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Detection of an open reading frame related to the CMS-associated *urf-s* in fertile Petunia lines and species and in other fertile *Solanaceae* species*

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Summary. In Petunia, a mitochondrial (mt) locus, *S-Pcf*, has been found to be strongly associated with cytoplasmic male sterility (CMS). The *S-Pcf* locus consists of three open reading frames (ORF) that are co-transcribed. The first ORF, termed *Pcf*, contains an unidentified reading frame *urf-s* that has been detected so far only in sterile Petunia lines and sterile somatic hybrids. In the study described here, a *urf-s*-related sequence was detected in seven different normal fertile Petunia lines and species as well as in additional members of the *Solanaceae* family by means of the polymerase chain reaction. The *urf-s*-related sequence identified in the fertile lines was termed *orf152*. In Petunia the nucleotide sequence of *orf152* was found to be identical to the corresponding part of *urf-s*. However, the genome organization around *orf152* was found to be different from that of *urf-s*. These results indicate that: (1) at least part of the *urf-s* sequence is present in fertile lines and species of Petunia and in other *Solanaceae* species; (2) the *orf152* sequence of Petunia is not part of the *Pcf* ORF. The relevance of these findings to a better understanding of the evolution of the *S-pcf* locus in (S) cytoplasm in Petunia is discussed.

Key words: CMS – Petunia – *urf-s* – *orf152*

Introduction

Cytoplasmic male sterility (CMS) is a maternally inherited trait in higher plants that causes the failure of the processes essential for the production of functional pollen grains. In Petunia, CMS is expressed as the failure to produce pollen grains during gametogenesis (Frankel and Galun 1977; Izhar 1975, 1984). A mitochondria (mt) DNA sequence, the *S-Pcf* locus, has been found to be strongly associated with CMS in Petunia.

Evidence for this association is the following: (1) the mt *S-Pcf* DNA segregates together with sterility in somatic hybrids (Boeshore et al. 1985; Clark et al. 1988), but is lost in fertile revertants; and (2) the nuclear gene (*Rf*), which restores fertility in CMS lines, also reduces the expression level of a CMS-associated 25-kDa polypeptide (Hanson et al. 1989; Nivison and Hanson 1989). The recent results of Wolf-Littman et al. (1992) also corroborate the strong association of *S-Pcf* with CMS in somatic hybrids of Petunia by showing that the entire *S-Pcf* locus is necessary to cause CMS.

The *S-Pcf* locus consists of three adjacent open reading frames (ORF). The first one is a chimera that consists of parts of the genes for ATP synthase proteolipid subunit 9 (*atp9*) and cytochrome oxidase subunit II (*coxII*), both of which are defective as compared to the normal *atp9* and *coxII*, and an unidentified reading frame *urf-s* (Young and Hanson 1987). This fused gene was termed *Pcf*.

urf-s is transcribed and translated into a 25-kDa polypeptide (Nivison and Hanson 1989); it can thus be termed *urf25* (Lonsdale and Leaver 1988). So far, *urf-s* has been found to be unique to CMS lines. The other two ORFs that reside on the *S-Pcf* locus contain the genes for NADH dehydrogenase subunit 3 (*nad3*) and ribosomal protein subunit 12 (*rps12*). *Nad3* and *rps12* are co-transcribed with the *Pcf* ORF. The coding regions of the *nad3* and *rps12* genes appear to be normal in comparison with the homologous genes from fertile Petunia (Gualberto et al. 1988; Rasmussen and Hanson 1989). However, in fertile Petunia lines, *Pcf* is not present, thus *nad3* and *rps12* are located downstream of a different regulatory sequence.

In order to understand what recombination and rearrangement events created the current version of the chimeric *Pcf* ORF, we took upon ourselves to identify and study its origin. Recently, a 20-kDa polypeptide was identified in mitochondria isolated from both fertile and sterile Petunia lines (Nivison and Hanson 1989). This 20-kDa polypeptide is recognized by antibodies pro-

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duced against a synthetic polypeptide whose sequence corresponds to the sequence of the internal direct repeat of the *urf-s* sequence. These results led us to believe that at least part of the *urf-s* sequence is present in fertile Petunia lines. In the present article we describe the identification of a *urf-s*-related sequence in fertile lines and species of Petunia and in other members of the *Solanaceae* family. We show that, in contrast to the situation in CMS lines, in fertile Petunia lines *urf-s* is not part of a *Pcf* ORF.

Materials and methods

Plant material

The following fertile Petunia species and lines were used: *P. parodii*, L.S.M., *P. atkinsonia*, *P. axillaris*, Fries, *P. violaceae*, Lindl. and lines Rosy Morn and 3704 of *P. hybrida* (Hook) Vilm. *P. uruguay* is a line collected by R. Frankel in South America. The sterile Petunia line used was *P. parodii* line 3688 with (S) cytoplasm. Fertile *Lycopersicon esculentum* (tomato), *Capsicum annuum* (pepper) and *Solanum tuberosum* (potato), randomly picked, were also used.

DNA preparation

Plant DNAs were extracted from leaves by the CTAB method of Doyle and Doyle (1990). The DNA extraction procedure was conducted while taking the appropriate precautions to avoid DNA contaminations that might interfere with the PCR reaction (Kwok and Higuchi 1989).

Polymerase chain reaction (PCR)

PCRs were performed using a Perkin Elmer Cetus, DNA thermal cycler. The conditions were those recommended by the Promega Corporation, Madison, Wis. The denaturation, annealing and extension times were 1 min, 2 min and 4 min at 96 °C, 55 °C and 72 °C, respectively. The enzyme Taq polymerase was purchased from Promega. The oligonucleotides, synthesized by an Applied Biosystem synthesizer, were purchased from Biotechnology General, Rehovot, Israel.

DNA transfer and hybridization

The previously cloned *urf-s* fragment (Young and Hanson 1987) was excised from agarose gels and [^{32}P]-radiolabeled by the random priming method of Feinberg and Vogelstein (1983) using a kit from Boehringer-Mannheim, Federal Republic of Germany. The PCR-amplified DNA was transferred from agarose gels onto Hybond-N nylon membranes (Amersham) and fixed by UV cross-linking.

Hybridization conditions were according to the membrane manufacturer's instructions. The hybridization membranes were washed twice for 30 min in 0.1% SDS, 0.1 × SSPE (0.15 *M* NaCl, 10 m*M* NaH_2PO_4, 1 m*M* EDTA pH 7.7) at 50 °C and 65 °C, respectively, and then subjected to autoradiography.

DNA sequencing

The PCR-amplified fragments derived from three different PCR reactions were cloned into the vector Bluescript KS$^+$. Each clone was sequenced using the dideoxy method of Sanger et al. (1977).

Results and discussion

The PCR technique was used to amplify a *urf-s*-related sequence in total DNA extracted from leaves of seven fertile Petunia species and lines that are genetically different from each other and which do not contain nuclear fertility restoration alleles (Izhar 1984). The sequences and positions of a pair of 20-nucleotide-long synthetic oligonucleotide primers (a, b) complementary to the 5′ and 3′ ends of the *urf-s* sequence used are presented in Fig. 1. The resultant PCR-amplified fragments were gel fractionated and hybridized with a *urf-s* probe (see Materials and methods). The hybridization results that are presented in Fig. 2A indicate that in six out of the seven fertile lines tested, an amplified fragment the size of *urf-s* hybridized with the *urf-s* probe. It is interesting to note that in different cases either one or two bands are observed in the CMS and fertile Petunia lines tested. It is possible that the lower amplified fragment is missing one (or more) of the quadruple tandem repeats present in *urf-s* (M. R. Hanson, personal communication). This possibility is currently under investigation. In the case of *P. axillaris* (Fig. 2A, lane e), a *urf-s*-related fragment was not detected in this PCR reaction, but was detected later using different DNA preparations. We are currently using Southern blot analysis of these seven fertile lines to determine whether this *urf-s*-related sequence is located in the nucleus, chloroplast or mitochondria.

The PCR-amplified fragments resulting from three different PCR reactions of *P. hybrida* line 3704 were

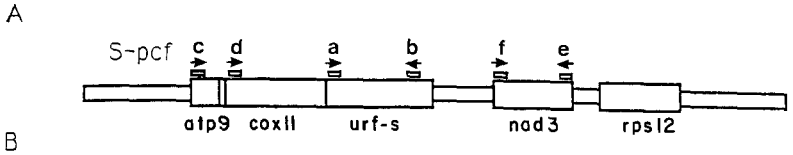

Fig. 1 A, B. The positions and the sequences of the oligonucleotide primers used for PCR amplification of the appropriate *S-Pcf* locus sequences. **A** Positions of the primers; *a* and *b* are the 5′ and 3′ homologous primers of *urf-s*, respectively, *c* is the 5′ primer of *atp9*, *d* is the 5′ primer of *coxII*, *e* is the 3′ primer of *nad3*, *f* is the 5′ primer of *nad3*. **B** Sequences of the oligonucleotide primers (*a*–*f*) mentioned above (5′ to 3′ direction)

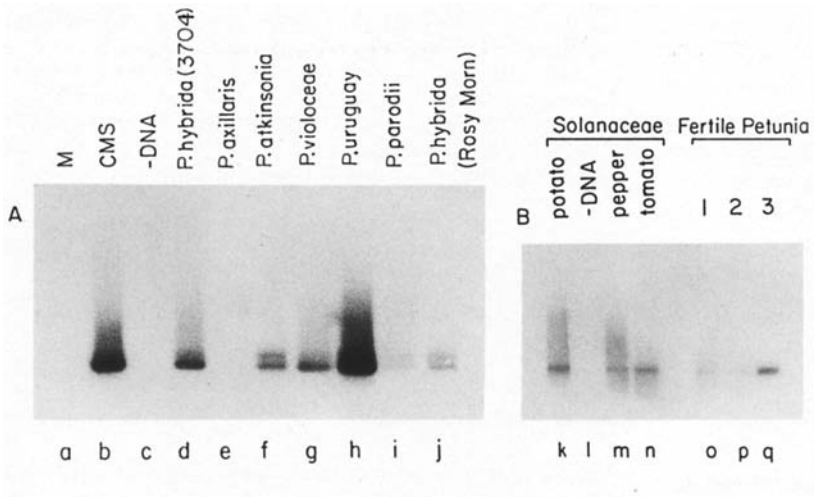

Fig. 2 A, B. PCR amplification of *urf-s* in: **A** seven fertile species and lines (*lanes d–j*) and a CMS line (*lane b*) of Petunia; **B** pepper, tomato and potato (*lanes k, m, n*) and three Petunia plants of *P. hybrida* line 3704 (*lanes o–q*). Amplification was done using the *a* and *b* oligonucleotide primers shown in Fig. 1. The fertile and sterile Petunia lines and species as well as the other *Solanaceae* species used are described in the Materials and methods. The amplified fragments were hybridized with a cloned *urf-s* probe as described in the Materials and methods. *M* Size markers, *–DNA* minus DNA control

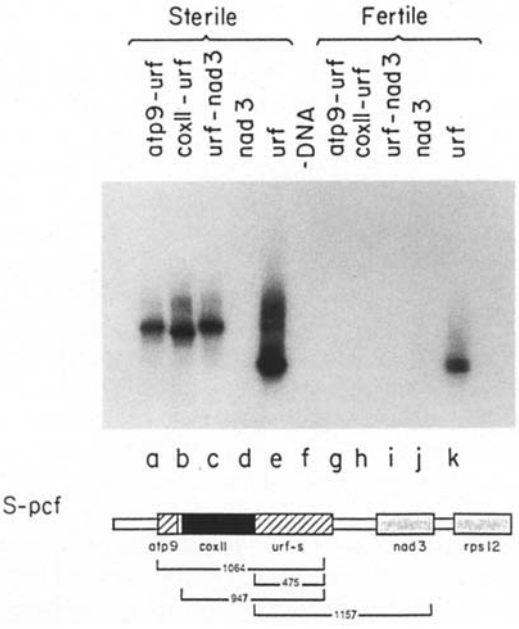

Fig. 3. PCR amplification of *atp9-urf*, *coxII-urf*, *urf-nad3*, *nad3* and *urf* sequences in sterile (*lanes a–e*) and fertile (*lanes g–k*) Petunia lines. *–DNA* minus DNA control. Amplification was done using the CMS *P. parodii* (3688) and the fertile *P. hybrida* (3704) lines and the respective *c-b*, *d-b*, *a-e*, *f-e* and *a-b* oligonucleotide primers shown in Fig. 1. The amplified fragments were hybridized with a cloned *urf-s* probe as described in the Materials and methods

cloned and sequenced. Their sequence (data not shown) was found to be identical to the corresponding part of *urf-s* (Young and Hanson 1987). This *urf-s* related sequence, which was detected by PCR in fertile Petunia lines, is an ORF potentially coding for 152 amino acids and is now designated *orf152*. As in the case of *urf-s*, which is part of the chimeric *Pcf* ORF, the PCR-amplified *orf152* does not begin with a methionine. We are currently working on the characterization of its 5′ and 3′ flanking sequences.

In order to find out whether, as in the case of CMS Petunia, the *orf152* sequence is part of the chimeric *Pcf* ORF (see Fig. 3), we used the PCR technique for the amplification of the appropriate *atp9-urf*, *coxII-urf*, and *urf-nad3* fragments (see Fig. 1). The sequences of the synthetic oligonucleotide primers used are also presented in Fig. 1. Figure 3 shows the hybridization results of the amplified *atp9-urf*, *coxII-urf*, *urf-nad3* and *urf* fragments of a CMS line and a fertile line with a cloned *urf-s* probe. In the CMS *P. parodii* line 3688, the appropriate *atp9-urf*, *coxII-urf*, *urf-nad3* and *urf* fragments were amplified; the sizes are 1,060, 950, 1,157 and 475 bp, respectively, as expected. These amplified fragments hybridized with the *urf-s* probe. However, under the same reaction conditions only an amplified *orf152* sequence was detected in the fertile *P. hybrida* line 3704.

These results indicate that the *atp9* and *coxII* sequences of the oligonucleotide primers shown in Fig. 1 B (c, d) are not present in the 5′ position to *orf152* in fertile Petunia lines. In other words, the genome organization around *orf152* is different from that of the *Pcf* in the *S-Pcf* locus.

Assuming that the *orf152* sequence, which is present in fertile lines, is the origin of *urf-s*, the characterization and mapping of the *orf152* flanking sequences would enable us to understand the recombinational rearrangement events that led to the formation of the chimeric *Pcf* ORF and eventually to the mt *S-Pcf* locus. These studies are being carried out in our laboratory at present.

Our data indicating the presence of *orf152* in additional members of the *Solanaceae* family (Fig. 2 B) together with it being conserved in seven fertile Petunia lines and species raise the possibility that *orf152* is an essential DNA sequence.

As mentioned before (Hanson et al. 1989; Nivison and Hanson 1989) two antibodies produced against syn-

thetic peptides whose sequences are based on the *urf-s* sequence (Young and Hanson 1987) recognize both a 25-kDa and a 20-kDa protein. The 20-kDa protein is expressed in both (S) and normal cytoplasms of Petunia at similar levels. It is thus tempting to suggest that the 20-kDa protein found in normal tissues is a product of the newly described *orf152* sequence. Furthermore, the presence of the *urf-s* related sequence in pepper, tomato and potato, in addition to petunia, suggests that it might be an essential sequence coding for an essential peptide. To clarify this and other possibilities, further studies of this sequence (including its characterization and physical mapping) and its stability in evolution is currently being done in our laboratory.

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