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Interaction of the mitochondrial S-*Pcf* locus for cytoplasmic male sterility in *Petunia* with multiple fertility-restoration genes in somatic hybrid plants

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Summary. The Cytoplasmic Male Sterility (CMS)-associated region in *Petunia*, the S-Pcf locus, was defined by the analysis of recombinant mitochondrial genomes of somatic hybrid plants resulting from a fusion of protoplasts from CMS and fertile lines. The presence of the S-Pcf locus was shown to correlate with the CMS trait in stable somatic hybrids and in other CMS *Petunia* lines. A small population of unstable, sterile somatic hybrids was also generated in this fusion, most of which underwent cytoplasmic segregation in subsequent generations. Stable revertants of such sterile somatic hybrids were shown to lose the S-Pcf locus. In this paper we present a molecular and genetic analysis of unstable progenies of an unstable, sterile somatic hybrid plant derived from the same fusion experiment. Both male-sterile and fertile progenies of this somatic hybrid plant have shown continuous segregation of fertile and male-sterile progenies. All segregants in this line contained, and transcribed, the S-Pcf locus. Genetic analysis indicated the presence of various levels of multiple nuclear fertility-restoration genes in this group of progenies. These findings consolidate the association between the S-Pcf locus and the CMS trait in *Petunia*. It also shows that the restoration of fertility by the multiple nuclear gene system does not affect the transcription of the S-Pcf locus and that the presence of an intact S-Pcf locus is necessary in order to maintain the potential sterility in the cytoplasm.

Key words: *Petunia* – Cytoplasmic male sterility – Somatic hybrids – Mitochondrial DNA – Fertility-restoration genes

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Introduction

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

The CMS-associated region of *Petunia* was defined by the analysis of recombinant mitochondrial genomes of somatic hybrid plants produced by fusing protoplasts from a CMS line (*Petunia parodii*; line 3688) and a fertile line (*Petunia hybrida*; line 3704) (Izhar et al. 1983; Schlicter 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) and the region was further defined by Boeshore et al. (1985). The locus, named S-Pcf (Hanson et al. 1989), was shown to contain three adjacent open reading frames: Pcf, the *Petunia* CMS-associated fused gene (Young and Hanson 1987) and the genes for NADH dehydrogenase subunit 3 (nad3) and the small ribosomal subunit protein 12 (rps12) (Rasmussen and Hanson 1989). The three genes are cotranscribed (Young and Hanson 1987; Rasmussen and Hanson 1989). The Pcf gene consists 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).

Over 4000 somatic hybrid plants were generated in the original protoplast fusion experiment, about 97% of which were stable fertile, about 2% were stable male-sterile and about 1% showed unstable behavior (Izhar et al.

1983). The unstable somatic hybrid plants sorted out somatically or in subsequent generations. It has been shown that some unstable male-sterile somatic hybrids, which reverted to fertility, have lost the S-Pcf locus (Clark et al. 1988). However, two unstable somatic hybrids, 33-1 and 13-194, identified by Izhar et al. 1983 and Schlicter (1983), showed peculiar behavior. They were phenotypically sterile for over 2 years, but upon crossing segregated male-sterile and fertile progenies. This instability has continued over several generations and into the present (Izhar et al. 1983; Wolf et al. 1988). In this study we report on a molecular and genetic analysis of the progenies of line 13-194. The data strongly corroborate the former molecular and genetic evidence showing that the S-Pcf locus in *Petunia* is apparently the sequence causing the CMS phenotype. The results show the presence of an intact S-Pcf locus in all progenies tested and point to the existence of nuclear multiple fertility-restoration genes in this system.

Materials and methods

Plant material

Line 13-194 originated in a protoplast fusion experiment (Izhar et al. 1983; Schlicter 1983). The parental lines were: (1) A CMS line 3688, having a *P. parodii* (L.S.M.) nuclear background and a sterile cytoplasm which was introduced by repeated backcrosses. (2) Line 3704, a normal fertile line of *P. hybrida (Hook) Vilm.* Both the original lines were tested and found to have no male fertility-restorer genes (Ettinger-Paltin 1981; see also Izhar 1984). A fertile tetraploid line 6698 was obtained by colchicine treatment of F_1 plants resulting from the cross 3704 × 3699, 3699 being a normal fertile line isonuclear to 3688 (Izhar et al. 1983). A sterile tetraploid line, 6697, was obtained by colchicine treatment of F_1 plants of a cross between 3688 × 3704. Plants of lines 6698 and 6697 were used as tester lines in crosses with tetraploid somatic hybrid plants. All the somatic hybrid plants referred to in this paper, as well as plants of lines 6698 and 6697, were tetraploids with 4n = 28 chromosomes (Izhar et al. 1983).

Hybridization probes

Plasmid clones encompassing the *Petunia* mitochondrial S-Pcf locus were provided by M. R. Hanson (Young and Hanson 1987). Specific probes were constructed by subcloning the various fragments into pUC9 and pIB131 vectors (International Biotechnologies, Inc.), as shown in Fig. 1. Whole plasmid DNA was labelled by nick-translation (Rigby et al. 1977). 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 and 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 manufacturer's directions.

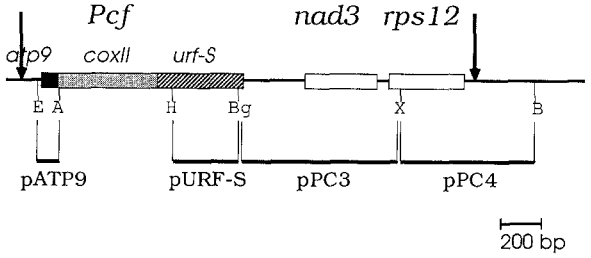

Fig. 1. The *Petunia* mitochondrial S-*Pcf* locus. The structure of the region is according to Young and Hanson (1987) and Rasmussen and Hanson (1989). *Arrows* indicate the start and end sites of the major RNA transcript. Specific probes were constructed by digestion with restriction enzymes (*A*, *Acc*I; *B*, *Bam*HI; Bg, *Bgl*II; *E*, *Eco*RI; *H*, *Hin*dIII; *X*, *Xho*I) followed by cloning into pUC9 or pIBI31 vectors

RNA analysis

Leaf tissue (up to 5 g) was ground in liquid nitrogen and immediately transferred into five 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 −20 °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, reprecipitated with 0.3 M NaOAc/ethanol and washed with 70% ethanol. Anthers were collected from buds corresponding to early prophase developmental stages. Anther 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 4 V/cm for 5–6 h, soaked in 20 × SSC for 30 min and the RNA was transferred to nitrocellulose membranes (Schleicher and Schuell) in 20 × 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 F_5 plants of line 13-194 was digested with *Bam*HI and hybridized with probes from the S-Pcf locus (see Fig. 1) and a maize coxII probe

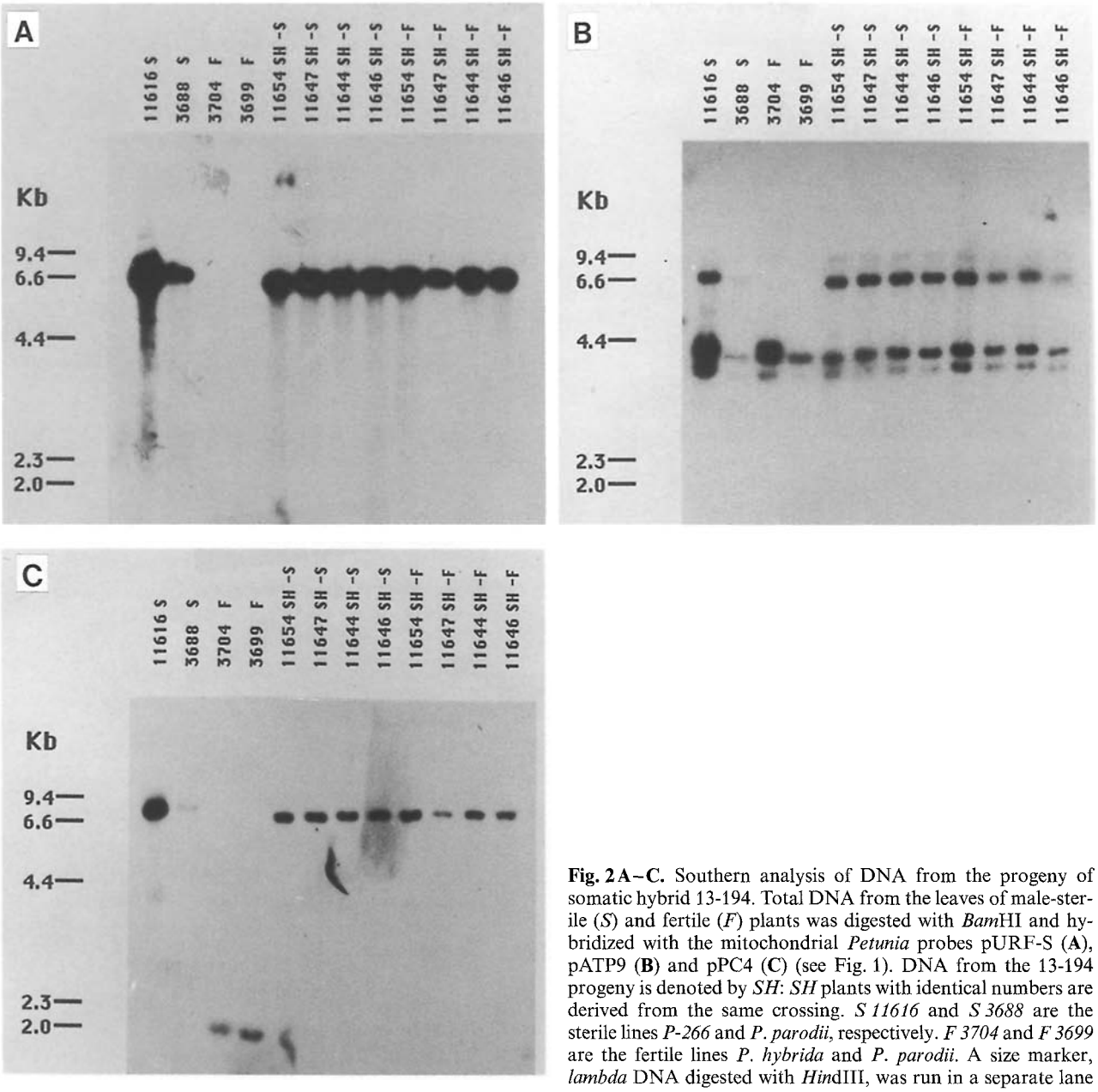

Fig. 2A–C. Southern analysis of DNA from the progeny of somatic hybrid 13-194. Total DNA from the leaves of male-sterile (*S*) and fertile (*F*) plants was digested with *Bam*HI and hybridized with the mitochondrial *Petunia* probes pURF-S (**A**), pATP9 (**B**) and pPC4 (**C**) (see Fig. 1). DNA from the 13-194 progeny is denoted by *SH*: *SH* plants with identical numbers are derived from the same crossing. *S 11616* and *S 3688* are the sterile lines *P-266* and *P. parodii*, respectively. *F 3704* and *F 3699* are the fertile lines *P. hybrida* and *P. parodii*. A size marker, *lambda* DNA digested with *Hin*dIII, was run in a separate lane

(pZmE1, Fox and Leaver 1981). The results are shown in Fig. 2: pURF-S hybridizes specifically with the Pcf gene (Wolf et al. 1988); a single, 6.6 kb *Bam*HI fragment is detected in *Petunia* CMS lines and is absent in fertile lines (Fig. 2A, first four lanes). All the probes from the S-Pcf locus detect the 6.6 kb fragment in male-sterile lines, showing that this fragment encompasses the whole region (Fig. 2A-C). The S-Pcf atp9 segment is identical to the amino-terminus of the normal atp9 gene (Young and Hanson 1987); therefore, hybridization with pATP shows additional band(s) due to the presence of normal atp9 gene(s) in both male-sterile and fertile mitochondria (Young et al. 1986; Fig. 2B, first four lanes). The intensity of these additional bands and the 6.6 kb band seem comparable, indicating that the atp9 and the S-Pcf genes are in a similar stoichiometry. pPC4 detects the nad3/rps12 region, only one copy of which is present in the *Petunia* mitochondrial genome: in male-sterile plants it is part of the S-Pcf locus and resides on the 6.6 kb *Bam*HI fragment, whereas in normal genomes it resides on a 1.8 kb *Bam*HI fragment (Rasmussen and Hanson 1989; Fig. 2C, first four lanes).

The hybridization patterns obtained for male-sterile and fertile plants of line 13-194 were identical in all cases, and similar to those of the male-sterile lines (Fig. 2, A–C). Equivalent results were obtained for the pPC3 and

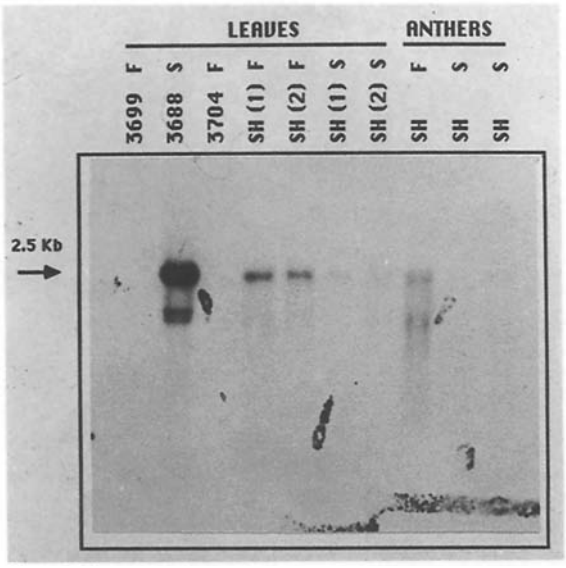

Fig. 3. Northern analysis of RNA from the progeny of somatic hybrid 13-194. Total RNA from the leaves and anthers of male-sterile (*S*) and fertile (*F*) plants (about 10 μg RNA per lane) was separated on a 1% formaldehyde-agarose gel and probed with pURF-S (Fig. 1). RNA from the progeny of 13-194 is denoted by *SH. F 3699*, and *F 3704* are the fertile lines *P. parodii* and *P. hybrida*, respectively. *S 3688* is the sterile line *P. parodii*

pZmE1 probes, and for other restriction enzymes (data not shown). The results show that the Pcf gene and the entire S-Pcf locus are present in all 13-194 progeny, and in seemingly the same abundance, disregarding the phenotype. The probes of the nad3/rps12 region, pPC4 (Fig. 2C) and pPC3 (data not shown), detected only the 6.6 kb band, showing that there is no indication for an additional normal arrangement of these genes in the 13-194 genome.

RNA. The RNA from the CMS line 3688 reacted with pURF-S, showing a band at 2.5 kb (as well as an additional band at about 1.4 kb); the RNA from the fertile lines 3699 and 3704 did not react with this probe (Fig. 3). A similar 2.5 kb Pcf transcript has been previously shown using S1 nuclease protection analysis (Young and Hanson 1987). RNA from the leaves of both male-sterile and fertile plants of line 13-194 reacted with pURF-S to yield a pattern similar to that of the CMS line (Fig. 3). To adjust for mitochondrial RNA content, the blot was rehybridized 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, 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 amount of Pcf RNA in the developing anthers of male-sterile and fertile plants of the line 13-194 is also comparable (Fig. 3).

Genetic studies

The original somatic hybrid 13-194 was sterile (Izhar et al. 1983). Upon crossing with the tester line 6698, it yielded 13 sterile plants and two fertile ones. Plants in this line continued to show instability and were still segregating after five generations; both male-sterile and fertile plants segregated male-sterile and fertile progenies. However, cuttings from male-sterile plants gave rise to sterile plants only, and cuttings from fertile plants to fertile plants only. The proportion of fertile plants in the population increased with each generation of inbred crosses. (For example, out of 2609 F_4 plants, 1788 (69%) were fully fertile.)

Whereas in parental lines and in stable somatic hybrids the plant phenotype was either fully fertile or completely sterile, in the 13-194-derived population partially fertile plants with varying amounts of pollen were present.

To find out whether the segregation in this line is due to a sorting out of mitochondria of the segregation of nuclear genes, we conducted the following experiment: fertile and male-sterile F_5 plants of line 13-194 were pollinated with two sources of pollen: (1) Fertile plants of line 13-194, and (2) Fertile plants of line 6698; these plants were all tetraploid and did not contain any obvious fertility-restoration genes (as tested by crosses). Also some fertile F_2 plants of line 13-194 were used as a pollen source in test crosses with the sterile 6697 CMS tester line.

Over 1,000 plants were obtained. They were classified as fully fertile (F), partially fertile (F^2 or F^3) or completely sterile (S) according to the amount of pollen (see Izhar 1978). The results are summarized in Table 1. Table 2 compares the progenies of individual 13-194 plants pollinated with the two alternative sources of pollen. The data show that by a simple cross it was possible to manipulate the level of fertile vs sterile progenies. Whereas the crosses within line 13-194 (selfing of F13-194 and crosses of S13-194 × F13-194) showed segregation similar to that of previous generations (data not shown), the use of a stable fertile line (6698) as pollinator caused a marked shift towards sterility. This indicates the presence of nuclear restoration genes in the progeny of 13-194. This restoration involves a multiple number of genes similar to that described by Izhar (1978). The restoration genes were present in both the female and male gametes of line 13-194. Different levels of nuclear fertility-restoration genes are present in the progenies of 13-194. It is clear that even male-sterile plants of line 13-194 contain restoration genes (19.1% of the progeny of the test-cross S13-194 ×

Table 1. Test crosses of the progenies of plants of line 13-194. Progenies of fertile (F13-194) and male-sterile (S13-194) plants of line 13-194 were pollinated with two sources of pollen, fertile 13-194 plants and stable fertile plants of line 6698. The resulting plants were classified as fully fertile (F), partially fertile (F^2 or F^3) or completely sterile (S) according to the amount of pollen

Cross	No. of progeny (%)				Total
	F	F^2	F^3	S	
F13-194 × F13-194	90 (81.1)	19 (17.1)	2 (1.8)	0 (0.0)	111
F13-194 × 6698	142 (45.2)	57 (18.2)	47 (15.0)	68 (21.7)	314
S13-194 × F13-194	234 (71.8)	32 (9.8)	31 (9.5)	29 (8.9)	326
S13-194 × 6698	54 (19.1)	14 (4.9)	46 (16.3)	169 (59.7)	283

Table 2. Test crosses of the progenies of individual plants of line 13-194. Fertile (F13-194, nos. 1–5) and male-sterile (S13-194, nos. 6–9) plants were pollinated with two sources of pollen, fertile 13-194 plants and stable fertile plants of line 6698. The crosses F13-194 × F13-194 were selfings. The resulting plants were classified according to the amount of pollen as in Table 1

Plant no.	XF13-194				X 6698			
	F	F^2	F^3	S	F	F^2	F^3	S
F13-194								
1	22	4	1	0	10	6	11	6
2	24	8	1	0	15	1	4	3
3	12	4	0	0	8	9	7	9
4	22	0	0	0	26	0	3	3
5	10	3	0	0	18	1	3	13
S13-194								
6	22	8	4	8	9	5	9	19
7	20	3	1	0	10	2	7	15
8a	16	8	6	6	0	0	3	46
8b	37	2	0	0				
9	34	8	10	9	9	2	1	16

6698 were fertile; Table 1), although in lesser amount than fertile plants (compare: 45.2% fertile progeny for F13-194 × 6698; Table 1). Interestingly, when F13-194 segregants where used as a pollinator on the CMS tester line 6697, they did not produce fertile plants, although several hundreds of progeny plants were observed. Since these were F13-194 F_2 plants, it is possible that they had not yet accumulated such a high number of restoration genes.

The sterility/fertility phenotype of some populations of line 13-194 was observed over a period of about 1 year. Typical changes in the expression of fertility to sterility occurred when the temperature in the greenhouse was above 25 °C; some of these plants reverted to fertility when temperatures went down toward 20 °C. The results (data not shown) were typical of the temperature sensitivity shown by the fertility-restoration genes in the earlier studies of Izhar (1978, 1984).

Discussion

The somatic hybrid 13-194 was generated in the same protoplast fusion experiment that led to the definition of the S-Pcf locus in *Petunia* (Izhar et al. 1983). The original 13-194 plant was male sterile, but upon crossing, it segregated male sterile and fertile progenies, which, although somatically stable, continued to segregate through subsequent generations. Unlike the progenies of stable somatic hybrids, the progenies of 13-194 exhibited quantitative variation in the amount of pollen and their sensitivity to ambient temperature.

The hybridization patterns of DNA from male-sterile and fertile 13-194 plants were similar and all plants contained the entire S-Pcf locus in seemingly the same stochiometric amount. The RNA hybridization patterns were also similar and the S-Pcf transcript was present in comparable amounts in both leaves and anthers of fertile and male-sterile 13-194 plants. The DNA and RNA patterns were indistinguishable from those of CMS lines. The molecular data point, therefore, to the presence of a seemingly intact S-Pcf locus in all 13-194 progenies. There was no evidence for an additional arrangement of the S-Pcf region or the existence of additional copies of a normal mitochondrial genome in these plants.

Pollination of male-sterile and fertile 13-194 plants with two alternative sources of pollen, fertile 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 that the restoration of fertility observed was of nuclear origin and due to an accumualtion of nuclear restoration genes. The parental lines in the original fusion experiment, line 3688 and line 3704, were shown, by regular crosses, to contain no restorers. However, only two plants out of more than 4,000 originated in this experiment, exhibited this peculiar segregation behavior. The accumulation of restorers can, therefore, be explained by a rare event, such as mutation or a certain segregation of genes, that occurred following the original protoplast fusion, and caused the level of the restoration genes to pass a restoration threshold. The fact that these plants are tetraploid somatic hybrids may also affect the accumulation of these genes. The changes in restoration illustrated in Tables 1 and 2, and the variability in the amount of pollen which was observed in line

13-194, are typical of a quantitative system and consistent with the *Petunia* multigenic, temperature-sensitive restoration system described earlier (Izhar 1978, 1984).

Only one CMS cytoplasm is known to exist in *Petunia* (Izhar and Frankel 1976; Izhar 1984). In addition to the above multigenic restoration system, it can be restored by a single dominant nuclear gene, *Rf* (Izhar 1984). It has been shown that the *Rf* restorer does not affect the abundance of the Pcf transcript (Young and Hanson 1987; Wolf, unpublished results). Fertile plants carrying the CMS cytoplasm and the *Rf* nuclear gene have, however, a much lower abundance of a 25 kDa URF-specific protein (Nivison and Hanson 1989). The restoration mechanism in line 13-194 does not seem to affect the arrangement or abundance of the S-Pcf locus or its transcription. We are presently investigating whether the protein level of the S-Pcf genes is affected in these plants. It is both important and interesting to see whether the two nuclear restoration systems affect the expression of the mitochondrial gene in the same way.

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 plants regenerated from tissue culture (Izhar 1984). "Reversion" from sterility to fertility in *Petunia* is, therefore, likely to be associated with the segregation of a mixed population of mitochondria (Clark et al. 1988). Both the genetic and molecular data in this paper imply 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. The present findings consolidate the association between the S-Pcf locus 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. Fertile plants containing the S-Pcf locus retained their potential for sterility, which could be recovered upon crosses.

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