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**Expression of Mitochondrial DNA Associated
with Cytoplasmic Male Sterility in *Petunia***

S. Izhar, M. Hanson

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EXPRESSION OF MITOCHONDRIAL DNA ASSOCIATED WITH CYTOPLASMIC MALE STERILITY
IN PETUNIA.

Investigators' Institutions

A.R.O. BET DAGAN, ISRAEL.

CORNELL UNIVERSITY, USA.

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C. Abstract

The ultimate goal of the proposed research was to identify the genetic information which specifies cytoplasmic male sterility (CMS) in one model plant species, and to understand how this genetic information results in abortion of normal pollen development.

The experiments focussed on a mitochondrial gene (the pcf gene) which was found by us to be associated with CMS in Petunia. The pcf gene was found to consist of an in-frame fusion of sequences homologous to ATP synthase subunit 9 (atp9), cytochrome oxidase subunit II (coxII), and an unidentified open reading frame (urf-s). In addition, a long 3' flanking region was identified. In order to gain insight into the structure of the coxII region present in pcf, two normal petunia coxII genes present in the fertile line 3704 have been sequenced (Pruitt and Hanson, 1989).

The lack of intron sequences within the coxII-homologous region of pcf suggests that this region may be a processed pseudogene (Pruitt and Hanson, 1989). In order to determine whether another gene is located 3' downstream to pcf, sequencing of this region was done. Sequencing has revealed two genes. Co-transcribed with pcf are the genes for subunit 3 or NADH dehydrogenase (nad3) and small ribosomal subunit protein 12 (rps12) (Rasmussen and Hanson, 1989). The co-transcribed nad3 and rps12 genes are the only copies of these genes present in the CMS line's mtDNA, while normal copies of atp9 and coxII are present elsewhere.

The petunia CMS-associated region is thus a complex locus (termed S-pcf) comprised of three different co-transcribed genes: pcf, nad3, and rps12. The association between this complex locus and CMS was further substantiated by the following data:

- 1) Reversion to fertility in a progeny of the unstable somatic hybrid petunia plants was found to be associated with the loss of the S-Pcf locus.
- 2) Fertile progeny of an unusual sterile somatic hybrid (13-194) which gave rise to both fertile and sterile progenies was found to contain the S-Pcf locus.

Genetic analysis revealed the presence of a multigenic nuclear restorer system. Both the sterile and the fertility restored plants transcribed the pcf gene into a 2.5 kb RNA (Wolf et al., in preparation).

D. Objectives of the original research proposal

The main goal of the proposed research was to establish the correlation between the mt pcf gene and CMS in petunia.

The specific objectives were as follows:

1. Characterize the normal counterparts of the fused pcmsf gene's components.
 - a. Sequence the normal coxII gene from CMS mt genome.
 - b. Clone and sequence the fertile line's coxII.
 - c. Further analyze the URF region of the pcmsf gene.
 - d. Analyze the 3' flanking region of the pcmsf gene
 - e. Sequence, in a fertile petunia line, sequences homologous to this 3' flanking region.
2. Study the expression of the abnormal fused gene and homologous sequences.
 - a. Analyze gene expression by RNA isolation followed by S1 protection.
 - b. In situ hybridization with reproductive tissues.

E. Body of the report

F. Description of cooperation

The mitochondrial DNA clone libraries were established from parental and somatic hybrid material supplied by the ARO laboratory. The DNA sequencing and S1 protection studies, as described in the research plan, were carried out in the Cornell lab.

S. Izhar supplied somatic hybrids for molecular analysis and gene action work in Hanson's laboratory. Mitochondrial DNA probes were supplied by Hanson's laboratory to Izhar's laboratory for further analysis of segregants of somatic hybrids and for the expression of the *Pcf* gene in *E. coli*.

The cooperation between the two laboratories produced a joint publication (included in this report).

The cooperation between the Volcani and Cornell laboratories still continues in the good tradition of this project. The consultation, planning the work and supplying plant material, probes and information also continue.

S. Izhar visited the Hanson's laboratory and during this visit the results obtained and the work plan were discussed.

This cooperation produced the continuation of this BARD project and the submission of a new proposal to BARD by the two P.I.S. including Dr. N. Firon from the Volcani.

G. Evaluation of the research achievements with respect to the aims of the original research proposal and emphasizing the benefit to agriculture.

The main goals of the proposed research were to determine whether the *Pcf* is the *cms* gene in petunia and to characterize its activity in the anther.

The results obtained so far indicate very strongly that almost absolute association exists between the *Pcf* gene and CMS:

- (1) The DNA segregates with sterility in somatic hybrids.
- (2) Male-fertile progeny derived from three different male-sterile somatic hybrid plants did not exhibit the mitochondrial DNA

(mtDNA) arrangement which has previously been correlated with cms in Petunia (Clark et al., 1988). The cms-associated mtDNA arrangement was present in the male-sterile predecessors of these fertile revertants. Thus, it is concluded that the loss of the S-pcf locus is associated with reversion to fertility in the progeny of the unstable somatic hybrid petunia plants.

- (3) In addition to the fertile revertants, progeny of an unusual stable sterile somatic hybrid gave rise to both fertile and sterile progenies. In further generations, segregation of fertile and sterile progeny continued.

The unique thing about this plant material was that also some fertile progeny segregated fertile and sterile progenies. These fertile plants contained the S-pcf locus. Genetic analysis revealed the presence of a multigenic nuclear restorer system (Wolf et al. in preparation) even though nuclear restorer genes were not identified in the original parental lines. This unique case strengthens the association of the S-pcf locus with the CMS phenomenon in that the only fertile plants which sorted out fertile progenies were somatic hybrids with heteroplasm and restorer genes in the nuclear background.

- (4) The same nuclear gene which restores fertility also affects Pcf expression at the polypeptide level (Nivison and Hanson 1989; Hanson et al., 1989).
- (5) Furthermore, detailed mapping of the mt genome showed that the Pcf region is the only region different between normal and CMS cytoplasms (Hanson et al., 1990).

In order to understand why and how the presence of the CMS-associated S-Pcf locus confers sterility it was suggested to characterize the normal counterparts of the fused pcmsf gene's components.

Two normal petunia coxII genes present in the fertile line 3704 have been sequenced (Pruitt and Hanson, 1989). Close examination of the sequence of normal coxII and the normal atp9 sequences reveals homology which may have been responsible for the fusion of atp9 sequences to coxII sequences in Pcf.

Furthermore, the lack of intron sequences within the coxII-homologous region of pcf suggests that this region may be a processed pseudogene

(Pruitt and Hanson, 1989).

In order to determine whether another gene is located 3' downstream to Pcf sequencing of this region was done.

Sequencing has revealed two genes. Co-transcribed with pcf are the genes for subunit 3 of NADH dehydrogenase (nad3) and small ribosomal subunit protein 12 (rps12) (Rasmussen and Hanson, 1989). The co-transcribed nad3 and rps12 genes are the only copies of these genes present in the CMS line's mtDNA, while normal copies of atp9 and coxII are present elsewhere. The coding region of the nad3 and rps12 genes appear normal in comparison to the homologous genes from fertile petunia and other organisms (Rasmussen and Hanson, 1989). However, in the fertile petunia line 3704, pcf is not present, and nad3 and rps12 are located downstream of a different regulatory sequence. Thus the petunia CMS-associated region is actually a complex locus (termed S-pcf) comprised of three different co-transcribed genes: pcf, nad3, and rps12.

So far, urf-s (which is included in the Pcf gene) was found to be unique to sterile lines and sterile somatic hybrids and was not detected in fertile lines and stable fertile somatic hybrids by Southern hybridization (Wolf, et al. in preparation).

In a search for the origin of urf-s, an urf-s related sequence (termed urf-f) was identified in a fertile petunia line (F5) which does not contain fertility restorer genes. Urf-f was detected 5' to nad3. However, in contrast to urf-s, atp9 and coxII sequences were not detected 5' to urf-f. These results suggest that the mt genome organization around the urf sequence in the fertile F5 line is different from that of the CMS lines and from that of all the other fertile lines and fertile wild species examined. These results raise the possibility that F5 (originated from South America - the source of the Petunia genus) is related to the wild progenitor of CMS petunia.

With respect to our proposal to study the expression of the abnormal fused gene and homologous sequences.

Transcript mapping of both atp9 and S-pcf by S1 nuclease protection revealed three 5' transcript termini at identical positions in both genes. In suspension culture RNA of CMS lines, the transcripts terminating closest to the start codon of these genes are the most abundant. S1 protection experiments also revealed that pcf, nad3 and rps12 are co-transcribed (Rasmussen and Hanson, 1989); Hanson et al,

1990). More detailed analysis of the 3' transcript termini has revealed the presence of two termini (Hanson et al; 1990). Thus, there could be as many as six transcripts of S-pcf differing in length in suspension culture RNA. However, whether transcripts terminating at all three 5' points also exhibit both 3' termini is not known.

Transcript analysis has also revealed large differences in abundance of S-pcf transcripts in plants carrying different nuclear backgrounds. Further analysis of transcripts, comparing different tissues and Rf lines, is in progress.

Transcript analysis of the S-Pcf locus in the fertility restored line 13-194 revealed the presence of a 2.5 kb transcript (Wolf et al., in preparation). This line contains a multigenic restoration system. The amount- of the Pcf RNA in the CMS and fertility restored plants was found to be comparable both in leaves and anthers.

In situ hybridization experiments of reproductive tissues with Pcf probes were not done yet.

Probes have already been constructed for use in these experiments. It was possible to construct the proper probes for these experiment only after the information about the S-pcf locus became available. We plan to do the experiments in the near future.

Antiserum to the URF-S-specific synthetic peptide did detect a protein present in the CMS parental line that was absent from the normal fertile line (Hanson et al., 1990). This 25-kd protein was present in CMS somatic hybrids, but missing from fertile somatic hybrids examined (Nivison and Hanson, 1989). The 25-kd protein is much reduced in abundance in lines containing at least one Rf allele (Hanson et al., 1990).

In addition to recognizing the 25-kd CMS-specific protein, the anti-URF-S antiserum recognizes a 20-kd protein which is present in both fertile and CMS lines.

Both the A.R.O. and Cornell laboratories continue the studies of the expression of the S-pcf locus at the polypeptide level.

A fragment of the S-pcf locus was expressed in E.coli in order to produce the urf-s peptide to be used for the production of antibodies (Roth, 1988). Transformed cells of E. coli expressed the urf-s polypeptide which also interacted with antibodies produced against an urf-s synthetic polypeptide.

Benefit to agriculture

In this project the association of the mt S-pcf locus with CMS in petunia has been established. The S-pcf locus has been characterized and found to contain three ORFs.

A basis for studying the biochemistry and physiology of CMS is now available.

This information will be important in future studies and in the induction of CMS in other crop species.

This study also forms the basis for the study of the mechanism of nuclear fertility restoration of CMS in petunia. The use of male fertility restoration genes is essential in those crops in which the reproductive parts are the economical yield.

H. List of publications

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Wolf, O., Clark, E., Soferman, O., Tabib, Y., and Izhar, S. (1989). A unique mitochondrial gene region associated with CMS in Petunia: Molecular and genetic evidence. XII EUCARPIA Congress, Gottingen, Germany.

Wolf, O., Soferman, O., Izhar, S. The presence of restorer genes in fertile segregants of petunia somatic hybrids which contain the S-Pcf locus. in preparation.

Figure 3.
THE URF-S REGION HYBRIDIZES
SPECIFICALLY WITH CMS RNA

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- Lifshitz I. (1986). Isolation and Characterization of the Mitochondrial Genome in Petunia. MSc. Thesis, Hebrew Univ. of Jerusalem, Israel (in Hebrew, with English summary).
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Looking for the CMS progenitor in Petunia

N. Firon, O. Soferman, Y. Tabib and S. Izhar

In our search for the fertile progenitor mtDNA that gave rise to the CMS cytoplasm, we have recently identified an urf-s related sequence in a fertile petunia line (F5) which does not contain fertility restorer genes. So far, urf-s was found to be unique to sterile lines and sterile somatic hybrids.

The urf-s related sequence in the fertile F5 line was detected in four plants of the same fertile line, using the polymerase chain reaction (PCR) technique. Using a pair of synthetic oligonucleotides flanking the urf-s sequence we obtained in both a CMS line and F5 DNA, an amplified fragment, 470 bases long. This amplified urf-s fragment (termed urf-f) hybridizes with a cloned ³²p-labelled genomic urf-s sequence at high stringency (Figure 1, A, B). Atp9 and coxII sequences are not detected 5' to urf-f in this fertile line, as opposed to a CMS line (Figure 2), suggesting that the pcf ORF is not present in F5 line.

The urf-f sequence is found to be located 5' to nad3. The urf-nad3 fragment which was PCR-amplified, comigrates with the corresponding fragment derived from a CMS plant line. This sequence hybridizes to an urf-s probe as well as an nad3 probe (Figure 1).

These recent findings are also important for the study of the evolution of the CMS-associated S-pcf locus.

These results suggest that the mt genome organization around the urf sequence in the fertile F5 line is different from that of the CMS lines and from that of all the other fertile lines and fertile wild species examined. These results raise the possibility that F5 (originated from South America - the source of the Petunia genus) is related to the wild progenitor of CMS petunia.

Work is in progress in our laboratory to characterize the urf-f fragment and its possible transcriptional and translational products.

Figure 1. Identification of an urf-S related sequence in the fertile petunia line F5.

URF, URF-NAD, ATP9-URF, and COXII-URF fragments were PCR amplified using total DNA of the fertile F5 line of petunia. The PCR amplified fragments were hybridized to (A.) - a cloned NAD3 probe and (B.) - a cloned URF probe. The probes were cloned from a CMS petunia mtDNA.

Figure 2. PCR-amplification of URF, ATP9-URF and COXII - URF fragments in a CMS petunia line.

The PCR-amplified fragments were separated by gel electrophoresis, blotted into nitrocellulose and hybridized with an urf-S probe.

The effect of multiple nuclear fertility restoration genes on fertile segregants of somatic hybrids which contain the S-Pcf locus.

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Summary

The Cytoplasmic Male Sterility (CMS)-associated region in *Petunia* was defined by the analysis of recombinant mitochondrial genomes of somatic hybrid plants resulting from fusion of protoplasts from CMS and fertile lines. The presence of this region was shown to correlate with the CMS trait in stable sterile somatic hybrids and in other *petunia* lines. A small population of unstable sterile somatic hybrids was also generated in the fusion experiment most of which segregated in the following generations. It was recently shown that stable fertile progeny of such sterile somatic hybrids did not contain the CMS-associated region. In this paper we present molecular and genetic analysis of an unstable progeny of an unstable sterile somatic hybrid plant derived from the same fusion experiment. Both male-sterile and fertile progenies of this somatic hybrid has shown continuous segregation to fertile and male-sterile plants. All plants in this line contain and transcribe the CMS-associated mitochondrial region. Genetic analysis show the presence of multiple nuclear fertility restoration genes in this group of progenies from the original somatic hybrid plant. These findings consolidate the association between the CMS-associated region and the CMS trait in *Petunia*. It also show that the system of multiple nuclear fertility restoration genes does not affect the transcription of the CMS-associated region. This paper also presents data which show that there is no alteration in the mitochondrial CMS-associated region in the fertility - restored progenies mentioned above.

Key words: *Petunia* - Cytoplasmic male sterility - Somatic hybrids - mitochondrial DNA - Fertility restoration genes.

Introduction

Cytoplasmic Male Sterility (CMS) has been shown to be coded for in the mitochondrial genome (Hanson and Conde 1985; Pring and Lonsdale 1985). However, only in a number of plant species, notably maize and *Petunia*, an association of specific mitochondrial sequences with the CMS phenotype could be made (Hanson et al. 1989).

The CMS-associated region of *Petunia* was defined by the analysis of recombinant mitochondrial genomes of somatic hybrid plants constructed by fusing protoplasts from a CMS line (*Petunia parodii* ; line 3688) and a fertile line (*Petunia hybrida* , line 3704; Izhar et al. 1983). Comparison of the mtDNA restriction fragments identified a region of DNA arrangement present in the sterile parent and sterile stable somatic hybrids but absent from the fertile parent and fertile stable somatic hybrids. This first observation was made by Lifshitz (1986) analysed and defined by Boeshore et al. 1985. This region was shown to contain three adjacent open reading frames: Pcf (*Petunia* CMS-associated fused gene), consisting of sequences homologous to segments of the genes for ATP synthase subunit 9 (atp9) and cytochrome oxidase subunit II (coxII) and an unidentified reading frame (urf-S) (Young and Hanson 1987); nad3, the gene for NADH dehydrogenase subunit 3; and rps12, the gene for the small ribosomal subunit protein 12 (Rasmussen and Hanson 1989). The three genes are co-transcribed (Young and Hanson 1987; Rasmussen and Hanson 1989). It may be important to emphasize that the whole locus is regulated by the atp9 promoter.

Over 4000 somatic hybrid plants were generated in the previous protoplast fusion experiment, about 95% of which were stable fertile, about 1% were stable male sterile and about 1% showed unstable behavior (Izhar et al. 1983). Some of the unstable somatic hybrid plants sorted out during the following generations; it has been shown that such sterile hybrids which reverted to fertility, have lost the CMS-associated region (Clark et al. 1988). However, two unstable somatic hybrids, 33-1 and 13-194, showed peculiar behavior: They were phenotypically sterile for over two years, but upon crossing they segregated male sterile and fertile progenies. This instability continued over several generations (Izhar et al. 1983; Wolf et al.

1988). In the present study we bring a molecular and genetic analysis of the progeny of line 13-194. The data strongly corroborate the former molecular and genetical evidence showing that the CMS- associated region in *Petunia* is apparently the sequence causing the CMS phenotype.

Materials and methods

Plant material. Line 13-194 originated in a protoplast fusion experiment (Izhar et al. 1983). The parental lines were: 1) A CMS line 3688, having a *Petunia parodii* (L.S.M.) nuclear background and a sterile cytoplasm which was introduced by repeated backcrosses. 2) Line 3704, a normal fertile line of *Petunia hybrida* (Hook) Vilm. Both lines were tested and found to have no male fertility restorer genes (Ettinger-Paltin 1981). A fertile tetraploid line 6698 was obtained by colchicine treatment of plants resulting from crossing lines 3704 x 3699, 3699 being a normal fertile line isonuclear to 3688 (Izhar et al. 1983). The original somatic hybrid 13-194 was sterile and, upon crossing with 6698, yielded sterile and fertile plants. Line 13-194 continued to show instability and was still segregating after five generations.

Hybridization probes. Plasmid clones encompassing the *Petunia* mitochondrial CMS-associated region were provided by MR Hanson (Young and Hanson 1987). Specific probes were constructed by subcloning the various fragments into pUC9 and pIBI31 (International biotechnologies, Inc.), as shown in Fig.1. Whole plasmid DNA was labelled by nick-translation (Rigby et al. 1977). Alternatively, plasmid inserts were isolated from preparative gels by electroelution and labelled by the method of random priming (Feinberg and Vogelstein 1983). Radiolabelling kits were obtained from Amersham and International Biotechnologies, Inc. Radiolabelled nucleotides were purchased from Amersham and New England Nuclear.

DNA analysis. Total DNA was prepared from fresh leaf tissue by a modification of the method of Murray and Thompson (1980). Restriction endonuclease-digested DNA was separated on 1% agarose gels and

transferred to nitro-cellulose membranes (Schleicher & Schuell). The transfer, hybridization and autoradiography were done according to standard procedures (Maniatis et al. 1982). Restriction endonucleases were purchased from Pharmacia and United States Biochemical and used according to the manufacturers' directions.

RNA analysis. Leaf tissue (up to 5 g) was ground in liquid nitrogen and immediately transferred into 5 volumes of RNA extraction buffer containing equal volumes of phenol (saturated with 0.1 M Tris, pH 8.8) and LSTE buffer (150 mM LiCl, 5 % SDS, 50 mM Tris, pH 8.8, 5 mM EDTA). After shaking for 10 min, 0.1 volumes of chloroform were added and the phases separated by centrifugation at 10,000 rpm for 15 min in a SS-34 rotor. The organic phase was back-extracted with LSTE buffer and the combined aqueous phase was extracted with an equal volume of phenol-chloroform (1:1) followed by an extraction with chloroform. The RNA was precipitated overnight at -200 C with 0.05 volumes of 1 M acetic acid and 0.7 volumes of ethanol. The precipitate was washed with 70% ethanol, dried and suspended in 2 ml of water; an equal volume of 4 M LiCl was added and the samples were kept on ice for 4 h. The pellet was suspended in water, re-precipitated with 0.3 M NaOAc/ethanol and washed with 70% ethanol. Anthers were collected from buds corresponding to early prophase developmental stages. Anther tissue RNA was prepared essentially as described above, using 2 ml of the extraction buffer; centrifugations were performed in Eppendorf tubes. All final RNA preparations were dissolved in water containing 1 mM DTT and 1 unit /ml RNasin (Promega). Denatured samples were run on 1% agarose minigels and stained with ethidium bromide to assess the integrity of the RNA. For Northern analyses the RNA was denatured and separated on 1% agarose-formaldehyde gels as described (Maniatis et al. 1982). Gels were run at 4V /cm for 5-6 h, soaked in 20X SSC (1X SSC is 0.15 M NaCl, 0.015 M NaCitrate) for 30 min and the RNA was transferred to nitro-cellulose membranes (Schleicher and Schuell) in 20X SSC, overnight. The blots were dried and baked in a vacuum 80°C oven. Hybridization was according to Maniatis et al. (1982).

Results

Molecular studies

DNA. Total DNA isolated from sterile and fertile F_2 plants of line 13-194 was digested with BamHI and hybridized with probes from the CMS-associated region (Fig. 1). Identical hybridization patterns were observed for male-sterile and fertile plants of line 13-194 (Fig. 2A-C; also with the pPC3 and coxII probes, data not shown). The pURF-S probe, shown to hybridize specifically with the Pcf gene (Wolf et al. 1988; Fig. 2A four last lanes), detected a single, 6.6 Kb fragment in all the progeny of line 13-194, sterile and fertile (Fig 2A). Hybridization with other probes from the CMS-associated region detected the same 6.6 Kb fragment in all plants of line 13-194 (Fig. 2 B,C). This BamHI fragment is characteristic for *Petunia* CMS genomes (Fig. 2 A-C, S 3688 and S 11616) and is absent in fertile lines (Fig. 2 A-C, F 3699 and F 3704). These results show that the Pcf gene and the CMS-associated region are present in all 13-194 progeny. The pATP9 probe contains the CMS-associated atp9 segment, which is almost identical to that of the normal atp9 gene (Young and Hanson 1987). Hybridization resulted in multiple bands due to the presence of normal atp9 gene(s) in both male-sterile and fertile mitochondria (Young et al. 1986; Fig. 2B). The intensity of these bands and that of the CMS-associated 6.6 Kb band seems comparable, indicating that they are in a similar stoichiometry. There is no difference in the abundance of the CMS-associated region in the DNA of male-sterile and fertile plants of line 13-194 or in parental CMS. The pPC4 probe detects the nad3/rps12 region. *Petunia* mitochondrial genomes contain only one copy of the nad3/rps12 genes; in male-sterile plants they are part of the CMS-associated region and reside on the 6.6 Kb BamHI fragment, whereas in normal genomes they reside on a 1.8 Kb BamHI fragment (Rasmussen and Hanson 1989; Fig. 2C four last lanes). A single 6.6 Kb fragment was detected in all progeny of the 13-194 line and, therefore, there was no evidence for additional normal mitochondrial genomes in these plants (Fig. 2C).

RNA.

The RNA from both sterile and fertile 13-194 plants as well as the RNA from the parental CMS line 3688 showed a 2.5 Kb band hybridizing with pURF-S (Fig. 3). A Pcf transcript of a similar size has been previously shown using S1 nuclease protection analysis (Young and Hanson 1987). The RNA from the fertile Petunia lines 3699 and 3704 did not react with the pURF-S probe. To adjust for mitochondrial RNA contents, the blots were re-hybridized with pATP9; this probe revealed an atp9 transcript of about 500 bp in all lanes (data not shown). Somewhat higher amounts of both Pcf and atp9 transcripts were detected in the samples from the fertile 13-194 plants as compared to those from sterile 13-194 plants (Fig. 3), probably due to more mtRNA in the fertile samples. The amount of Pcf RNA in the leaves of male-sterile and fertile plants of the progeny of 13-194 seems, therefore, to be comparable. The same is true for the anthers: the amount of Pcf RNA in the developing anthers of male sterile and fertile plants of the line 13-194 is also comparable.

Genetic studies

The original somatic hybrid 13-194 was sterile. Upon crossing with 6698, it yielded 13 sterile plants and 2 fertile ones (Fig. 4). Later crosses were within the line. The line continued to show instability and was still segregating after five generations. Both male-sterile and fertile plants produced both male-sterile and fertile progenies. This is the only case in Petunia where fertile plants gave rise to sterile progeny. The percentage of fertile plants in the population increased with each generation of inbred crosses, so that the majority of the plants in F_5 were fertile. Somatically, however, the plants in line 13-194 were stable: cuttings from male-sterile plants gave rise to sterile plants only, and cuttings from fertile plants to fertile plants only. Whereas in parental lines and in stable somatic hybrids the plants' phenotype was either full fertility or complete sterility, the 13-194 population contained also partially fertile plants with varying amounts of pollen. To find out whether the segregation in this line is due to a sorting out of mitochondria or segregation of nuclei, we

conducted the following experiment:

Fertile and male-sterile F_5 plants of line 13-194 were pollinated with two sources of pollen: a) Fertile 13-194 plants; b) The progeny of line 6698. Although these plants vary in their nuclear backgrounds, all are stable fertile tetraploids and should not contain restorers, as both parental lines of 6698 (3704 and 3699) did not contain restorers. The crosses yielded over a thousand plants, which were classified as fully fertile (F), partially fertile (F_2 or F_3) or completely sterile (S) according to the amount of pollen. The results are summarized in Table 1. Tables 2 and 3 compare the progenies of individual 13-194 plants pollinated with the two alternative sources of pollen. Whereas the crosses within the line 13-194 (selfing of F_{13-194} and $S_{13-194} \times F_{13-194}$) showed segregation similar to that of previous generations (data not shown). Using a stable fertile line as pollinator caused a marked shift towards sterility. This indicates the presence of nuclear restorers in the progeny of 13-194. The restoration must involve more than one nuclear gene and is not dominant.

Discussion

The somatic hybrid 13-194 was generated in the protoplast fusion experiment that led to the definition of the CMS-associated region (Izhar et al. 1983). Originally male-sterile, upon crossing it segregated male-sterile and fertile progenies, which continued to segregate through the following generations. The plants were somatically stable and, unlike the progenies of other somatic hybrids, exhibited quantitative variation in the amount of pollen. The hybridization patterns of DNA from male-sterile and fertile 13-194 plants seemed similar and all contained and expressed the CMS-associated region *Pcf/nad3/rps12*. Only one arrangement of the *nad3/rps12* region could be detected, making the possibility of more than one mitochondrial genome in these plants quite unlikely. Pollination of male-sterile and fertile 13-194 plants with two alternative sources of pollen, plants of the 13-194 line and stable fertile tetraploids, showed that the ratio of sterile plants in the populations is dependent on the source of pollen. Crosses with stable

fertile plants yielded a significantly higher proportion of male-sterile plants as compared with crosses within the 13-194 line. This indicates very strongly a restoration of nuclear origin. The genetic and molecular data, therefore, indicate that the same mitochondria are present in male-sterile and fertile 13-194 plants and that the phenotype is determined by the presence or absence of restorers in the nucleus.

In *Petunia* a single CMS cytoplasm exists (Izhar and Frankel 1976; Izhar 1984), which can be restored either by a single dominant nuclear gene, Rf or by a multi-gene, temperature-sensitive nuclear restorers (Izhar 1984). The parental lines in the original fusion experiment, line 3688 and line 3704, were shown to contain no restorers. Crosses of male-sterile stable plants using pollen from fertile 13-194 plants did not restore their fertility in F_1 (data not shown). This phenomenon is expected with the multigenic restoration system known in *Petunia* (Izhar 1978). The changes in restoration illustrated in Tables 1-3 and the variability in the amount of pollen which was observed in line 13-194 are typical to a quantitative system.

On the molecular level, the restoration mechanism in line 13-194 does not seem to affect the abundance of the CMS-associated region or its transcription. Similar results were found for the action of the single dominant restorer Rf (Young and Hanson 1987; Wolf unpublished results). We presently investigate whether the translational level is affected. Elucidation of the restoration mechanism in this line will help us to understand the molecular events causing CMS in *Petunia*.

The CMS in *Petunia* is highly stable with respect to expression and transmission. Unlike maize CMS, no spontaneous reversions have been observed in intact plants or in normal plants regenerated from tissue culture (Izhar, 1984). This can be explained in part by the recent findings of Rasmussen and Hanson (1989) that the only copy of the *nad3/rps12* genes in the CMS mitochondrial genome is co-transcribed with the *Pcf* gene, using its promoter. "Reversion" from sterility to fertility in *Petunia* is, therefore, likely to be associated with either segregation of mitochondria or a change in cytoplasmic-nuclear interactions. It is theoretically possible that the 13-194 recombinant mitochondria acquired new characteristics that enabled them to be

restored by 13-194 restorers. However, restriction enzyme digestions with a number of enzymes followed by hybridization with several probes did not reveal any changes between the 13-194 and the parental CMS-associated region.

The CMS-associated region was originally defined by the analysis of recombinant mitochondrial genomes of stable somatic hybrids of a CMS and a fertile lines (Boeshore et al. 1985; Lifshitz, 1986). A small population of unstable somatic hybrids was also generated in this experiment. Recently it has been shown that fertile progeny derived from three different male-sterile somatic hybrids, which contained the CMS-associated region, have lost it upon reversion to fertility in F_2 (Clark et al. 1988). The progeny exhibited fragments characteristic to the normal nad3/rps12 arrangement and the reversion was stable since only fertile F_3 progeny was produced. The loss of the CMS-associated region in these plants seems, therefore, to be correlated with an irreversible loss of male-sterility and the results are compatible with segregation of mitochondria. The present findings consolidate the association between the CMS-associated region and CMS in Petunia. These results strengthen the hypothesis that the Pcf/nad3/rsp12 complex is necessary for the induction of CMS. In all cases where this region was absent the plants were fertile and had no potential for sterility. In the presence of the CMS-associated region the plants were either sterile, or fertile in the presence of restorers. Fertile plants containing the CMS-associated region retained their potential for sterility, which could be recovered upon crosses.

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