchased from Boehringer Mannheim.

Total cellular RNA was extracted from young umbels using the modified AGPC method. After the first isopropanol precipitation described in the original procedure (Chomczynski and Sacci 1987) the RNA pellet was re-hydrated and LiCl re-precipitated (Gelvin et al. 1991).

DNA cloning

The mtDNA library of petaloid line 2163A was constructed in Bluescript II KS phagemid vector (Stratagene). The ligation mixture contained 4 μg *Bam*HI- (MBI Fermentas) -digested mtDNA, 2.1 μg *Bam*HI-digested and dephosphorylated (shrimp alkaline phosphatase from USB) vector DNA and 2 U T4 DNA ligase (Boehringer Mannheim). The ligation mixture was incubated for 3 h at 20°C, then for 10 h at 12°C and subsequently transformed into *E. coli* strain TG-1 using the $CaCl_2$ method (Sambrook et al. 1989). The resulting library was screened with a PCR-amplified fragment of the beet *atp9* gene. Colony lifts were prepared on Hybond N filters (Amersham Life Science). For hybridisation details see the description of *RNA gel blot analysis* that follows. The insert size of the positive clones was determined using *Bam*HI digestion.

[1] The nucleotide sequence data are available in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers AJ009697 (*atp9-1*), AJ009982 (*atp9-2*) and AJ009824 (*atp9-3*)

DNA amplification and sequencing

PCR was performed in 25-μl aliquots containing: 10 m*M* TRISHCl, pH 8.8, 50 m*M* KCl, 0.08% P-40, 2 m*M* $MgCl_2$, 0.2 m*M* each of the four dNTPs, 0.22 m*M* each primer, 20 ng of template genomic DNA and 1 U *Taq* polymerase (MBI Fermentas). PCR was carried out in an MJ Research PTC-200 thermal cycler programmed as follows: an initial cycle 94°C for 5 min; 35 cycles of 92°C for 45s, 57°C for 45s and 72°C for 1.5 min; followed by a final cycle of 72°C for 10 min. Primer sequences were as follows (5' → 3'):

A) TTC AGT TCT TTG ATT CAT TC,
B) ATT GAC TTC TGA TCG TGA CT,
C) GCG CAA TAC TTC TTC GTG AA,
D) CAT TCC GAC CTC GAT ATG TG,
E) TCA GAA GAA TCA GAC CCT TG.

DNA sequencing was performed using dye terminator premixes purchased either from Amersham Life Science (Thermo Sequenase Dye Terminator Cycle Sequencing Pre-mix Kit) or Perkin Elmer (ABI Prism BigDye Terminator Cycle Sequencing Kit). Sequencing reactions were set up and cycled according to the manufacturer's recommendation. Reaction products were resolved and analysed using the ABI Prism 377 DNA Sequencer.

MtDNA gel blot analysis

MtDNAs were digested with restriction endonucleases (MBI Fermentas) under conditions recommended by the supplier. RNase A was also included in the reaction mixture at a concentration of 0.1 mg/ml. Restriction fragments were electrophoresed on 1% agarose gels run overnight at 2 V/cm. Approximately 5 μg of mtDNA was loaded on each lane. DNA separations were capillary-blotted onto positively charged nylon membranes (Boehringer Mannheim) and then UV-crosslinked according to standard procedures (Sambrook et al. 1989). The filters were hybridised overnight at 68°C in the Boehringer standard hybridisation buffer containing 25 ng/ml DIG-labelled DNA probe. The membranes were subsequently processed according to the supplier's instructions. Hybridisation signals were visualised using a chemiluminescent detection system (Boehringer Mannheim). Detailed recipes for DIG-related techniques are given in *DIG System User's Guide for Filter Hybridization* (Boehringer Mannheim 1995).

The *atp9* probe was labelled with DIG-11-dUTP (alkali labile, Boehringer Mannheim) using PCR. The reaction mixture contained 0.07 m*M* DIG-11-dUTP, 0.13 m*M* dTTP and 1 ng of unlabelled DNA (a PCR-amplified fragment obtained from the genomic template). All other components of the reaction were the same as in conventional PCR (see above).

RNA gel blot analysis

Total RNA (30 μg) was fractionated on a standard formaldehyde gel (1.2% agarose) and transferred to GeneScreen Plus membrane (Du Pont) by capillary blotting (Sambrook et al. 1989). A 25-ng aliquot of PCR-amplified DNA probe was labelled with Redivue [^{32}P]-dCTP using the Rediprime DNA Labelling System from Amersham Life Science. Hybridisation was performed overnight at 50°C in 50% formamide, 7% SDS, 0.12 *M* Na_2HPO_4, 0.25 *M* NaCl. Membranes were washed twice for 5 min in 2 × SSC, 0.1% SDS at room temperature and then twice for 15 min. in 0.5 × SSC, 0.1% SDS at 50 °C.

Reverse Transcription (RT) PCR

One microgram of total RNA was used in a 10-μl reaction of first-strand cDNA synthesis driven by SuperScriptII reverse transcriptase (Gibco BRL) according to the proportions and conditions giv-

en by the manufacturer. The reaction was primed with the oligonucleotide: 5'-CTA CAT ATC ATA TCA T-3'. For PCR (see above) 1 µl of the cDNA preparation was used.

Results

Cloning and sequencing of *atp9* from petaloid cytoplasm

In order to isolate the carrot *atp9* gene we constructed a mtDNA library of petaloid cytoplasm. The library was screened with a fragment of beet *atp9* gene containing a 211-bp conserved 5' portion of the coding sequence. The Southern blot of *Bam*HI-digested mtDNA of petaloid carrots exhibits three signals, corresponding to fragment sizes of about 6, 7 and 8 kb (Fig. 1). Therefore, only those of the recovered positive clones that possessed an insert of 6 kb or more were considered to be true positives and subsequently *Hin*dII digested in order to eliminate duplicates. Finally, three clones were selected for primer walk sequencing: 340 (6 kb), 473 (7 kb) and 653 (7kb).

Inserts of the clones 473 and 653 contained a 270-bp open reading frame which was identified as the *atp9* gene on the basis of sequence homology. This ORF, given the designation *atp9-1*, is capable of encoding a polypeptide of 89 amino acid residues. The sequence corresponding to the first 74 amino acids revealed close homology to the respective fragment of *atp9* genes from other plant species - the sequence identity is of about 80% and 90% at the DNA and protein levels respectively. The remaining 3' stretch corresponding to the 15 C-terminal amino acids did not exhibit significant sequence similarity to any other gene, although some plant species also possess specific carboxy extensions of the conserved *atp9* 'core' sequence (Bland et al. 1986; Young et al. 1986; Schuster and Brennicke 1989; Xue et al. 1989; Bonhomme et al. 1989; Recipon 1990; Kazama et al. 1990; Kaleikau et al. 1990; Dell'Orto et al. 1993; Kato et al. 1995; Laser et al. 1995). The 5'-flanking region of *atp9-1* contains an *rrn5* gene coding for 5S rRNA (Fig. 2). The *rrn5* locus is situated on the same strand between positions -332 and -212 upstream of the *atp9* gene. An examination of the region downstream of the *atp9* sequence revealed a homology with a fragment of the *Arabidopsis thaliana* mitochondrial genome. The function of this fragment is unknown.

The clone 340 appeared to contain a truncated version of the *atp9* gene, further referred to as *atp9-2*. Its sequence forms a 147-bp ORF corresponding to a 48 amino acid polypeptide. The deduced protein sequence exhibits high homology to the N-terminal part of the *ATP9-1*. There are 3 amino acid substitutions in *ATP9-2* in relation to the respective fragment of *ATP9-1*. Corresponding DNA sequences show 83% identity. Genomic surroundings of the truncated gene differ completely from those found in clones 473 and 653 (Fig. 2). The sequence adjacent to the 5' end of *atp9-2* contains a region of homology to plant mitochondrial *orfB* genes (Hiesel et al. 1987; Quagliariello et al. 1990; Gualberto et al. 1991; Bonhomme et al. 1992; Krishnasamy and Makaroff 1993; Kubo and Mikami 1996,). About 220 bp downstream of the *atp9-2* an open reading frame of 618 nucleotides is located that was identified as the *nad6* gene.

Fig. 1 Southern blot analysis of *Bam*HI-digested carrot mtDNAs hybridised with the *Beta vulgaris atp9* probe. *Lanes: 1* Line 2158A (CMS petaloid), *2* line 2158B (fertile), *3* line 2163B (fertile)

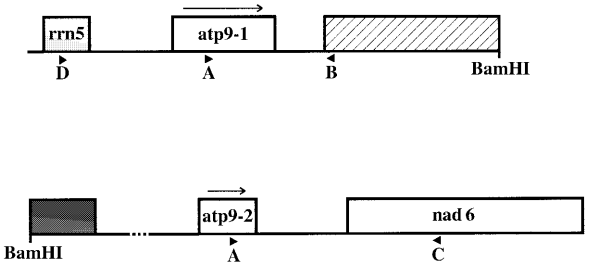

Fig. 2 Organisation of the *atp9* loci from carrot petaloid cytoplasm. *Open boxes* open reading frames for *atp9-1, atp9-2* and *nad6* as noted. *Shaded boxes, light shade rrn5* gene, *dark shade* a region of homology to *orfB, diagonal shade* a region of homology to the specific region of *Arabidopsis thaliana* mtDNA. *Arrows* indicate the direction of transcription, *arrowheads* mark the location of primers A, B, C and D. *Bam*HI Terminal *Bam*HI site of the clone

Occurrence of *atp9-1* and *atp9-2* sequences

Once the *atp9-1, atp9-2* and their flanking regions were determined it was possible to screen carrot germplasm for the presence of these sequences. Three primer PCRs were carried out to amplify fragments of both genes simultaneously in such a way that respective products could easily be differentiated from each other. The primer set consisted of one forward primer, (A) matching in the internal part of both carrot *atp9* sequences, and two reverse primers (B and C) anchored within flanking regions of *atp9-1* and *atp9-2*, respectively. The *atp9-1*-specifying fragment of 337 bp (approx. 0.34 kb) was expected for the primer pair A/B. The second combination A/C was to yield a product of 542 bp (approx. 0.54 kb) carrying a piece of *atp9-2*.

The screen involved a number of carrot accessions harbouring cytoplasms from various sources (Fig. 3). The longer PCR product corresponding to the *atp9-2* se-

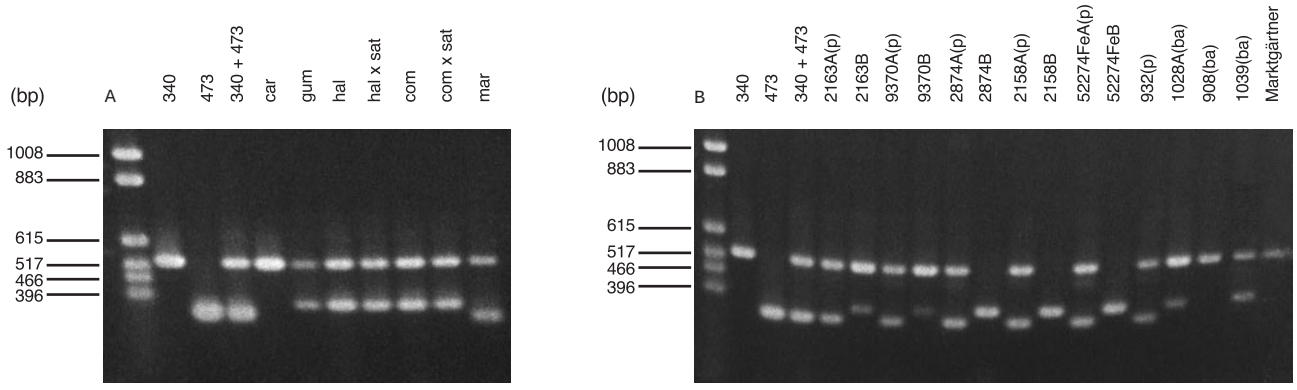

Fig. 3A, B PCR amplification of the *atp9-1* and *atp9-2* marker products. DNA of clones 340 and 473 was used as a positive control. **A** *car Daucus carota carota, gum D.c. gummifer, hal D. halophilus, hal × sat* hybrid *D. halophilus × D.c. sativus, com D.c. commutatus, com × sat* hybrid *D.c. commutatus × D.c. sativus, mar D.c. martimus*. **B** Carrot breeding material: *p* CMS petaloid line, *ba* CMS brown-anther line, *B* fertile line

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      L   F   A   L   M   M   A   F   L   I   L   S   V   F   Q
1   TTG TTT GCT CTA ATG ATG GCT TTT CTG ATC TTA TCT GTA TTC CAA   225
3   TTG TTT GCT CTA ATG ATG GCT TTT CTG ATC TTA TCT GTA TTC CAA   225
      L   F   A   L   M   M   A   F   L   I   L   S   V   F   Q

      I   Q   N   R   V   Y   I   E   Y   C   K   L   I   D STOP
1   ATT CAA AAT AGA GTC TAT ATC GAA TAT TGC AAG CTT ATA GAT TGA   270
3   ATT TAA AAT AGA GTC TAT ATC GAA TAT TGT AAG CTT ATA GAT TGA   270
      I STOP

1   TAG AAC TAC AAG GGT CTG ATT CTT CTG AAT AAG   -   -   -   -   303
3   TAG AAC TAC AAG GGT CTG ATT CTT CTG AAT AAG ATA GAT TGA TAG   315

1     -   -   -   -   -   -   -   -   -   - ATG ATA TGA TAT GTA   318
3   AAC TAC AAG GGT CTG ATT CTT CTG AAT AAG ATG ATA TGA TAT GTA   360
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Fig. 4 Sequence composition at 3′ end of carrot *atp9-1* (*1*) and *atp9-3* (*3*) genes. Predicted amino acid sequences are indicated *above atp9-1* and *below atp9-3*. Nucleotides are *numbered* from the deduced translation start of both *ap9* genes. Nucleotide substitutions are boxed. *Light* and *dark shading* denote the location of the repeat units. *Dashes* represent the region missing at the 3′ end of *atp9-1*. The position of the reverse primer E used for RT-PCR amplification is *underlined*

quence appeared to be present in most of the accessions studied regardless of their fertility phenotype. All CMS lines and wild *Daucus* species were found to possess this amplicon. Fertile lines differed with respect to this character, two of them also displayed the 0.54-kb product but the remainding three did not.

As far as cultivated carrot germplasm is concerned the *atp9-1* marking product was found exclusively in petaloid lines. It was also evident in the pattern obtained for *Daucus carota maritimus*. Other accessions, namely fertile and brown-anther lines as well as forms bearing *D.c. gummifer*, *D.c. commutatus or D. halophilus* cytoplasm, lacked the 0.34-kb fragment. Instead, all these accessions exhibited a longer product of 0.38 kb. No genotype was found exhibiting both the 0.34-kb and 0.38-kb fragments.

Isolation of the *atp9* gene from fertile cytoplasm

In order to identify the structural basis of the PCR polymorphism we directly sequenced the 0.38-kb product obtained for fertile line 2874. Similar to what was expected for the *atp9-1* marker product, the 0.38-kb fragment was found to be terminated by A and B primer sequences. Likewise, the whole of its sequence appeared to be almost identical to the respective region from petaloid cytoplasm and contained the 3’ portion of the *atp9* ORF together with the 3’ flanking region. However, this carboxy terminal part of the *atp9* ORF turned out to be shortened here due to the TAA stop codon situated at the position occupied by the third glutamine CAA triplet in the *atp9-1* ORF (Fig. 4). A direct repeat of 42 bp is another specific structural feature of the amplified fragment. The first repeat unit is located 30 bp downstream of the *atp9* stop codon and followed by the second unit without any spacing region. The sequence of the repeat unit is also present at the 3’ border of the *atp9-1* ORF but without any reiteration. Three C-terminal codons of the *atp9-1* ORF overlap this region.

Another PCR experiment was designed for the purpose of isolating the full-length *atp9* gene from fertile carrot cytoplasm. The forward primer D was matched within the *rrn5* gene known to flank *atp9-1* sequence at the 5’ end, and B served again as an anchor for the 3’ flanking region. Using male-fertile line 2874 as a source

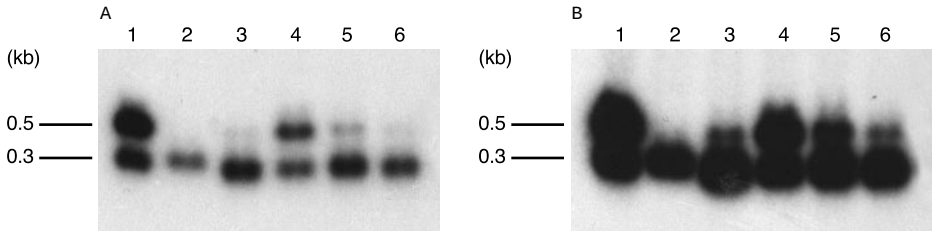

Fig. 5A, B Northern hybridisation analysis of the carrot *atp9* locus. *Lanes: 1* 2874A (sterile plant), *2* 2874B (fertile), *3–6* partially fertile plants of 2874A. **A** and **B** represent different exposures of the same blot

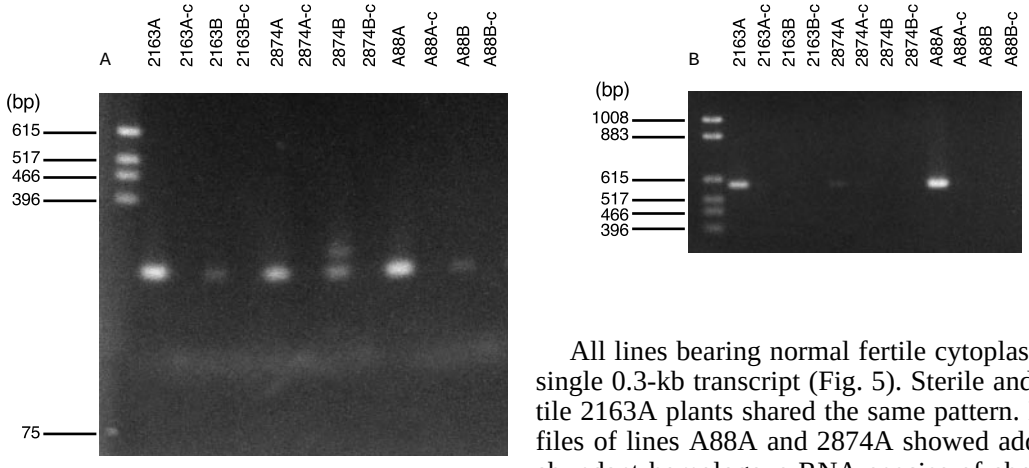

Fig. 6A, B RT-PCR analysis. **A** Amplification primers A and E, **B** amplification primers D and E. *c* Negative control lane (reverse transcriptase not added to the first-strand synthesis reaction)

of the genomic template, we obtained a fragment of 0.75 kb. It contained the sequence found previously in the 0.38-kb product together with the sequences identical to those of petaloid cytoplasm: the remaining 5' part of the *atp9* gene and its 5' flanking region extended to the D primer stretch (Fig. 4). The newly identified *atp9* ORF, further referred to as *atp9-3*, consists of 231 nucleotides which could encode a 76 amino acid polypeptide lacking the 13 C-terminal residues of *ATP9-1*. The successful amplification of the full-length gene from the fertile cytoplasm also provided evidence that both *atp9-1* and *atp9-3* are located in a similar genomic environment.

Transcription of *atp9* sequences

In a series of Northern blot experiments total umbel RNA separations were probed with the amplified fragment of the *Beta vulgaris atp9* gene previously utilised for library screening (see above). The following lines were included in the analysis: A88A, A88B, 2163A, 2163B, 2874A and 2874B. They carry petaloid (A) or fertile (B) cytoplasm in one of the three nuclear backgrounds specified as A88, 2163 and 2874. Due to the instability of the CMS trait, each petaloid line contained a certain fraction of partially male-fertile plants which were also examined.

All lines bearing normal fertile cytoplasm exhibited a single 0.3-kb transcript (Fig. 5). Sterile and partially fertile 2163A plants shared the same pattern. Northern profiles of lines A88A and 2874A showed additional, more abundant homologous RNA species of about 0.5 kb. As partially fertile A88A individuals demonstrated a pattern identical to that of the sterile plants, their counterparts from the line 2874A showed a reduced level of the 0.5-kb transcript in comparison with those exhibiting the stabile phenotype.

The same set of lines was also studied using an RT-PCR approach. First-strand cDNA synthesis was accomplished using the oligonucleotide anchored directly behind the repeat unit. Two forward primers were utilised: A, matching in the 5' region of *atp9* ORF, and D, covering a piece of the *rrn5* gene. The reverse primer E was complementary to the fragment of the repeat unit.

For each analysed line primer pair A/E yielded a product of approximately 0.2 kb (Fig. 6A). In the case of fertile cytoplasms this band corresponds to the primer extension proceeding from the repeat unit proximal to the *atp9* coding sequence. Fertile line 2874B showed an extra fragment of about 0.25 kb, which is expected when priming takes place within the distal repeat unit. Petaloid lines exhibited a relatively higher yield of the product than their fertile counterparts.

In another PCR experiment the same cDNA template samples were used with primers D and E. An amplification product was obtained only for petaloid lines (Fig. 6B). The size of approximately 0.6 kb was consistent with the one expected from the sequence data.

Discussion

Most types of CMS studied so far fall into Kaul's (1988) category of sporogenous male sterility where cases manifested with microsporogenesis or gametogenesis impair-

ment were assigned. In that context carrot petaloidy represents a distinct kind of alteration in which the cytoplasmic factor promotes a shift in the whole programme of flower development. Depending on nuclear genotype, the same sterility-inducing cytoplasm produces a range of phenotypes where stamens are replaced by petals or petal-like, bract-like or carpelloid structures. (McCollum 1966; Erickson et al. 1982; Kitagawa et al. 1994). Hence, petaloid carrots resemble certain mutants of nuclear homeotic genes (Börner et al. 1995). Petaloidy was found for the first time in this species in the early 1950s among wild American carrots (Thompson 1961). Later, the occurrence of petaloid plants in a wild population received from Sweden was reported by McCollum (1966). According to recent data all cultivated petaloid forms which have been investigated to date show the same mitotype (Szklarczyk 1997). This is likely to be a consequence of their common origin, although it can not be excluded that independent recombination events, resulting in the same mitotype, took place in distinct accessions.

In the course of this research two sequences of homology to *atp9* were identified in the mitochondrial genome of petaloids. On the basis of Southern hybridisation data two groups of fertile cytoplasms can be distinguished, possessing one or two sequences of homology to *atp9,* respectively (Szklarczyk 1997). As demonstrated in our PCR screen the difference can be attributed to the lack or presence of the truncated *atp9-2* version of the gene. With respect to the organisation of *atp9*, brown-anther carrots seem to be similarly structured as the latter group of fertile cytoplasms (data not shown). Interpretation of Southern profiles for carrot *atp9* sequences is often hindered due to low emission signals which may correspond either to local homologies or to substoichiometric *atp9* molecules (Small et al. 1987). The amplification product marking the presence of *atp9-1* appeared to be characteristic to petaloid forms as far as cultivated germplasm is concerned. Out of the five wild subspecies and species examined only cytoplasm of *Daucus carota maritimus* was found to share that amplification pattern. Interestingly, Nothnagel et al. (1997) report on the identification of a ‘petaloid-like’ plant from a population of *D. c. maritimus*. Its cytoplasm also appeared to confer this type of sterility after introduction into cultivated germplasm.

Northern profiles of the petaloid lines studied here show extensive differences in 0.5-kb transcript accumulation ranging from abundance (A88A) through reduced levels in partially fertile plants (2874A) to an apparent lack of that RNA species (2163A). All fertile lines lacked the 0.5-kb transcript. Our RT-PCR experiments provided evidence that in mitochondria of petaloids co-transcription of *atp9* and *rrn5* genes occurs. Therefore, it can be assumed that the 0.5-kb RNA represents a co-transcript of the *atp9* and *rrn5* genes. Previously, even larger *atp9* transcripts were identified specifically in petaloid plants (Szklarczyk 1997), i.e. the co-transcription of even more distant upstream sequences cannot be excluded. Accumulation of this transcript displayed by 2163A plants can be too low to be detected on Northern blots but sufficient for RT-PCR. Despite the location of the amplification primers within the repeat unit only one of the examined fertile lines exhibited RT-PCR products arising from both copies of reiterated sequences. This might be due to an altered editing pattern of *atp9* RNA which could affect correct priming in certain genotypes. Differential editing might also be responsible for divergent processing of the 0.5-kb transcript in petaloids as has been proposed for *orf107* transcripts of sorghum (Pring et al. 1998).

All known higher plant mitochondrial *atp9* genes share a highly conserved sequence corresponding to 74 amino acid residues. In some species this sequence occupies the whole open reading frame, while others exhibit N- or C-terminal extensions of that core sequence. Interestingly, no species were found having both the amino and carboxy extension of the *atp9* gene. In most plant *atp9* genes possessing the C-terminal extension the conserved sequence is followed by arginine CGA or glutamine CAA triplets which can be converted to the respective stop codons via C to U editing events. In the deduced carrot *ATP9-1* and *ATP9-3* sequences the core region also appears to be C-terminally elongated by 15 and 2 residues, respectively. The *ATP9-3* extension consists of two codons, for glutamine (CAA) and isoleucine. The *atp9-1* extension exhibits 13 additional codons, of which the first is again that for glutamine, so the editing of this may promote formation of *ATP9-3*-like protein.

The wide occurrence of the truncated *atp9-2* among fertile, cytoplasmic male-sterile and wild carrot accessions indicates that it cannot be associated with any of CMS systems. Fertile plants possessing *atp9-2* show only one homologous transcript which, according to RT-PCR data, arises from the *atp9-3* sequence. Therefore, it can be assumed that the *atp9-2* sequence remains untranscribed.

According to the recent report of Chahal et al. (1998) fertile plants were recovered from carrot petaloid CMS line. The reversion to fertility appeared to be cytoplasmically conditioned and accompanied by rearrangements of a 108-kb subgenomic circle present in the CMS line. Three RFLPs differentiating fertile and revertant cytoplasms from that of the sterile form were identified and mapped to that subgenomic molecule. One of those polimorphic fragments was detected with the *atp9* probe; it was present in the CMS plants but lacking in fertile and revertant cytoplasms. The authors suggest that the reversion which they found could have resulted from the amplification of submolar sequences of the fertile cytoplasm type. We find it to be more likely that the reversion is a consequence of a mutation or loss of the sterility-inducing sequence as in the case of T-cytoplasm maize (Levings 1993). This hypothesis also explains the results of Chahal et al. (1998) and correlates them with the CMS-specific organisation and expression of the *atp9* sequence reported here. Our data are in good agreement with, but cannot prove, the proposal that the *atp9-1* version of the gene is responsible for the CMS pheno-

type in carrots. The C to U conversion in the second glutamine triplet of the extension could be speculated to be a mechanism of fertility restoration. In this context, the reduced level of the 0.5-kb transcript in partially fertile individuals of line 2874A could only be a secondary symptom and hence not observed in restored plants with a different nuclear background. We cannot rule out another possible explanation, namely that *atp9-1* is represented only by the 0.5-kb transcript, while the 0.3-kb RNA would arise from an as yet undetected *atp9-3*-like version of the gene.

Acknowledgements The research was supported by the Polish State Committee for Scientific Research (grant no. 03910) to B.M. and by the Fond der Chemischen Industrie, Frankfurt/M., and Deutsche Forschungsgemeinschaft, Bonn, to T.B. M.S. received a fellowship from the German Academic Exchange Service (DAAD). The experiments comply with the current laws of Germany and Poland.

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