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A transcriptional alteration on the *atp9* gene is associated with a sunflower male-sterile cytoplasm

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Abstract The transcriptional patterns of six mitochondrial genes were studied in different nuclear backgrounds for four sunflower cytoplasmic male sterility (CMS) lines. Probes coding for *cob*, cox*I*, *coxII* and *atp6* did not detect differences between the CMS lines and male-fertile lines. *atpA* transcripts seemed to be dependent on the cytotype but were not related to the CMS trait in the genotypes studied. However, the *atp9* probe detected transcripts of 500 and 1,100 nt in the CMS-PEF1, while the fertile forms showed a single transcript of 650 nt. Nuclear genes that restore fertility specifically rectified this alteration, suggesting that modified *atp9* transcripts are associated with CMS in PEF1 sunflower. Cloning and sequencing of *atp9* from CMS-PEF1 and comparison with the corresponding male-fertile gene showed the insertion of a 0.5-kbp sequence in the 3' UTR of the gene that may be at the origin of the PEF1-CMS and the altered transcripts detected in this study.

Keywords *Helianthus annuus* · Mitochondria · RNA · CMS · *atp9*

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Introduction

Cytoplasmic male sterility (CMS) is a maternally inherited trait in which plants fail to produce functional pollen. In plant CMS lines the male-sterile trait appears to be associated with mitochondrial DNA (mtDNA) rearrangements, resulting in the expression of chimeric genes believed to interfere with normal pollen development. Male fertility can be restored by the introduction of nuclear fertility-restoring genes that compensate for this deficiency (reviewed in Schnable and Wise 1998).

Around 30 sources of CMS have been detected in sunflower, arising from inter- or intraspecific crosses and spontaneously in natural populations (listed in Crouzillat *et al*. 1991; Horn and Friedt 1999). Some of these cytotypes have been differentiated by their response to nuclear restorer genes and/or by applying restriction fragment length polymorphism (RFLP) methods to mtDNA (Crouzillat *et al*. 1994). Experimental evidence obtained in different plant species suggests that numerous mtDNA recombination events characterize each CMS. However, the first mapping studies in which mtDNA from sunflower CMS-PET1 was compared to an isonuclear fertile line showed a high similarity between them (Siculella and Palmer 1988).

The rearrangement of mitochondrial genes can lead to alterations in transcription patterns and, in some cases, recombination events have resulted in existing mitochondrial genes being altered, thereby producing modified proteins (e.g. *coxI* in sorghum, Bailey-Serres *et al*. 1986). Other consequences are alterations in regulatory sequences (e.g. *coxII* in *Beta vulgaris*, Senda *et al*. 1991) and the generation of a chimeric open reading frame (ORF). In sunflower, the creation of a new open reading frame (*orf H522*) that is cotranscribed with *atpA* in CMS-PET1 has been reported (Köhler *et al*. 1991; Laver *et al*. 1991). The gene product of *orf H522* is a 16-kDa polypeptide associated with CMS-PET1 (Horn *et al*. 1991), which is also detected by *in organello* translation in nine additional male-sterile cytoplasms (Horn *et a*l. 1996).

Table 1 Description of the sunflower CMS used in this study

CMS	Cytoplasm origin	Nucleus from line	Reference
PET1	*H. petiolaris*	HA89	Leclercq 1969
BOL1	*H. bolanderi*	PAH2	Serieys 1984
PEF1	*H. petiolaris* ssp *fallax*	HA89	Serieys 1984
ANL2	*H. annuus* ssp *lenticularis*	HA89	Heiser 1982

Table 2 Summary of the hybridization patterns of mtRNA from different CMS and fertile lines analyzed with six mitochondrial probes

[a] f, Fertile; S, sterile
[b] Transcript sizes are in kilobases (kb)

Line	*coxI*	*coxII*	*cob*	*atp6*	*atpA*	*atp9*
fHA89[a]	1.7[b]	1.2	2.1	1.3	2.2 + 2.0	0.65
fPAH2	1.7	1.2	2.1	1.3	1.6	0.65
sBOL1	1.7	1.2	2.1	1.3	1.6	0.65
sPEF1	1.7	1.2	2.1	1.3	2.2 + 2.0	0.5 + 1.1
sANL2	1.7	1.2	2.1	1.3	1.6	0.65
sPET1	1.7	1.2	2.1	1.3	2.2 + 2.0 2.8 + 3.0	0.65

Recently, several sunflower CMS lines have been analyzed for the presence of a new ORF associated to this trait and for the presence of CMS-specific, mitochondrially-encoded proteins (Horn and Friedt 1999). However, this study failed to detect the specific 16-kDa protein in several cytotypes (e.g. PEF1) and, hence, in these cases the molecular basis of the CMS remains unknown. We used a different experimental approach here in an attempt to detect transcriptional alterations that may correlate with the CMS trait in three sunflower cytotypes not yet characterized at the molecular level. In addition, we investigated the organization of mitochondrial genes that may be at the origin of such altered transcripts and describe a rearrangement at the *atp9* locus of CMS-PEF1.

Materials and methods

Plant material

Two male-fertile sunflower (*Helianthus annuus*) inbred lines (HA89 and PAH2) and four CMS lines were used in this study. The CMS genotypes are described in Table 1. For each CMS, two nuclear restored forms (F2 and F5) were also examined. The CMS lines have the same mitochondrial genome as their restored forms but they lack nuclear fertility-restoring genes. The plant material was kindly provided by F.Vear (INRA Station d'Amelioration des Plantes, Clermont Ferrand, France) and H. Serieys (INRA Station d'Amelioration des Plantes, Mauguio, France).

RNA and DNA analyses

Mitochondrial RNA (mtRNA) was isolated from 10-day-old etiolated hypocotyles as reported by Chirgwin *et al.* (1979). Electrophoresis on 1.5% denaturing gels, blotting on Nylon N membranes and hybridization were carried out as described previously (de la Canal *et al.* 1991).Transcript sizes were estimated by comparison with the BRL RNA Ladder (0.24–9.5 kb).

MtDNA was prepared from leaves according to the procedure of Crouzillat *et al.* (1987). Methods for restriction endonuclease analysis, blotting and hybridization have been described by Crouzillat *et al.* (1994).

The following mitochondrial probes were used: *coxI* (Isaac *et al.* 1985), *coxII* (Fox and Leaver 1981), and *cob* (Dawson *et al.* 1984) from maize; *atp6*, *atp9* and *atpA* from sunflower were provided by Dr. H. Recipon (Université Paris XI, Orsay, France); the a*tp9* probe was a 0.5-kbp *Ava*I fragment containing the coding region and part of the 3' UTR from fertile sunflower HA89 (Recipon 1990). The probes were radioactively labelled using a random priming kit (Amersham, Les Ulis, France).

Cloning and sequencing

Sunflower mtDNA was digested with *Hind*III and fractionated by electrophoresis on a 0.8% agarose gel. Fragments of about 4.1 kbp were recovered by electroelution and ligated into pUC18, and the resulting recombinant plasmids were used to transform *E. coli*. Transformants were selected on the basis of their hybridization to the sunflower *Ava*I fragment (*atp9* probe). A positive clone containing the 4.1-kbp fragment was automatically sequenced using a gene walking strategy.

Results

The transcriptional pattern of six mitochondrial genes has been studied by Northern hybridization in two male-fertile sunflower inbred lines and four CMS lines. Table 2 summarizes the results obtained. A probe encoding *coxI* detected a single transcript of 1,700 nucleotides (nt) which appeared in all the samples tested. Probes coding for *coxII*, *cob* and *atp6* gave similar results, detecting transcripts of about 1,200, 2,100 and 1,300 nt, respectively. In agreement with Siculella and Palmer (1988), hybridization with *atpA* showed two altered transcripts for CMS-PET1, in addition to those seen in the isonuclear fertile line (fHA89); the CMS-BOL1 and ANL2 showed a different *atpA* transcript of about 1,600 nt. However, a transcript of the same size was observed in the male-fertile PAH2, suggesting that it may be a consequence of a specific nuclear-cytoplasm interaction unrelated to CMS.

Major differences in mitochondrial transcripts were observed with the *atp9* probe, which detected a single mRNA of about 650 nt in the male-fertile genotypes and all the CMS tested except PEF1, where two transcripts of about 500 nt and 1100 nt were present. Furthermore, when two CMS-PEF1 nuclear-restored genotypes were

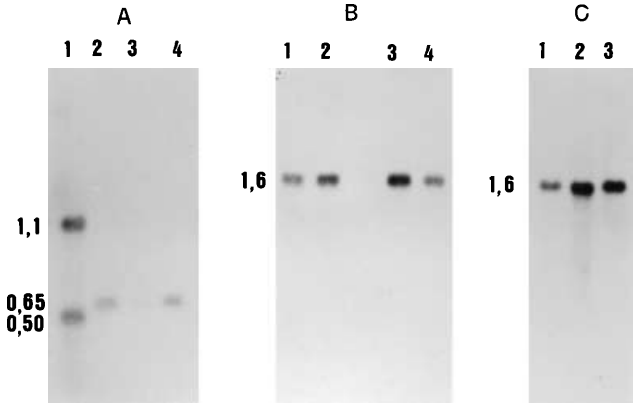

Fig. 1A–C Northern hybridization analysis of the following sunflower genes: *atp9* from CMS-PEF1 (**A**), *atpA* from CMS-BOL1 (**B**), and *atpA* from CMS-ANL2 (**C**). MtRNA was separated on a 1.5% agarose-formaldehyde gel, blotted, and probed with the corresponding probes. Quantitative variation in the levels of the transcripts is the result of differences in the amount of RNA loaded in adjacent lanes. Transcript estimated sizes are given in kilobases (kb). **A** *lane 1* CMS-PEF1, *lane 2* nuclear restored CMS-PEF1 (F_2), *lane 3* nuclear-restored CMS-PEF1 (F_5), *lane 4* male-fertile HA89. **B** *lane 1* CMS-BOL1, *lane 2* nuclear-restored CMS-BOL1 (F_2), *lane 3* nuclear-restored CMS-BOL1 (F_5), lane 4 male-fertile PAH2. **C** *lane 1* CMS-ANL2, *lane 2* nuclear-restored CMS-ANL2 (F_2), lane 3 nuclear-restored CMS-ANL2 (F_5)

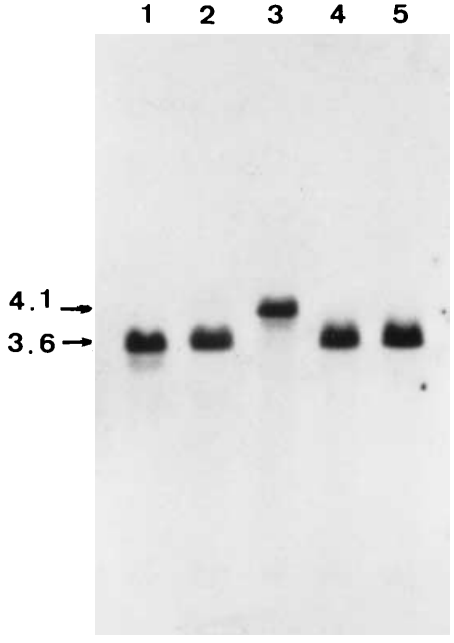

Fig. 2 Southern hybridization analysis of mtDNA from fertile sunflower and CMS lines restricted with *Hind*III and probed with *atp9*. *Lanes: 1* Male-fertile HA89, *2* CMS-PET1, *3* CMS-PEF1, *4* CMS-BOL1, *5* CMS-ANL2. Estimated fragment sizes are indicated in kilobases (kb)

tested, the *atp9* transcript detected was similar to that present in male fertile line (Fig. 1 A) and the other CMS line. Thus, the PEF1 *atp9* transcript varied in its response to nuclear restorer genes, suggesting its involvement in the CMS phenotype. On the other hand, this was not the case for the *atpA* transcript of CMS-BOL1 and ANL2, which showed the same transcription pattern in fertility-restored genotypes (Fig. 1B, C).

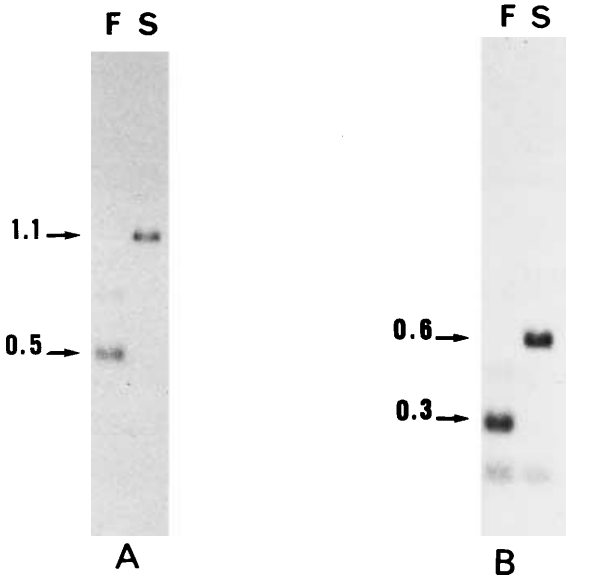

Fig. 3A, B Southern hybridization analysis of cloned *Hind*III fragments containing the *atp9* gene from male-fertile HA89(*F*) and CMS-PEF1 (*S*). *Hind*III fragments were digested with *Ava*I (**A**) or *Hae*III (**B**) and probed with *atp9*. Estimated fragment sizes are indicated in kilobases (kb)

To investigate the origin of the transcriptional alteration observed in CMS-PEF1 we studied the structure of the *atp9* gene in the CMS lines and a male-fertile genotype. MtDNA was digested with different restriction enzymes, blotted, and hybridized to an *atp9* probe. Figure 2 shows that *atp9* hybridized to a 3.6-kb *Hind*III fragment in the male-fertile line and all the CMS lines tested except PEF1; in the latter case a fragment of about 4.1 kbp was detected. Hence, *Hind*III fragments carrying the *atp9* gene from the male-fertile line HA89 and the CMS-PEF1 were cloned and their restriction patterns compared. Hybridization studies were performed with an *Ava*I probe containing all of the coding region of the male-fertile sunflower *atp9* and its 3' non-coding region (Recipon 1990). This probe recognized the 0.5 kbp *Ava*I fragment in the male-fertile genotype, but a larger fragment of about 1.1 kbp was detected in PEF1 (Fig. 3A). In addition, when *Hind*III fragments were digested with *Hae*III, different fragments hybridizing to the *atp9* probe were detected in both samples (Fig. 3B). These results confirmed the existence of rearrangements in the vicinity of the *atp9* locus of CMS-PEF1.

To establish more precisely the alteration present in CMS-PEF1, we sequenced the complete 3,931-bp *Hind*III fragment (accession number AF258785). Blast search analyses have shown that this mtDNA region contains a nucleotide sequence homologous to the ATPase subunit 9 gene (*atp9*) from several plant species, followed by a longer region homologous to NADH-ubiquinone oxidoreductase chain 5 *(nad5)* genes (Fig. 4). A de-

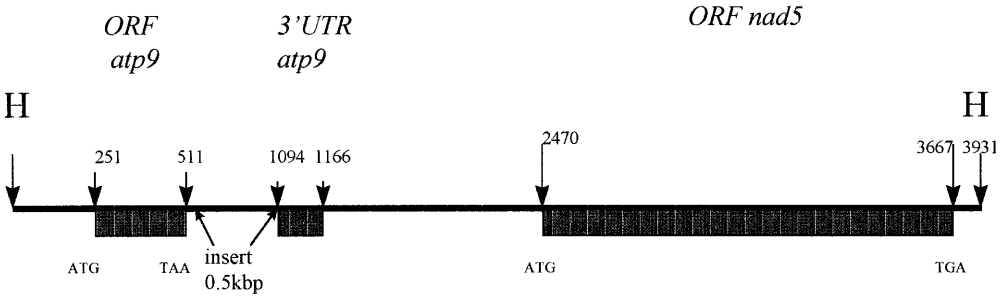

Fig. 4 Schematic representation of the CMS-PEF1 mtDNA *Hind*III fragment containing the *atp9* and *nad5* genes. Numbers indicate the relative positions (in base pairs) starting from the *Hind*III site

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ATGTTAGAAGGTGCAAAACTAATAGGGGCCGGAGCTGCTACAATTGCTTC      50
AGCGGGAGCTGCTATCGGTATTGGAAACGTCCTTAGTTCCTCGATTCATT     100
CCGTGGCTCGGAATCCATCATTGGCTAAACAATCATTTGGTTATGCCATT     150
TTGGGCTTTGCTCTAACCGAAGCTATTGCATCGTTTGCCCCAATGATGGC     200
CTTTCTGATCTCATCCGTATTCCGATCAAAGAATCAAAGAAAGAAGGTTG     250
AAGTTTCATAAAGCAAGCACCTCTTTCCAGCCTTAGGAAAAATGGAACCA     300
GATACATTCTTAAAGGAATGCGCTTTGCCTTACACAATATAAAAAAGAAA     350
AAGAGAATAAAAATAGAAAGATGAAGGAACAAAGTTGACACAATCCCTTT     400
CTTTCCGTTGGTCAACAACCAACAAAACAAAATCGTTTAGTTCTTCATCG     450
TACAGGAAGGCCTCTCTTTCTGTATGGGGGGAAGAAGACTCTAAAATAAA     500
ATGGAGAGAAACATTCCGGAATGGGTGGACGCCTTCACCACAGACATCAG     550
AGTGGTTTTTTGTTTTCTTTTCCTCCAAGGGCTCTTCGTGGTCTACCTTT     600
TTATCGCTCTTTTCGTCCATAGAAAGGATCGAAAAGAGCTTTCCACTCAG     650
GCATTTAGTACGGGGGGCAGTCTCAAATTTCATTATTCTTGGAAGGGTGT     700
CTTTCTCGAAATCAAACACCCCGGTTTGATTTCGAGAAGACACCCTTCTC     750
AGGGGGGCGAACCCCCGTCTCCGCCTTTAGGAGATTGAACGGCAGAAGGT     800
TGAAGTTTCATAAAGCAAGCACCTCTTTCCAGCCTTAGTCAAATAGCCAC     850
CGGAGGCAAGGGGCGCAGTTCTTAAACAGAAGAATGGCGATAAAGAGGAA     900
ATGATTCAAATTCAAG                                       916
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Fig. 5 Nucleotide sequence of *atp9* mtDNA from CMS-PEF1. Regions homologous to *atp9* from male-fertile sunflower are indicated in *bold.* The start and stop codons are indicated with *double underlining*. The *single underlined* sequence indicates the region also present in several sunflower mitochondrial genes

tailed analysis of the region containing the *atp9* gene showed that it displays 99% identity in the coding region with the previously reported sunflower male-fertile *atp9* gene (accession number X51895) but that it is interrupted by a 0.5-kbp insertion immediately downstream of the coding region (Figs. 4, 5). The inserted sequence is followed by the 3′ non-coding region of *atp9*, which exhibits homology (93% identity) with the corresponding nucleotide sequence of the male-fertile gene. Interestingly, part of the inserted sequence (underlined in Fig. 5) is homologous to common sequences present in several sunflower mtDNAs available in the Genebank (e.g. *cob*, *atpA*, *coxIII*) and may be implicated in site-specific recombination in mtDNA.

Finally, sequence analyses showed that the coding region of the *nad5* gene is located 2,220 bp downstream from the ATG codon of the rearranged *atp9* gene of CMS-PEF1, on the same strand. This sequence encodes for a predicted 399 amino acid protein that highly homologous (95%) to the reported exon a from *nad5* mitochondrial genes.

Discussion

Numerous investigations have established a correlation between mitochondrial rearrangements and the CMS phenotype, and usually these rearrangements also involve mitochondrial genes leading to altered transcription patterns. However, none of the six mitochondrial probes tested in this study detected altered transcripts correlated to CMS in the BOL1 and ANL2 sunflower cytotypes. Northern analysis of the *atpA* gene of these CMS lines showed that the transcript pattern is dependent on the genotype. Even if both CMS lines showed different *atpA* transcripts compared to the male-fertile line HA89, nuclear genes that restore fertility have no effect on their transcription patterns. Therefore, *atpA* appears not be implicated in the CMS phenotype of BOL1 and ANL2. In addition, as no novel polypeptides associated to these cytotypes have been detected (Horn and Friedt 1999), the molecular origin of these CMS cytotypes remains unknown. Among the three novel sunflower CMS studied here, only PEF1 showed transcriptional alterations that may be associated to CMS. In fact, the *atp9* gene of PEF1 showed two transcripts different from that found in the fertile lines and in the nuclear restored PEF1. The nature of the CMS-PEF1 nuclear-restorer genes is unknown, but Serieys and Vincourt (1987) observed the segregation of at least two dominant, independent and complementary restorer genes.

In sunflower CMS-PET1, defined rearrangements at or near the *atpA* locus correlate with CMS-associated differences in its pattern of transcripts (Siculella and Palmer 1988). However, Horn *et al*. (1991) reported that the *atpA* protein from fertile sunflower and the male-sterile lines PET1 and Baso showed similar, if not identical, molecular mass, isoelectric point, and peptide fingerprinting. According to their results a novel 16-kDa polypeptide present in the CMS and not *atpA* may play a role in CMS. In these cytotypes, besides one main transcript of the *atpA* gene in the fertile lines, the male-sterile lines showed additional larger transcripts. However, this is not the case of CMS-PEF1 studied here, where the *atp9* transcript seen in the fertile lines is absent and two novel RNA molecules appeared. In addition, *in organello* translation assays did not detect dif-

ferences in the protein pattern of CMS-PEF1 compared to the male-fertile HA89 (Horn and Friedt 1999), suggesting that there is no novel chimeric ORF at the origin of this sterility. From the sequence analyses performed in this work, no novel chimeric ORF could de deduced in the *atp9* locus but, instead, the existence of rearrangements in the 3' non-coding region of the *atp9* gene may be at the origin of the transcriptional alterations. The insertion of a 0.5-kbp sequence in the 3' UTR of *atp9* from CMS-PEF1 may explain the approximately 500-bp increase in the size of one of the transcripts detected in the CMS compared to the fertile cytotype. However, the fact that the 5' regulatory sequence of *atp9* from the male-fertile line has not been described, precludes a comparative analysis. Hence, its potential contribution in the determination of the sterile genotype cannot be excluded.

The central role of abnormal *atp9* transcripts in the CMS trait has been recently demonstrated by Hernould *et al.* (1998), who showed that in transgenic tobacco, expression of an unedited copy of *atp9* resulted in male sterility. Rearrangements of plant mtDNA often involve part of *atp9* sequences (e.g. Tang *et al.* 1996; Zabala *et al.* 1997). Also, sunflower CMS3 cytotype mtDNA contains a unique rearrangement of loci that involves the *atp9* gene (Spassova *et al.* 1994). Despite this evidence, to our knowledge this is the first report on rearrangements leading to CMS-associated altered *atp9* transcripts reverted by nuclear-restorer genes.

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