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Analyses of mitochondrial DNA structure and expression in three cytoplasmic male-sterile chicories originating from somatic hybridisation between fertile chicory and CMS sunflower protoplasts

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Abstract Cytoplasmic male-sterile (CMS) chicories have been previously obtained by somatic hybridisation between fertile industrial chicory protoplasts and CMS sunflower protoplasts. In this study, we compared three different CMS chicory cybrids that originated from three different fusion events. The cybrids were backcrossed with different witloof chicories in order to transfer the three male-sterile cytoplasms from an industrial chicory nuclear environment to a witloof chicory nuclear context. Southern hybridisation, using different mitochondrial genes as probes, revealed that the three cybrid mitochondrial genomes were different and that they were stable throughout backcrossing generations regardless of the pollinator. However, pollinators were found to influence floral morphologies – with one being able to restore fertility – showing that nuclear context can affect the sterility of the cybrids. PCR and RFLP analyses revealed that the *orf522* sequence, responsible for CMS in PET1 sunflower, was present in two out of the three cytoplasms studied, namely 411 and 523, but was absent from the other cytoplasm, 524. We thus concluded that *orf522* is not responsible for CMS in the 524 cybrid. Although the *orf522* gene is present in the 411 and 523 cytoplasms, it is probably not responsible for the sterile phenotype of these cybrids.

Key words Cybrids · *Cichorium intybus* L · *Helianthus annuus* L · Mitochondrial segregation · *orf522* · Cytoplasmic male sterility

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Introduction

Cytoplasmic male sterility (CMS) is a maternally inherited phenotype characterised by the inability of a plant to produce functional pollen. CMS can appear spontaneously, can be induced by inter- or intra-specific crosses and can be obtained by protoplast fusions leading to recombinations between the two parental mitochondrial (mt) genomes. After somatic hybridisation, the resulting cell, a heterokaryocyte, consists of a mixture of cytoplasms containing both types of chloroplasts, mitochondrias and nuclei. The most frequent situation, resulting from heterokaryocyte mitosis and wall formation, is the random distribution of mixed cytoplasms and reindividualised parental nuclei in each resulting daughter cell. Those cells are called cybrids (Pelletier and Chupeau 1984). Molecular evidence based on restriction analysis of some cybrid mtDNA, suggests that recombinations occur between parental mtDNA prior to cytoplasmic segregation in the somatic hybrids (Evans et al. 1983). It has been suggested that the intergenomic recombinations might be limited to certain repeated sequences (Clarck et al. 1986; Kemble et al. 1986; Kothari et al. 1986; Landgren and Glimelius 1994; Landgren et al. 1994). Several parameters could influence mitochondrial segregation, in particular differences in replication rates and protoplast origin (Landgren and Glimelius 1990, 1994; Landgren et al. 1994). Because CMS can be restored by nuclear genes, it is thought to result from nuclear-mitochondrial dysfunction.

A few CMS systems have become models for fundamental research into the processes leading to pollen abortion. CMS studies on maize (Levings 1993), petunia (Connett and Hanson 1994), sunflower (Laver et al. 1991) and *Brassica* (Bonhomme et al. 1991) have shown differences between fertile and CMS plants at the mitochondrial level. Based on comparisons between CMS, fertile and/or restored plants, CMS was found to be associated with mtDNA regions containing open reading frames (ORFs). These regions, considered to be CMS determinants, could originate from mtDNA recombina-

tions events leading to the creation of these novel ORFs transcribed and translated into novel polypeptides. In the case of alloplasmic CMS, these ORFs may already exist in the cytoplasm donor species, their expression being inhibited by their coexistence with nuclear restoration genes. Such silent CMS genes can be revealed in an alloplasmic situation after crosses and/or protoplast fusion. Restorer genes can induce the loss of the CMS determinant (He et al. 1995) or, as in most of the cases studied, act on its expression at the post-transcriptional level (Krishnasamy and Makaroff 1993; Tang et al. 1995; Monéger et al. 1994). CMS is conveniently used for hybrid seed production because it avoids manual castration. In the case of crops cultivated for seed production, restorer gene(s) are required in the pollinator to restore male fertility and allow self-pollination in the resulting hybrids. Studies on the restoration mechanism, which is under the influence of nuclear genes, indicates the influence of the nuclear genome on the function of mitochondria. In addition to agronomic applications, CMS offers a good material for fundamental studies on mitochondria and nuclear-mitochondrial interactions because it offers a rare case of mitochondrial variants presenting an easily observable phenotype.

In *Cichorium intybus* L. only nuclear male-sterile plants have been observed. Somatic hybridisation by protoplast fusion is a good way to induce mitochondrial modifications. Because CMS is usually associated with mtDNA modifications, Rambaud et al. (1993) performed protoplast fusions between fertile industrial chicory protoplasts (*Cichorium intybus* L. cv 'Magdebourg') and CMS PET1 sunflower protoplasts (*Helianthus annuus* cv 'Mirasol') with the aim of inducing or transferring CMS in chicory and thus obtaining CMS chicories. The 411-, 524- and 523-type plants, obtained after fusions, had flowers lacking anthers (411 and 524) or flowers with brown anthers unable to ensure pollination. First analyses determined the cytoplasmic nature of the three male sterilities and revealed mtDNA rearrangements (Rambaud *et al.* 1993). The 411 progeny, shown to contain the *orf522* sequence in its mtDNA structure, also presented instability (Rambaud et al. 1997).

Genetic analyses on PET1 CMS sunflower showed that CMS is correlated with a specific 17-kb mitochondrial fragment hybridising to the *atpA* gene and containing a new sequence, *orf522*, located downstream of the *atpA* gene (Laver et al. 1991). The *atpA-orf522* fragment can be detected in male-sterile and restored sunflowers (Laver et al. 1991; Köhler et al. 1991). The *orf522* is co-transcribed with the *atpA* gene to form an extra 3-kb transcript that hybridises to the *atpA* probe in addition to the 1.9 kb RNA found in fertile and CMS sunflower corresponding to the *atpA* monocistronic mRNA. The *orf522* translation product, a 15-kDa polypeptide, is found in CMS sunflower, regardless of the explant used for the analysis.

In this paper, we report the results of molecular investigations on mtDNA from three different cytoplasmic male-sterile cybrids containing the 411, 523 and 524 cytoplasms, respectively. They were backcrossed into witloof chicory to transfer the male-sterile cytoplasm from an industrial chicory nuclear context to a witloof chicory nuclear context.

The three cytoplasms originate from three different fusion events and were investigated to determine their origin, whether they exhibited stable profiles and whether they shared common features. In addition, analyses of floral morphology in different nuclear contexts could provide us with information on nuclear influences on the mitochondrial genome expression. Finally, we addressed the question of the *orf522* gene and its involvement in the CMS phenotype of the three different chicory cybrids.

Materials and methods

Plant materials

For each cytoplasm studied, one plant without anthers was chosen to be propagated by in vitro culture. These plants had already been crossed for two generations with an industrial chicory ('Pévèle') and then with different endivia chicory lines used as the male parent (ALG, BLH, Jupiter and β91) (Table 1). This *in vitro* culture gave rise to the first series studied ($I_{411\ a,\ b,\ c...}$, $I_{523\ a,\ b,\ c...}$, $I_{524\ a,\ b,\ c...}$).

The three plants chosen plants were then backcrossed for five generations with different witloof chicories used as pollinators (ALG, AXQ, BLH, Rub, and Jupiter). The siblings from the fifth generation of backcrosses, constituted the second series analysed ($V_{411\ a,\ b,\ c...}$, $V_{523\ a,\ b,\ c...}$, $V_{524\ a,\ b,\ c...}$). One particular 411 plant, backcrossed with the AXQ pollinator, segregated in CMS plants (411 AXQ ster.) and one fertile plant, considered to be a revertant 411 plant (411 AXQ rev.). *Cichorium intybus* L. cv 'Pévèle' and *Helianthus annuus* cv 'Mirasol' (CMS line) (provided by Ets. Florimond Desprez), the two species used for protoplast fusions, and the pollinators used for backcrosses, ALG, AXQ, BLH, Rub and Jupiter (provided by INRA, Versailles), were used as controls.

Methods

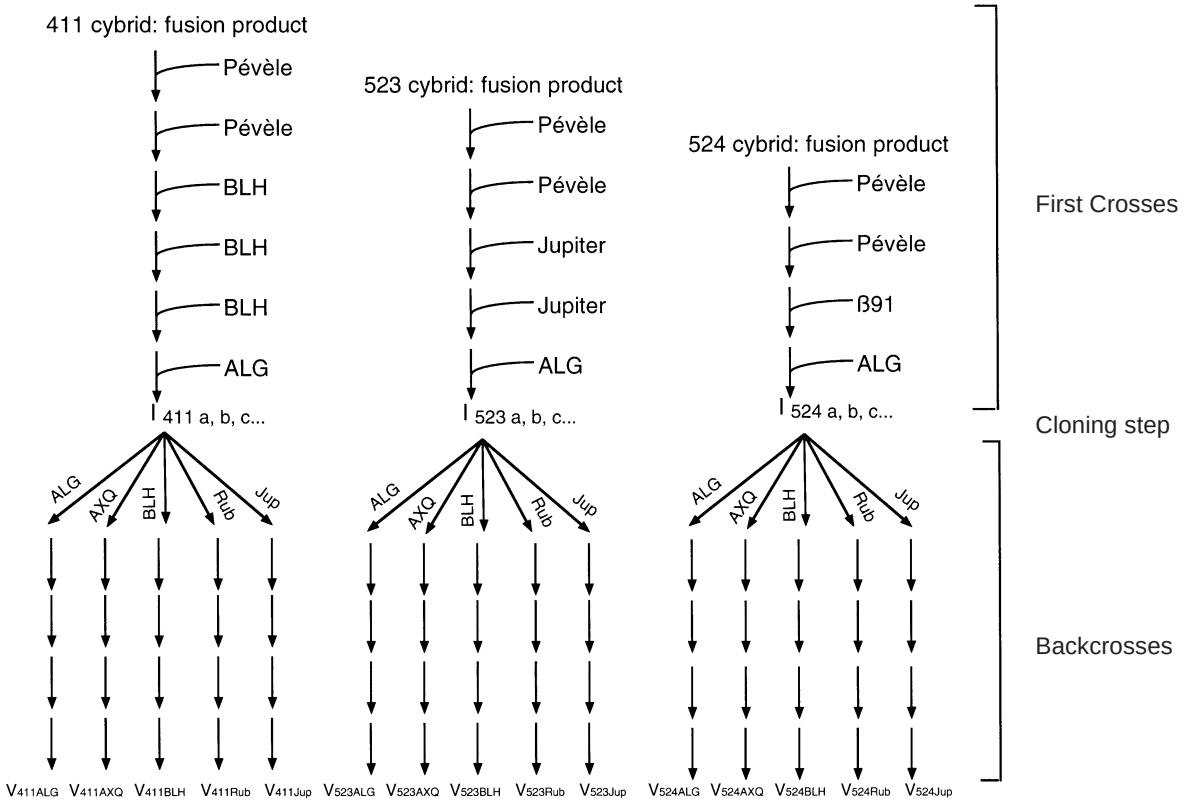
In vitro culture

For each plant propagated, leaf explants were surface-sterilised in calcium hypochloride (10 g/l) washed three times in sterile distilled and then placed on a solidified medium in petri dishes for callus proliferation (Rambaud et al. 1993). The calli were transferred onto a MS (Murashige and Skoog 1962) medium containing agar (5 g/l), major MS salts, Fe-EDTA at half strength (19.5 mg/l), sucrose (5 g/l), benzyladenine (0.1 mg/l) and Morel and Wetmore (1951) vitamins. After buds appeared, roots were induced by transferring the explants onto a Heller (1953) medium supplemented with half-strength MS Fe-EDTA and sucrose (10 g/l). During the in vitro culture**,** explants were cultivated at 24–20°C, with a light-dark cycle of 16 h:8h. The plantlets were then transferred into a greenhouse to produce the leaves used for total DNA extraction.

DNA isolation

Total DNA was extracted (Dellaporta et al. 1983) from chicory leaves and from 10-day-old dark-grown Mirasol sunflower seedlings.

Table 1 Three protoplast fusion products were the origin of three different CMS plants having either the 411, 523 or 524 cytoplasm. Those three CMS plants were first crossed differently. They were then propagated (I_{411}, I_{523}, I_{524}) to give the a, b, c… plants with the three different cytoplasms analysed in the RFLP experiments. The plants chosen for the propagating step were then backcrossed for five generations with different pollinators (ALG, AXQ, BLH, Rub, Jupiter). After five generations of backcrossing, siblings of each cytoplasm context and backcrossed with different pollinators were analysed in RFLP experiments



RNA isolation

Total RNA was extracted (Dean et al. 1985) from chicory buds and from 10-day-old dark-grown Mirasol sunflower seedlings.

Southern blot analyses

Total DNA (10 µg from each sample) was digested with restriction endonucleases (*Eco*RI, *Eco*RV, *Bam*HI and *Hin*dIII, Appligène), separated by agarose (0.8%) gel electrophoresis in 1× TAE buffer (40 m*M* TRIS-Acetate, 2m*M* EDTA tetra sodium salt) and blotted to nylon membrane (Hybond N, Amersham) by vacuum blotting (Vacugene XL) according to the manufacturer's instructions (Pharmacia Biotechnologies). After blotting, membranes were baked at 80°C for 2 h. Hybridisation was performed overnight at 42°C in 50% formamide, 0.2% (w/v) SDS, 1× Denhardt's solution [2% BSA (w/v), 2% PVP (w/v), 2% ficoll 400 (w/v)], 5× SSC (0.75 *M* NaCl, 0.375 *M* trisodium citrate pH 7), 20 m*M* NaH_2PO_4 /Na_2HPO_4 and 100 µg/ml denatured salmon sperm DNA. Mitochondrial probes were radiolabelled by random priming using the T_7 Quick Prime kit (Pharmacia) and then purified on a G50 Sephadex column (Pharmacia). Washes were performed at 45°C in 6×SSC-0.1% SDS (w/v) for 10 min, 2×SSC-0.1% SDS (w/v) for 15 min and 0.2×SSC-0.1% SDS (w/v) for 30 min. The membranes were then autoradiographed using Hyperfilm-MP Amersham, at –80°C with an intensifying screen. Before the next hybridisation, membranes were stripped in 0.1% (w/v) boiling SDS.

Northern blot analysis

Total RNA (20 µg from each sample) was mixed with 50% (v/v) of loading buffer [30% formamide (v/v), 9% formaldehyde 37% (v/v), 5% 10×MOPS (v/v), 3% glycerol (v/v), 2% bromophenol Blue (v/v), 0.25% ethidium bromide (v/v)], heated for 10 min at 65°C and cooled on ice. RNA samples were separated under denaturing conditions on a 1.5% (w/v) agarose gel in 1× MOPS buffer (20 mM MOPS pH 7.0, 5 m*M* sodium acetate, 1 m*M* EDTA pH 8.0), 0.6 M formaldehyde and transferred to a nylon membrane (GeneScreen, Dupont) by capillary blotting overnight in 20× SSPE. After blotting, membranes were UV-crosslinked (120,000 µJ/cm^2). Hybridisations were performed overnight at 42°C in 1×SSPE, 50% formamide (v/v), 1 m*M* EDTA pH 8.0, 0.1% SDS (w/v), 5×Denhardt's and 100 µg/ml denatured salmon sperm DNA. Mitochondrial probe preparation and washes were performed as for Southern analyses except that washes were performed with SSPE instead of SSC.

Reverse transcription (RT)

Total RNA (50 µg) was treated with 10 U of RNase-free DNase (Boehringer) at 37°C for 30 min, extracted with phenol/chloroform and ethanol-precipitated. The treated RNA (25 µg) was used for the reverse transcription in a reaction which contained 1.25 mM of each dNTP, 80 U RNasin, 10 m*M* DTT, 50 m*M* TRIS-HCl pH 8.3, 75 m*M* KCl, 3 m*M* $MgCl_2$, in a total volume of 50 µl, in the presence of 200 U of Super Script™ RNase H$^-$ reverse transcriptase (Gibco BRL). The mixture was incubated for 2 h at 37°C. The reaction was stopped by an incubation of 5 min at 100°C.

Fig. 1a–d Different floral morphologies of the 411, 523 and 524 cybrid lines backcrossed with different pollinators. **a** ALG chicory with normal blue anthers and pollen, **b** flower without anthers from CMS chicories backcrossed with ALG, AXQ and Rub pollinators, **c** flower with brown anthers lacking pollen from CMS chicories backcrossed with BLH pollinators, **d** Flower with nearly normal fertile morphology, blue anthers with less pollen than the normal fertile pollinator from CMS chicories backcrossed with Jupiter pollinator

Polymerase chain reaction (PCR) amplification

The reaction mixture used for PCR amplification contained either 2 µl of reverse transcription reaction or 75 ng of total DNA, 10 pmol of each primer, 0.2 m*M* of each dNTP, 2.5 m*M* $MgCl_2$, 5 µl of the 10×buffer supplied with the *Taq* polymerase (Eurobio) which was used at a concentration of 2.5 U per reaction, in a total volume of 50 µl. Amplification was performed in a Trio Thermoblock (Biometra) thermocycler programmed for a initial step of 3 min at 94°C; 30 cycles of 30 s at 94C, 30 s at 60°C, 1 min at 72°C; and a final step of 5 min at 72°C. For *atpA* gene amplification the following oligonucleotides hybridising the 5' and the 3' end sequence were used: GGA ATT CTC TCC GAG AGC TGC TG (A); GTG TAA GGC AAA GCG CAT TCC (B). For the *orf522* PCR amplification the following oligonucleotides were used: CCC CCT CCC TGG TGG ATC CGG CG (C) or ATG CCT CAA CTG GAT AAA TTC ACT TAT TTC (D) hybridising to the 5' end, CCC TCT ATG AGT ACC GTT CTC TCA CG (E) or TGA GTA CCG TTC TCT CAC GAG TTG AAG (F) hybridising to the 3' end.

DNA sequencing

The 800-bp fragment amplified after PCR was cloned in the pTAg plasmid. The ligation was performed with the LigATor kit (R & D Systems, Abingdon, UK). DNA sequencing was performed by the dideoxy chain termination method of Sanger *et al.* (1977) using the protocol supplied with the "Thermosequenase" kit (Amersham, Pharmacia Biotech.). Database searches were performed with Genbank/EMBL.

Mitochondrial gene probes

The *coxI* probe, corresponding to the maize *coxI* gene, and the *orf522* probe were kindly provided by C.J. Leaver. The *coxII*, *coxIII*, *atpA*, *atp6*, *atp9*, *cob*, *rrn18 and rrn26* probes, corresponding to the respective maize genes, were kindly provided by W. Hauswirth (Muise and Hauswirth 1992; McCarty et al. 1988). For the *coxI* probe, a 3.95-kb *Eco*RI-*Bam*HI fragment cloned in pAT153 was prepared by plasmid extraction. The other probes used were prepared by PCR amplification on mitochondrial gene containing plasmids using the universal primers pTAg forward and reverse.

Results

Flower morphology

Male-sterile chicories were obtained by protoplast fusions between fertile chicory protoplasts and CMS sunflower protoplasts. In the first crosses, floral morphological segregation was observed. Each plant chosen for the propagating step, in our analyses, exhibited antherless flowers. Because of the instability of the 411 cytoplasm and the floral segregation observed after the first crosses, flowering of each cybrid was observed to ensure that the plants analysed were male-sterile. The $I_{411\ a,\ b,\ c...}$ plants presented either antherless flowers or flowers with rudimentary anthers. The $I_{524\ a,\ b,\ c...}$ plants presented completely antherless flowers. The $I_{523\ a,\ b,\ c...}$ plants present-

ed either antherless flowers or flowers with brown anthers.

In the fifth generation siblings, the floral morphologies varied according to the pollinator used for the backcrosses; however, in one single family, the floral morphologies were identical. This was true for all of the cybrids studied. When each of the three cybrids were backcrossed with AXQ, Rub and ALG, identical flowers without anthers were observed (Fig. 1b). When the cybrids were backcrossed with BLH, flowers with brown anthers were observed (Fig. 1c). When V_{411}, V_{523} or V_{524} antherless plants were backcrossed with Jupiter, the progeny exhibited a brown-anther flower morphology. After the second backcross with Jupiter, the flower morphology reverted to normal blue anthers with viable pollen in all three cybrids. In this case, male fertility was restored as those plants could undergo self-pollination (Fig. 1d). It was thus concluded that the pollinators used for backcrossing influence the floral morphologies. This result indicates that the nuclear contexts used for backcrosses do influence the phenotype. Even though Jupiter seems to restore male fertility in all three cybrids, there was no indication that the 411, 523 and 524 cytotypes were identical.

Molecular analyses of the mtDNA

Characterisation of the three cytoplasm mtDNAS

A restriction fragment length polymorphism (RFLP) analysis was performed on $V_{411\ ALG}$, $V_{523\ ALG}$ and $V_{524\ ALG}$ siblings and determined that the three cytotypes were different. Some probes did not show any differences between the three cytotypes and fertile chicory, such as the *coxI* and *atp9* probes. However, when the *coxII* gene was used as a probe, analyses made on the $V_{411\ ALG}$, $V_{523\ ALG}$ and $V_{524\ ALG}$ siblings after *Eco*RI digestion indicated differences between the three cytotypes (Fig. 2a–c). All plants analysed exhibited the 2.5-kb sterile sunflower fragment. $V_{523\ ALG}$ and $V_{524\ ALG}$ also possessed the 6-kb fertile chicory fragment, but $V_{411\ ALG}$ did not. The 7-kb fragment observed in $V_{411\ ALG}$ and $V_{524\ ALG}$ siblings was absent in $V_{523\ ALG}$. On the contrary, a 3.5-kb fragment observed in $V523_{\ ALG}$ and $V_{524\ ALG}$ siblings, was absent in $V_{411\ ALG}$.

Some differences could also be observed between the three cytoplasms with respect to the *atpA-orf522* region. This RFLP analysis revealed a persistence of the *orf522* gene in the 411 cybrid. The 523 cybrid possessed the *orf522* sequence, while the 524 cybrid did not hybridise with this sequence. In order to confirm the hybridisation results, we performed PCR analysis using oligonucleotides hybridising with the 5’ and the 3’ ends of the *orf522* gene. These analyses also showed that the 524 cybrid did not contain the *orf522* gene in amounts that were undetectable with the RFLP analysis. Both 411 and 523 cybrids amplified a fragment identical in length to that amplified for CMS sunflower (Table 2). On the contrary, no amplification signal could be observed for the fertile chicory, ‘Pévèle ’, or for the 524 cybrid (Table 2).

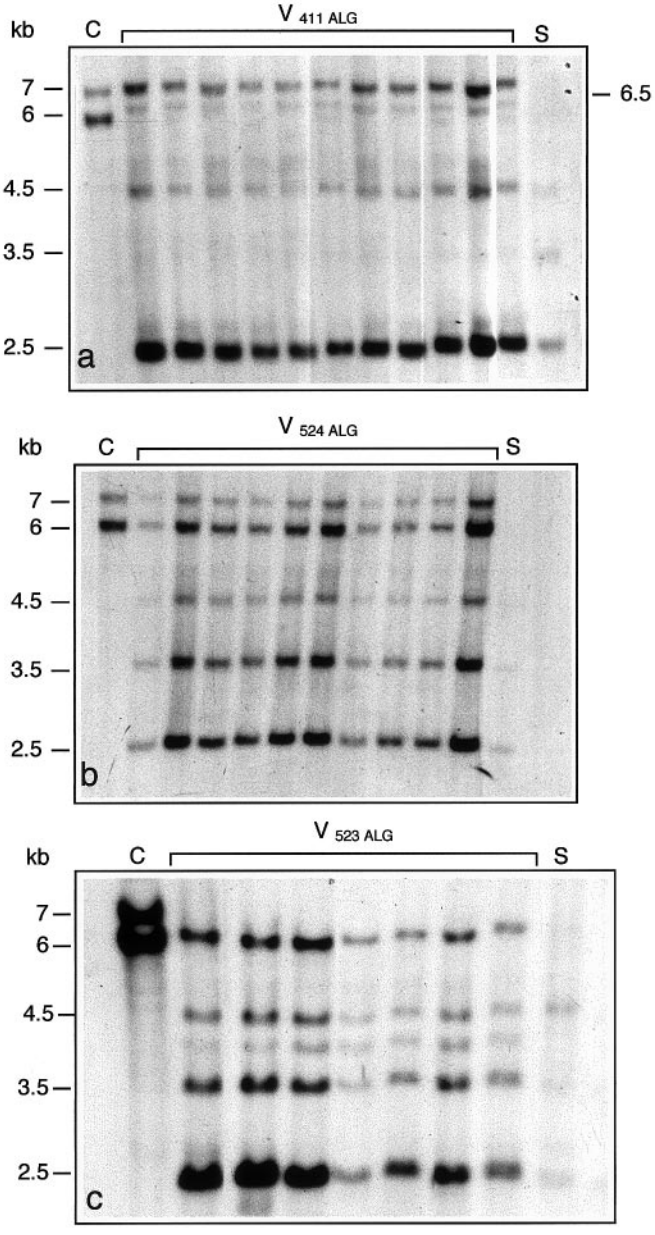


Fig. 2a–c Southern blot analysis of total DNA from the parental species (*C* chicory, *S* sunflower) and the CMS chicories backcrossed for five generations with the ALG pollinator. DNA was digested with *Eco*RI and hybridised with the mitochondrial *coxII* probe. **A** $V_{411\ ALG}$ siblings, **b** $V_{524\ ALG}$ siblings, **c** $V_{523\ ALG}$ siblings. Fragment sizes are given in kilobases (kb)

MtDNA rearrangements occurred in the atpA-orf522 *region*

In sunflower, *orf522* is cotranscribed with the *atpA* gene (Laver et al. 1991). By implementing PCR, using one oligonucleotide hybridising with the 5’ *atpA* end (A) and a second one hybridising with the 3’ *orf522* end (E), we

Table 2 Detection of the *orf522* sequenc, and corresponding mRNA, in cybrids (411, 523, 524), fertile chicory (C) and CMS sunflower (S) by PCR and RTPCR. The *orf522* sequence was amplified from DNA or cDNA using primers C and E, the *AtpA-orf522* CMS sunflower sequence was amplified using primers A and E, *AtpA* was amplified using primers A and B

		Cytotype				
		411	523	524	C	S
Amplified fragments						
cDNA-RTPCR (a)	*orf522* (522-bp)	+[a]	+	–	–	+
	atpA-orf522 (2.2-kb)	+/– (8/24)	–	–	–	+
	atpA-orf522 (800-bp)	+	+	–	–	–
DNA-PCR (b)	*atpA*	+	+	+	+	+
	orf522	(+)	+	–	–	+

[a] +, Amplification of the corresponding fragment; –, absence of the corresponding fragment; (+), weak amplification of the corresponding fragment; +/–, amplification of the corresponding fragment from only some of the samples analysed.

Sterile, revertant and restored 411 plants were tested. (a), plants issued from in vitro culture (I); (b), plants analysed after five generations of backcrosses (V)

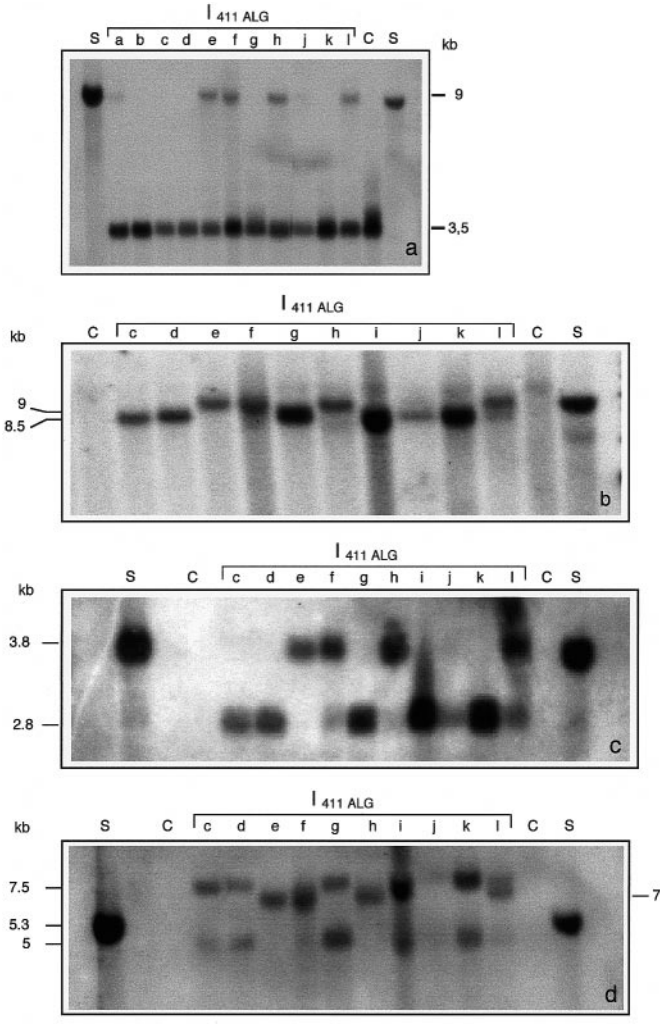


Fig. 3a–d Southern blot analysis of total DNA from the parental species (*C, chicory, S* CMS sunflower) and the 411 CMS chicory cybrids originating from in vitro culture. DNA was hybridised with *atpA* (**a**) or *orf522* (**b**, **c**, **d**) after *Hin*dIII (**a**, **b**), *Eco*RI (**c**) or *Eco*RV (**d**) digestion

amplified a 2.2-kb fragment. In the 523 cybrid, the 2.2-kb fragment was never observed after PCR amplification. On the other hand, this fragment was amplified from the DNA of 8 out of 24 I_{411} plants (Table 2). Moreover, a 800-bp fragment was amplified from the mtDNA of the 411 and 523 plants (Table 2). This result showed that in the vicinity of *orf522*, 411 cybrid plants displayed heterogeneity.

AtpA and *orf522*, used as probes, hybridised with the same fragment when sunflower total DNA was digested either by *Eco*RV (Figs. 3d, 4c) or *Hin*dIII (Fig. 3a, b), with the hybridisation fragment sizes being 5.3-kb and 9-kb, respectively. When total DNA of the $I_{411\ ALG}$ plants were hybridised with the *atpA* and *orf522* probes, the 11 plants tested could be separated into two different groups. In the first group, 7 of the 11 plants (a, b, c, d, g, j, k) possessed only the fertile chicory fragments when the *atpA* gene was used as a probe after *Hin*dIII digestion (Fig. 3a) and hybridised with the *orf522* probe on fragments different from those of the CMS sunflower (Fig. 4–d). After *Hin*dIII digestion (Fig. 3a), the second group of plants (e, f, h, l) presented two *atpA* hybridisation fragments: the first fragment corresponding to that of the fertile chicory, and the second corresponding to that of the CMS sunflower fragment. After *Eco*RI and *Hin*dIII digestions, these 4 plants showed the *orf522* CMS sunflower hybridisation fragment (Fig. 3b–c), but after *Eco*RV digestion, the second group possessed a 7-kb fragment hybridising with the *orf522* probe (Fig. 3d) instead of the 5.3-kb sunflower fragment.

A similar analysis was performed on the 523 plants (I_{523} and V_{523}) and V_{411} plants and revealed patterns identical to those observed for the I_{411} first group. When total DNA was digested with *Bam*HI (Fig. 4b), the *orf522* gene hybridised with a 2.3-kb fragment in sunflower and I_{523ALG} plants corresponding to the 3' end of the *orf522* sunflower gene (Fig. 5). The 1.2-kb fragment corresponding to the 5' end of the sunflower *orf522* gene

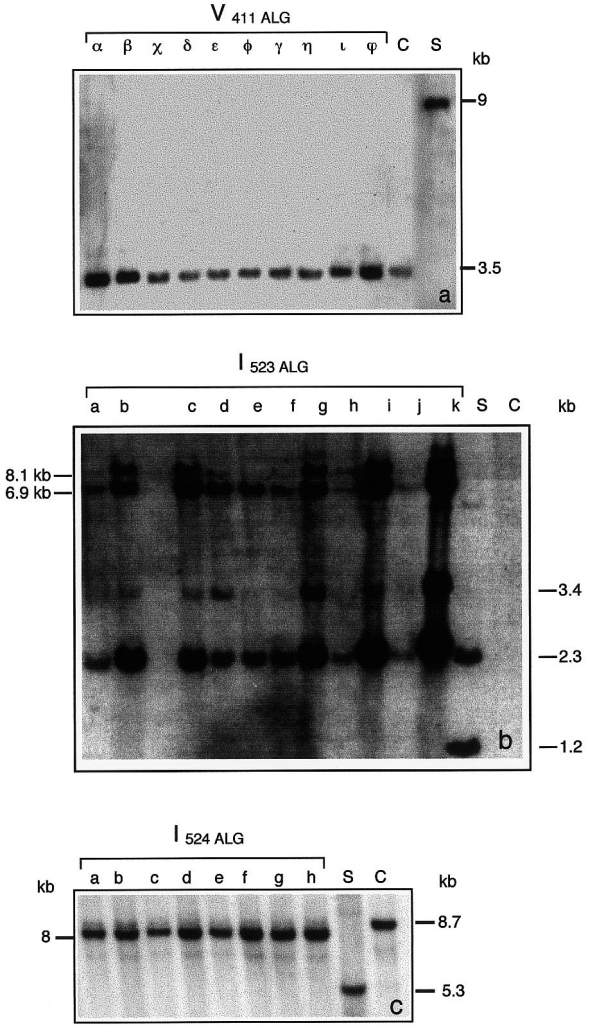


Fig. 4a–c Southern blot analysis of total DANN from the parental species (*C* chicory *S* CMS sunflower) and the CMS chicory cybrids. **a** Total DNA from 411 cybrids, backcrossed for five generations with the ALG pollinator, hybridised with the *atpA* probe after *Hin*dIII digestion, **b** total DNA from 523 cybrids, originating from the in vitro culture, hybridised with the *orf522* probe after *Bam*HI digestion, **c** total DNA from 524 cybrids, originating from the in vitro culture, hybridised with the *atpA* probe after *Eco*RV digestion

was not found in I_{523ALG} plants, but rather *orf522* hybridised to two, three or four other fragments, showing a 5' end of the *orf522* gene being rearranged in plants carrying the 523 cytoplasm.

All of these results suggested that the 411 cybrid, originating from protoplast fusion, had received at least the sunflower *atpA-orf522 Hin*dIII fragment and that this fragment had undergone modifications, eliminating the sunflower *atpA* gene. This region stabilised, as shown by the profile homogeneity observed after 5 generations of backcrosses (Fig. 4a). With respect to the 523 cybrid, it seems to have integrated only the *orf522* sequence. Unlike in the 411 cybrid, the *atpA* CMS sunflower fragment was never observed in the 524 cybrid, however the *atpA* region had undergone modifications as shown by the new fragment observed after *Eco*RV digestion (Fig. 4c).

Due to the lack of the *atpA* sunflower gene in the 411 and 523 cytotypes and as a 800 bp fragment was amplified from the 411 and 523 cybrid plants, we wanted to understand the origin of this 800 bp fragment. The amplified fragment was sequenced. It contained the sequence of the 5' PCR primers immediately followed by the 265 bp inverted repeated sequence found between *atpA* and *orf522* in the sunflower and finally the total *orf522* sequence (Fig. 5b).

Did the other parts of the mitochondrial genome stabilise?

We wanted to determine whether the standardisation of the mitochondrial patterns observed in the vicinity of the *atpA* and *orf522* genes was a general feature of this mitochondrial genome. When total DNA from $V_{411\ ALG}$ and $V_{523\ ALG}$ sibling plants was digested by *Hin*dIII and hybridised with *cob*, 1 plant with each cytoplasm (respectively η and φ) presented a profile different from those of the other plants (Fig. 6b,c) while the 524 plants displayed a stable profile with the *cob* probe (Fig. 6a).

This result showed that the 411 and 523 cytoplasms still exhibited a slight heterogeneity in the vicinity of the mitochondrial *cob* gene. Homogeneity had been reached in the *atpA* region. The only other observable heterogeneity on Southern blots was due to the signal intensity of very weak bands; this heterogeneity was very difficult to interpret. RFLP analyses also revealed that the three cytoplasms presented recombined profiles in the *atp6*, *cob*, *rrn18* and *rrn26* regions (Fig. 6 and data not shown). On the whole, our observations argue in favour of a standardisation in the mtDNA structure of each cytoplasm.

Did the nucleus influence mtDNA rearrangements?

To determine whether the nucleus had any interaction with mitochondrial stabilisation, we performed a RFLP analysis on the fifth backcrossed generation with several pollinators (ALG, AXQ, BLH, Rub and Jupiter). Regardless of the pollinator used for backcrosses, all of the CMS chicories tested exhibited the same profile in each cytoplasm context, as shown in Fig. 7 for the 411 cybrids and the *atpA* probe. Furthermore in comparing the mitochondrial profiles obtained for the different witloof pollinators used (with seven different probes), we observed profiles identical to industrial chicory profiles.

RFLP analyses were also performed with 411 AXQ sterile, 411 AXQ revertant and 411 Jupiter restored cybrids, AXQ pollinator and CMS sunflower using the *atpA* and *orf522* probes. No difference could be observed between the 411 sterile, revertant and restored cybrids. All

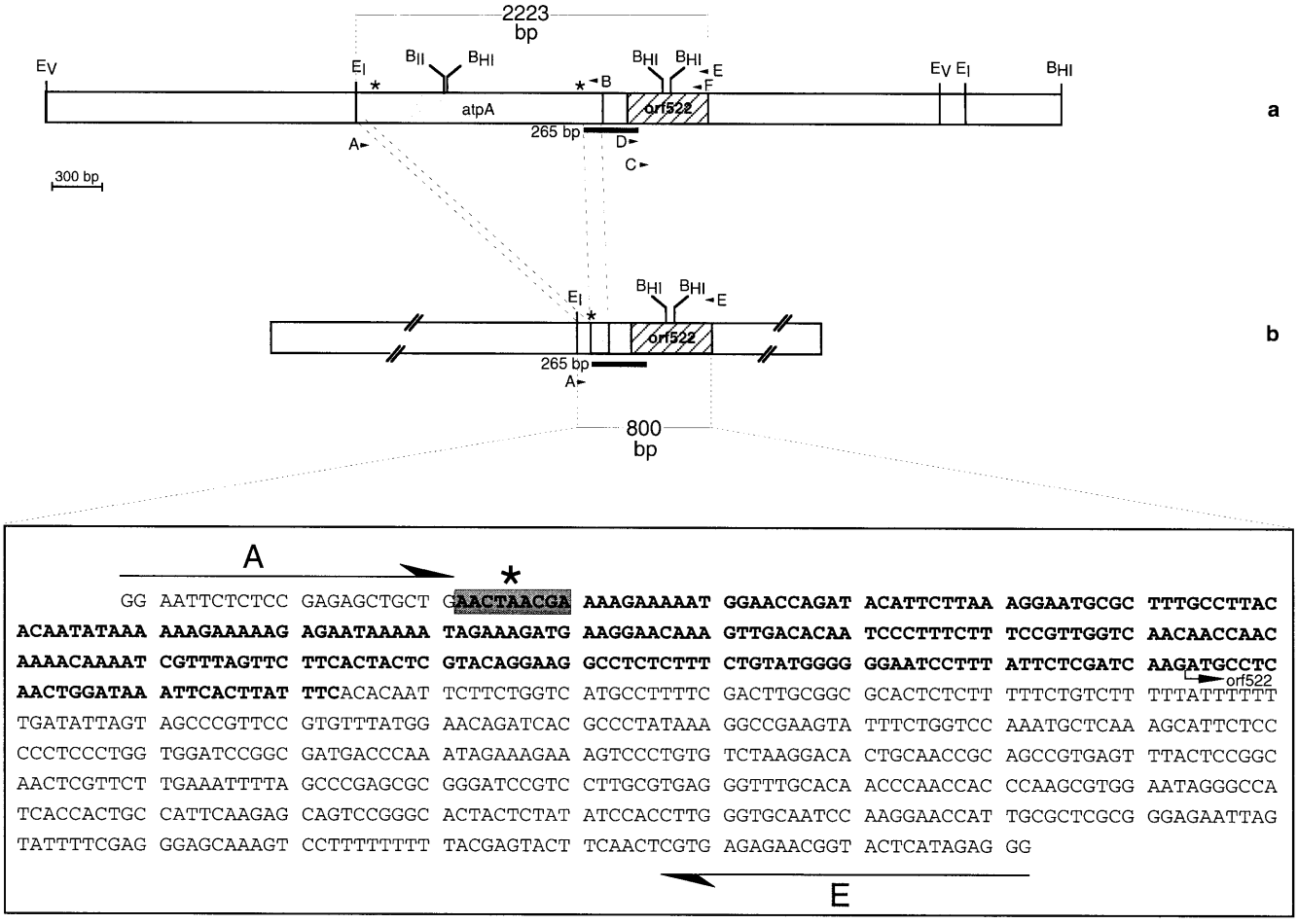


Fig. 5 **a** Restriction map of the *atpA-orf522* CMS sunflower region. E_I *Eco*RI restriction site, E_V *Eco*RV restriction site, B_{HI} *Bam*HI restriction site, B_{II} *Bg*/II restriction site. **b** Nucleotide sequence of the 800-bp fragment amplified by PCR using primers A and E. This reaction gave a 2.2-kb fragment with CMS sunflower DNA but amplified a 800-bp fragment with the 411 and 523 cybrids DNA. *Arrows* indicate the primers used for the amplification reaction. *Bold type* indicates the 265-bp inverted repeated sequence found in the CMS sunflower (Laver et al. 1991). The star indicates a 9-bp repeated sequence found in *atpA* sunflower gene in position 25 after the *atpA* ATG codon. The same sequence is found at the beginning of the 265-bp inverted repeated sequence

of these plants presented the same pattern (described above for V_{411} plants), which was different from that of the sunflower (data not shown).

Expression of the *orf522* gene in the 411 and 523 chicory cybrids

To investigate whether *orf522* was expressed or not, we performed a first RT-PCR using oligonucleotides A and B, which hybridise with the 5' and 3' *atpA* gene ends, respectively. For all experiments, a control reaction was included where reverse transcriptase was omitted. This ensured that the amplification obtained in the RT-PCR reactions was in fact from cDNA and not from contaminating mtDNA. In order to compare the results, we included a PCR on DNA in the experiment. All of the plants analysed amplified the *atpA* gene, which confirmed the expression of this gene and the validity of the experimental conditions. When RT-PCRs were performed using oligonucleotides hybridising with the 5' and 3' *orf522* ends, different levels of amplification were observed. As expected from the results obtained on mtDNA, the fertile AXQ chicory and the 524 CMS cybrid did not amplify *orf522*. The CMS sunflower and the 523 CMS cybrid amplified the *orf522* sequence at a similar level from cDNA as from DNA. Finally, plants with the 411 cytotype amplified the *orf522* from their cDNA more faintly than from DNA, whatever their phenotype (sterile or fertile) or nuclear background (Table 2).

To verify these results, we hybridised a Northern blot, using the same RNA samples, with the *orf522* and *atpA* sequences (Fig. 8a–b). The mRNA from the fertile witloof AXQ chicory, the 411 AXQ revertant, the 411 AXQ CMS, the 411 ALG CMS and the 524 CMS chicories hybridised with the *atpA* probe at different intensities. The 411 restored cybrid exhibited a smaller *atpA* RNA than the other chicories. The 523 cybrid chicory presented two hybridisation signals, with the smaller RNA giving a stronger signal than the larger one (Fig. 8a). Concerning the hybridisation with the *orf522* sequence used as probe, only CMS sunflower gave a signal. The other

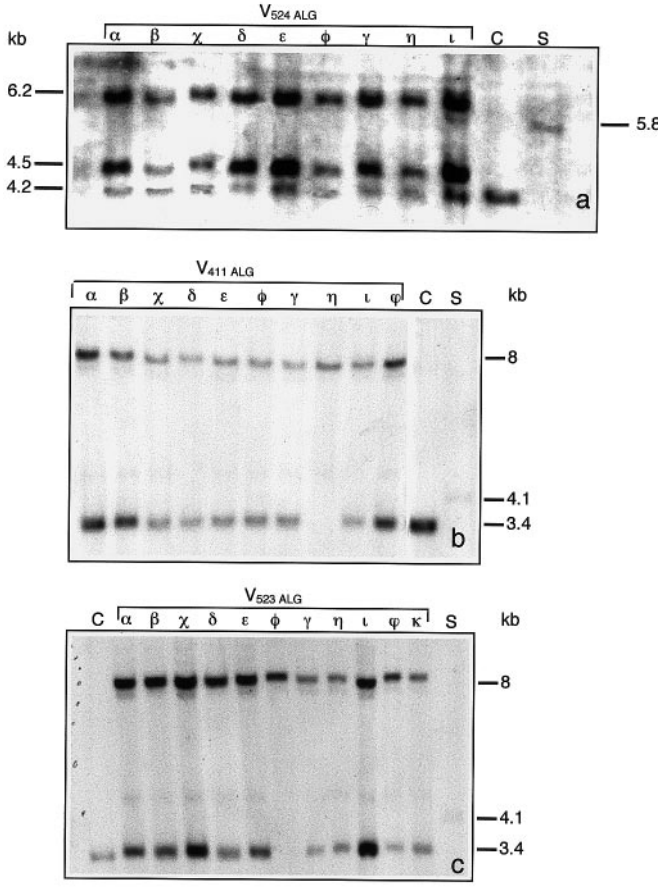


Fig. 6a–c Southern blot analysis of total DNA from the parental species (*C* chicory, *S* sunflower) and the CMS chicories with the mitochondrial *cob* probe). **a** Total DNA digested by *Eco*RV. $V_{524\ ALG\ \alpha-\iota}$ 10 siblings originating from the 524 cybrid plant. **b** Total DNA digested by *Hin*dIII. $V_{411\ ALG\ \alpha-\varphi}$ 10 siblings originating from the 411 cybrid plant. The η plant does not exhibit the 3.4-kb fragment. **c** Total DNA digested by *Hin*dIII. $V_{523\ ALG-to\ \kappa}$ 11 siblings originating from the 523 cybrid plant. The ϕ plant does not exhibit the 3.4-kb fragment. Fragment sizes are given in kilobases (kb)

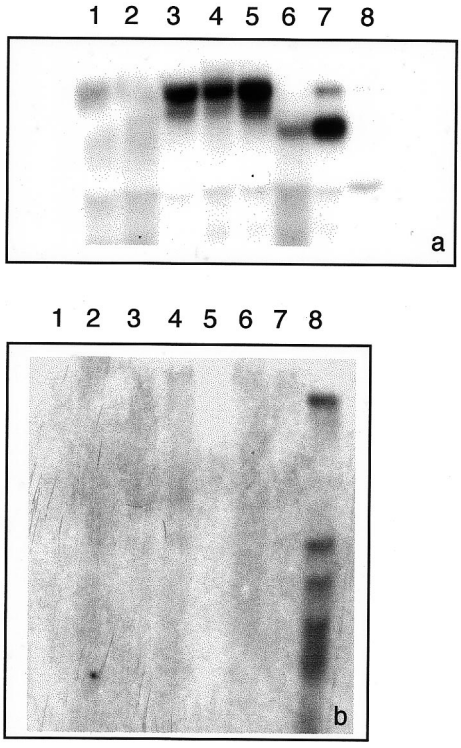


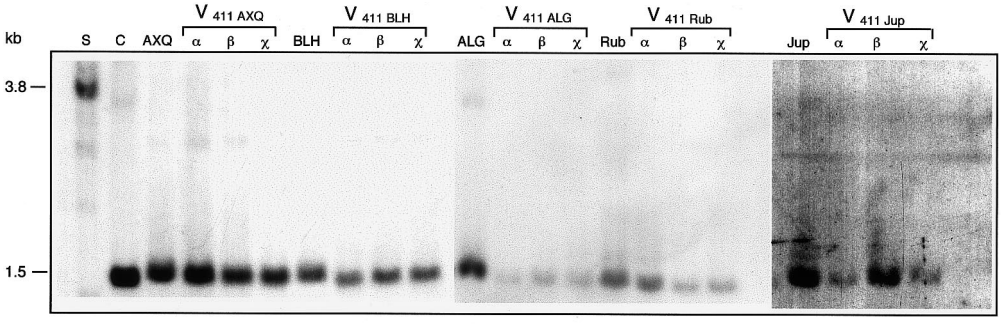
Fig. 8a, b Northern blot analysis of *atpA* (**a**) and *orf522* (**b**) expression. *Lane 1* fertile AXQ chicory, *lane 2* 411 revertant cybrid backcrossed with AXQ pollinator, *lane 3* 411 sterile cybrid backcrossed with AXQ pollinator, *lane 4* 411 sterile cybrid backcrossed with ALG pollinator, *lane 5* 524 sterile cybrid backcrossed with ALG pollinator, *lane 6* 411 fertile cybrid restored with the Jupiter pollinator *lane 7* 523 sterile cybrid backcrossed with ALG pollinator, *lane 8* CMS sunflower

plants analysed, including the 523 CMS cybrid, yielded no apparent signal (Fig. 8b).

Discussion

The symmetrical protoplast fusions show a tendency to maintain the chicory mitochondrial genome even when there is no pre-treatment to select this genome. Sunflower or new hybrid bands can be observed; however, the chicory bands are more frequently seen when all RFLP analyses are considered. It is possible that the chicory nuclear genome sends more of the necessary signals to the chicory mitochondrial genome than to that of sun-

Fig. 7 Southern blot analysis of DNA using *atpA* as the probe from the parental species (*C* chicory, *S* sunflower and the 411 CMS chicories backcrossed with five different pollinators [ALG, AXQ, BLH, Rub, Jupiter (Jup)] over five generations. Total DNA was digested by *Eco*RI. For the five pollinators used for backcrossing, 3 siblings ($V_{411\ AXQ\ \alpha-\chi}$, $V_{411\ BLH\ \alpha-\chi}$, $V_{411\ ALG\ \alpha-\chi}$, $V_{411\ Rub\alpha-\chi}$, $V_{411\ Jup\ \alpha-\chi}$) are analysed for each nuclear context. Fragment sizes are given in kilobases (kb)



flower. Alternatively, among all of the possible recombined genomes, only those which carry a majority of chicory mitochondrial genes will be selected to the chicory nuclear context. This can be interpreted as a nuclear-organellar incompatibility, as mentioned by Rose *et al.* (1990). Sunflower plants were never regenerated in the media used to cultivate protoplasts after fusion. This result suggests that our medium was not suitable for sunflower and that the sunflower nucleus was not maintained after fusion. Therefore, the sunflower mitochondria in the cybrids must have received chicory nuclear information. The same results were observed in somatic hybrids within the *Brassicaceae* family, in which culture conditions favoured *Brassica campestris* or *Brassica campestris*-like mitochondrias (Landgren and Glimelius 1994). Moreover, differences in mitochondrial replication rates could have emphasised the progressive elimination of the sunflower's mitochondrial genome, while recombination events maintained the sunflower sequences that were introduced into the chicory mitochondrial genome. The different mitochondrial structures observed when the three cybrids were compared and the polymorphism observed in the I_{411} plants could be explained by the random segregation of a heterogeneous mitochondrial genome population. On one hand, the polymorphism observed among the I_{411} plants could originate from the in vitro culture. Subjecting plant cells to stress, such as during tissue culture or somatic cell fusion, may trigger reorganisation of the mitochondrial genome. Changes in the restriction profiles are also not uncommon (Shirzadegan et al. 1991). In addition, Hartmann et al. (1989) have shown that amplification and segregation of rearranged mitochondrial genomes occur dynamically during tissue culture. On the other hand, polymorphism was noticed on the fourth 411 progeny which was backcrossed with industrial chicory (Rambaud et al. 1997). This polymorphism showed that the propagating step was not the only explanation for the polymorphism observed in the first series that we analysed. Although certain new mitochondrial gene structures observed at the beginning (after protoplast fusions) have disappeared, the structure modification(s) causing CMS were maintained, as the cybrid progenies remain male-sterile except when backcrossed with Jupiter.

It is interesting to note that the *orf522* gene has a different status in the three cytoplasms. In the 524 cybrid the gene is not present. From this result, we can infer that the 524 cybrid represents a novel CMS, different from the CMS of sunflower. The 411 cybrid received the entire *atpA-orf522* fragment during protoplast fusion. From the results obtained in this region, we hypothesised that at least the *Hin*dIII *atpA-orf522* CMS sunflower fragment was directly introduced into the 411 CMS chicory mitochondrial genome. Because the $I_{411\ e,\ f,\ h,\ l}$ plants have this fragment and a larger *Eco*RV *atpA-orf522* fragment, when compared to the sunflower, a recombination event between the *Hin*dIII 5' site and the *Eco*RV 5' site of the sunflower fragment could have occurred in the 411 chicory mitochondrial genome. It seems to have rearranged itself, progressively eliminating the sunflower *atpA* gene, whereas the entire *orf522* remained united with the 265-bp repeat characteristic of sunflower. In the 523 cybrid, the *orf522* sequence was introduced, but the sunflower *atpA* gene was never observed. We can not be certain whether this cybrid eliminated the sunflower *atpA* gene rapidly after receiving the *atpA-orf522* sunflower sequence so that it was never detected or whether the *orf522* sequence alone was introduced into that mitochondrial genome.

It is possible that the 411 and 523 cytoplasms have undergone the same events but at different rates. For the $I_{411\ c,\ d,\ g,\ i,\ j,\ k}$, I_{523}, V_{411} and V_{523} plants, two hybridisation fragments with *orf522* could be observed after *Eco*RV digestion (instead of one fragment in CMS sunflower). According to Fig. 5, a *Eco*RV site would not be expected within the 800-bp PCR fragment. The patterns obtained for the 523 cytotype, after *Bam*HI digestion and *orf522* hybridisation, show that the *orf522* 3' end is identical to that of the CMS sunflower but that the *orf522* 5' ends are different. We suppose that the 5' region of the *orf522* gene is involved in recombinations with large inverted repeated sequences different from the sunflower 265-bp repeat. Recombination through this inverted repeat gives rise to two isomeric forms of the master chromosome. Each isomeric form contains the entire mtDNA sequence complexity; however the genomic environment of the repeat is different and leads to mitochondrial DNA polymorphism. The two *Eco*RV 5' sites detected by hybridisation experiments could be located on both sides of one of these repeated sequences, whereas the other enzymes used (*Hin*dIII, *Eco*RI, *Bam*HI) would have sites inside the inverted sequence and thus would not reveal this isomerisation by hybridisation with the *orf522* probe.

Our results showed two rearrangement mechanisms that occurred simultaneously in the 411 cytotype; the first one eliminating the sunflower *atpA* gene and the second one giving rise to a duplication of the *orf522* sequence with a new molecular environment. Such mechanisms are well known in plant mitochondrial genomes (Backert et al. 1997) but are rarely observed (Fauron et al. 1992, Chétrit et al. 1992).

It is unlikely that *orf522* induces CMS in the 411 cybrid because: (1) the weak *orf522* transcript accumulation level observed after RT-PCR amplification is identical in sterile, revertant and restored 411 cybrid florets; (2) the 411 revertant plants show that the *orf522* sequence is present and expressed at the same level when compared to the 411 CMS cybrid; (3) the level of expression of the *orf522* gene, although detectable by RT-PCR experiments, is very weak, and corresponding mRNA was never detected in Northern experiments. Concerning the 523 cybrid, which presents a stronger RT-PCR *orf522* amplification signal, we might expect the *orf522* to be expressed at the same level as in the CMS sunflower. However, a mRNA for the *orf522* gene was never detected on Northern blots. The nature of the explant and the development stage (buds or seedlings) of the plant used

for the RNA extraction may be one of the factors explaining the different intensities of the fragment observed. Actually, tissue and developmental regulation of mitochondrial gene expression has been reported (Binder et al. 1996). This regulation can induce variations in mRNA abundance. Furthermore, the three cybrids were all restored by the same pollinator, suggesting a similarity in the sterility inducer. All of these observations together lead us to conclude that it is highly improbable that the *orf522* gene is responsible for male sterility in the 411 and 523 cybrids. In contrast to the study made on the *Brassicaceae* family (Sakai and Imamura 1992), in which mitochondrial genome stabilises rapidly, the mitochondrial genome of CMS chicory cybrids is not completely fixed after several generations of backcrosses. There is no evidence that the whole mitochondrial genome will stabilise. It is possible that a slight instability will remain in certain mitochondrial regions, with the mitochondrial genome structure being in balance between two or more configurations.

Because of three different stable profiles observed after *Eco*RI digestion and *coxII* hybridisation, RFLP analysis informs us that the three cybrids display three different mitochondrial genome structures. However, we can observe common features. The three cytotypes show identical profiles in the *atp6* and *rrn18* regions, even though they are different from the two parents. Identical mtDNA rearrangements strengthen the suggestion that intergenomic recombinations involve repeated sequences.

The nucleus does not seem to have any major influence on mitochondrial genome stabilisation. It seems rather that the pollinators influence cybrid floral morphology. Due to the fact that all of the mitochondrial structures were stabilised in the same way regardless of the pollinator used and because Jupiter pollinator restores the three cybrid male fertility, we can conclude that these flower phenotypic differences are the result of nuclear effects rather than of differences in mitochondrial structures. The Jupiter pollinator thus carries nuclear restoration gene(s). This could mean that determinants inducing CMS in the three cybrids are identical because they are restored by the same pollinator, even if their mitochondrial structures are different. Alternatively, if the CMS determinants are different in the three cytotypes, the Jupiter line must carry restorer genes for each CMS. If the cybrids carry the same CMS, we should observe common features for the three cybrid mitochondrial genomes, i.e. differences between the cybrids cytotypes, the fertile chicory control and the cybrid revertants.

A new path is now open to the study of the CMS chicory determinism. A smaller mRNA, corresponding to *atpA* expression, in the 411 restored cybrid, suggests that *atpA* could be involved in the male-sterility mechanism. A restorer gene transcriptional effect could be suggested. As no differences were observed between fertile and sterile 411 AXQ plants, a post-transcriptional mechanism of restoration could also be hypothesised. These are preliminary results and may lead to new investigations.

Due to the fact that CMS chicories present many mitochondrial rearrangements (*atpA*, *atp6*, *coxII*, *cob*, *rrn18*, *rrn26*, *orf522*), we are now studying mitochondrial protein profiles by in *organello* synthesis to determine if there are any differences in the protein profiles between revertant, sterile and restored cybrids.

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