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Nucleotide sequence and transcriptional analysis of a mitochondrial plasmid from a cytoplasmic male-sterile line of sunflower

L. de la Canal [1], D. Crouzillat [1], M.C. Flamand [2], A. Perrault [1], M. Boutry [2] and G. Ledoigt *

[1] Laboratory of Phytomorphogenesis, Blaise Pascal University, F-63038 Clermont Ferrand, France
[2] Physiological Biochemistry Unit, Catholic University of Louvain, Place Croix du Sud 1, B-1348 Louvain-la-Neuve, Belgium

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Summary. A mitochondrial plasmid of 1,939 bp (P2) from a cytoplasmic male-sterile line of sunflower has been cloned and sequenced. It presents 437 bp of near-perfect homology to the 1.4-kb mitochondrial plasmid P1 from sunflower. Sequences homologous to P2 were found in nuclear DNA. P2 was transcribed into a major 980-nucleotide (nt) RNA molecule and two minor transcripts of 570 and 520 nt. They were all transcribed from the same strand and within the region nonhomologous to P1. A single 5′ boundary and three 3′ termini were determined for P2 transcripts. The 5′ end is similar to a consensus sequence for plant mitochondrial genes. No evidence of translation products can be provided.

Key words: Mitochondrial plasmids – Mitochondrial DNA – Sunflower

Introduction

In addition to a large mitochondrial DNA molecule, mitochondria of some plant species contain small plasmid molecules of unknown function and origin (see Lonsdale 1989, for review). The mitochondrial genome of fertile sunflower (*Helianthus annuus*) consists of a 300-kb master circle, subgenomic circles resulting from intramolecular recombination (Siculella and Palmer 1988), and a 1,413-pb supercoiled plasmid (mt-plasmid P1) (Leroy et al. 1985; Perez et al. 1988; Crouzillat et al. 1989). About 20 cytoplasmic male-sterile (cms) lines of sunflower have been examined for the presence of mt-plasmids (Crouzillat et al. 1987, 1989). Thus, two other circular DNAs (P2 and P3) have been detected. mt-plasmid P2, about 1.8 kb in size, is present in *H. petiolaris fallax* cms (PEF 1), but is always accompanied by a small number of P1 molecules (ratio P1/P2: 1/1,000) (Crouzillat et al. 1989). Many sunflower lines studied contain at least one mt-plasmid, but no apparent relationship exists between their presence and cms.

Here we report the nucleotide sequence and the transcriptional analysis of mt-plasmid P2 from sunflower.

Materials and methods

Plant material

Seeds of fertile *Helianthus annuus* and the male-sterile cytoplasm *H. petiolaris fallax* (PEF 1) were kindly provided by F. Vear (INRA, Clermont-Ferrand, France). Both lines have a nuclear background HA89. PEF 1 arose from an interspecific cross between *H. petiolaris fallax* and *H. annuus* (Serieys and Vincourt 1987).

Mitochondrial DNA and RNA isolation

Mitochondria were isolated from sunflower leaves and DNA was extracted as previously described (Crouzillat et al. 1987). Mitochondrial RNA (mtRNA) was prepared from mitochondria isolated from 10-day-old etiolated hypocotyls, as described by Chirgwin et al. (1979), and purified by centrifugation through a CsCl cushion (Glisin et al. 1974).

Nuclear DNA isolation

Nuclear DNA was prepared from leaves following the method of Kiss and Solymosy (1987), including the use of citric acid in the isolation medium and flotation on a Percoll cushion.

Cloning and sequencing of mt-plasmid P2

The circular form of P2 was purified from mitochondria of PEF 1. The plasmid was linearized with EcoRI and cloned into M13 vectors. Full-length clones were subcloned using restriction fragments generated by HindIII and HaeIII. P2 was also cloned on a PstI site and sequenced across the EcoRI site. The nucleotide sequence was obtained for both strands by the dideoxy chain termination method (Sanger et al. 1977).

* To whom offprint request should be addressed

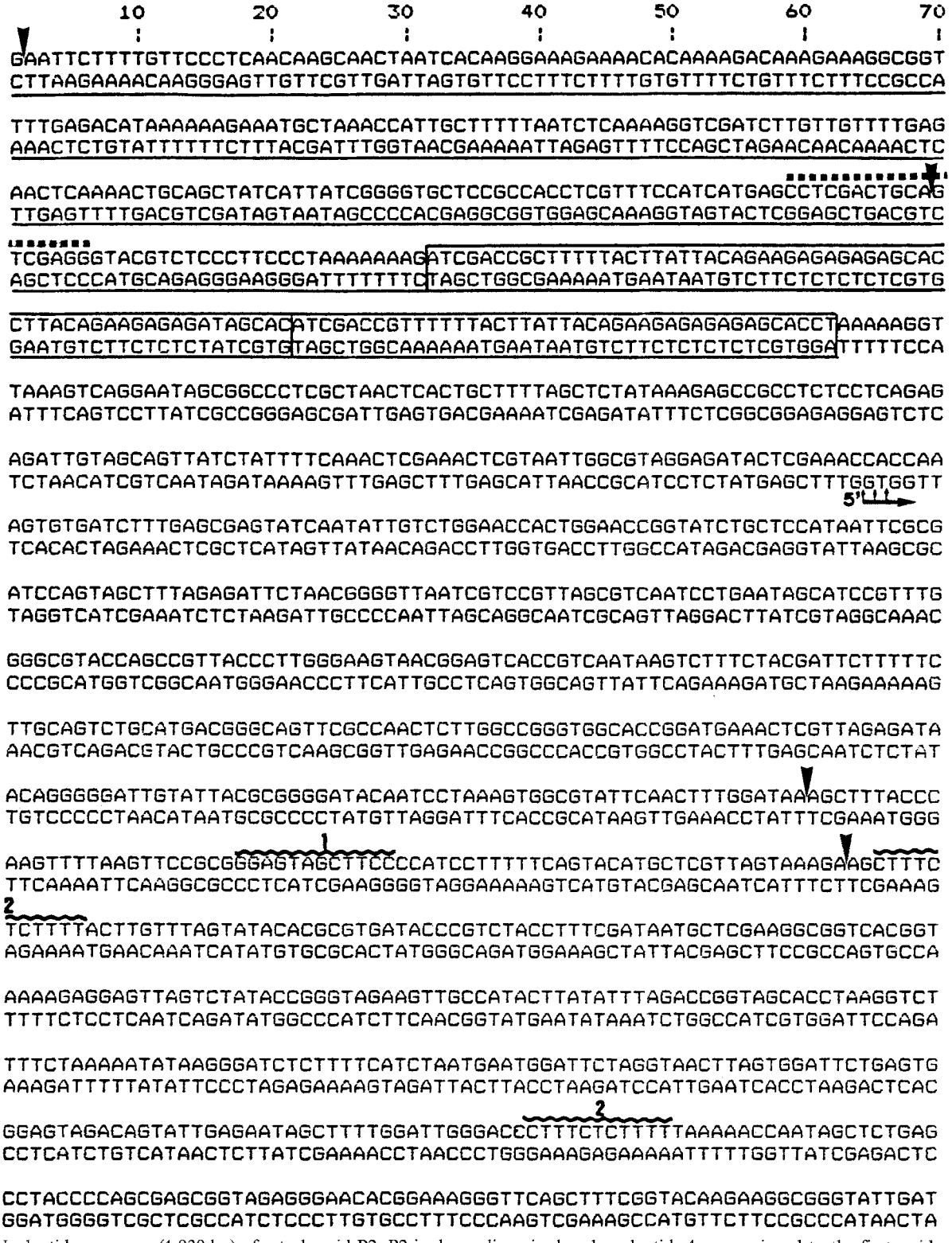
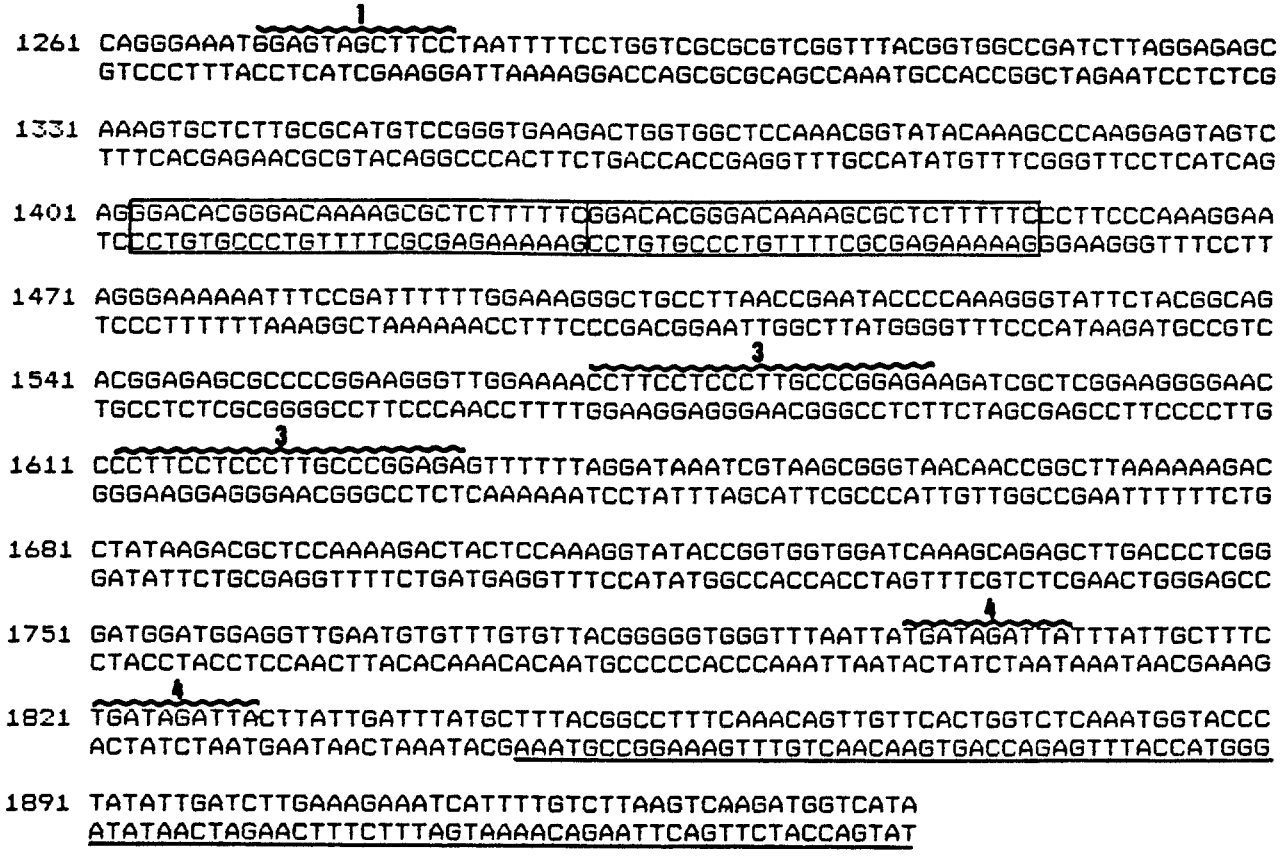


Fig. 1. Nucleotide sequence (1,939 bp) of mt-plasmid P2. P2 is shown linearized and nucleotide 1 was assigned to the first residue of the unique EcoRI site. Restriction sites PstI (nt 209) and HindIII (nt 830 and 903) are marked with ▼. The *horizontal arrow* shows the direction of transcription and *vertical arrows* indicate the position of the 5′ boundary of the transcripts. The tandemly repeated sequences are *boxed* and the region homologous to mt-plasmid P1 from sunflower is *underlined. Wavy overlines* identify direct repeated sequences and *the broken line* shows an inverted repeat. Bottom strand (called E3 in the text) is transcribed



Continuation of Fig. 1 (for legend see page 813)

RNA and DNA blotting

Total mtRNA (5 µg) was electrophoresed on a 2.2% agarose gel containing 2.2 *M* formaldehyde and then transferred to a Hybond N+ membrane (Amersham).

DNA was fractionated on a 0.7% agarose gel and transferred to Hybond N+. Procedures used for blotting were those recommended by the supplier.

Nucleic acid hybridization

DNA probes were radioactively labelled using random primers (Boehringer-Mannheim). Overnight hybridizations were performed at 42 °C in 5 × SSPE (0.9 *M* NaCl, 50 m*M* sodium phosphate, 5 m*M* EDTA), 50% formamide, 5 × Denhardt's solution, 0.5% SDS, and 20 µg/ml denatured herring sperm DNA. After hybridization, filters were washed as recommended by Hybond membranes. A final 0.1 × SSPE-0.1% SDS wash was always done.

S1 nuclease mapping

A synthetic primer was annealed to single-stranded DNA from the chosen clone and labelled with ^{32}P-dCTP using the Klenow fragment of DNA polymerase I. The probe was cut with the appropriate restriction enzyme and separated from the template by electrophoresis on a 4% polyacrylamide-urea gel. After electroelution, the probe was hybridized to 0.5 µg of mtRNA at 42 °C for 16 h in 10 µl of: 80% formamide, 0.4 *M* NaCl, 40 m*M* Pipes (pH 6.4), 1 m*M* EDTA. Different concentrations of formamide (70–85%) and different hybridization temperatures (42–55 °C) were tried without any noticeable effects. S1 nuclease digestion was performed with 75 units of enzyme in 150 µl of buffer [0.25 *M* NaCl, 30 m*M* NaAc, 1 m*M* $ZnSO_4$, 20 µg/ml denatured salmon sperm DNA (pH 4.6)] at 30 °C for 30 min. Protected fragments were precipitated and electrophoresed on a 5% polyacrylamide-7 *M* urea gel.

Results

Nucleotide sequence of mt-plasmid P2

The complete 1,939-bp sequence of P2 is displayed in Fig. 1. The overall G + C content is 44.8%. An 18-nt long palindromic repeat and many direct repeats occur in P2. Two tandemly repeated sequences were also observed.

From hybridization studies, a partial sequence homology between plasmids P1 and P2 from sunflower had already been observed (Crouzillat et al. 1987). The comparison of their nucleotide sequences indicates an extensive homology covering almost one-fourth of P2 length (Fig. 1). The homology of a 437-nt sequence surrounding the EcoRI site goes up to 99.1% (nucleotides 1846 to 342). This homologous region includes part of the tandem arrangement seen on the P1 sequence. In P1, these direct repeats consist of 2 units of 60 bp each, followed by a subunit of 40 bp. mt-plasmid P2 exhibits only 1 unit of 60 bp followed by the 40-bp sequence.

Transcription of P2

mtRNA was isolated from fertile and sterile PEF 1 sunflower and fractionated by electrophoresis in a 2.2%

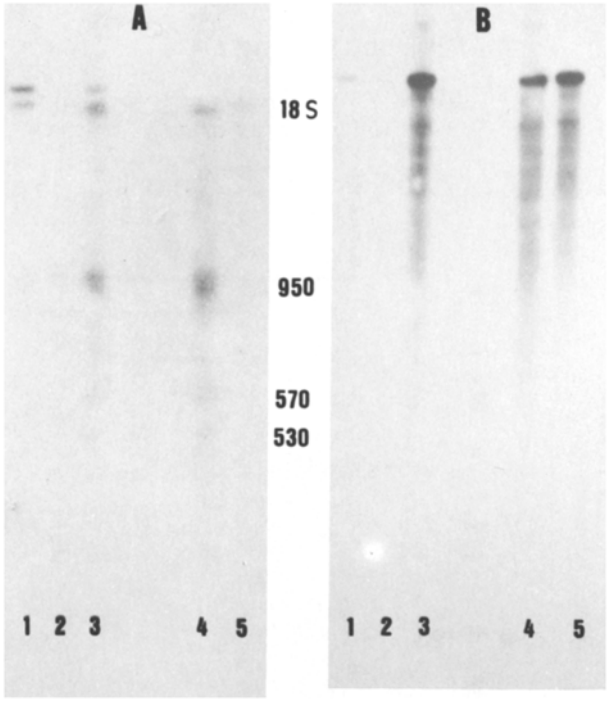


Fig. 2 A and B. Northern blots of mtRNA from fertile and sterile PEF 1 sunflower were hybridized to a P2 probe (**A**) or a maize *cob* probe (**B**). *Lanes 1:* 1 µg of PEF 1 mtDNA; *2:* 1 µg of PEF 1 mtDNA treated with 3 units of DNase I for 10 min at 20 °C; *3:* 5 µg of PEF 1 mtRNA; *4:* 5 µg of PEF 1 mtRNA treated with 3 units of DNase I for 10 min at 20 °C; *5:* 5 µg of fertile HA89 mtRNA. The 18S rRNA and the size of transcripts are indicated on the *right*

agarose gel under denaturing conditions. The 18S and 26S rRNA were visualized by ethidium bromide staining, suggesting a good integrity of mtRNA. Hybridization of Northern blots to a full-length P2 probe allowed detection of three transcripts only found in the sterile line PEF 1 (Fig. 2). The major transcript was approximately 950 nt long. Two other molecules of 570 and 530 nt are slightly seen in Fig. 2, but their presence was confirmed on overexposed films. mtRNA from the fertile line, which does not contain the P2 plasmid, did not show these molecules (lane 5). As a control, a *cob* probe (apocytochrome b) showed the same signals for both male-fertile and male-sterile lines (Fig. 2 B, lanes 3–5).

Treatment of RNA with DNase I (RNase free) did not change the transcript pattern of PEF 1; nonspecific hybridization to 18S rRNA was still observed after the treatment (Fig. 2 A, lane 4). On the other hand, when RNA samples were digested with RNase A prior to electrophoresis none, of the transcripts was detected (not shown).

Additional signals seen on Fig. 2 were due to a contamination of RNA with minicircular DNA, since signals of the same size (1950 and 1800 nt) were also seen with a mtDNA control (Fig. 2 A, lane 1). These signals, which disappeared by DNase I treatment, correspond to the L and OC form of P2.

Hybridization of Northern blots to strand-specific, full-length clones of P2 showed that the three transcripts were only detected with the E3 probe (from Fig. 1), giving the same pattern shown in Fig. 2 (data not shown).

Determination of 5′ termini of the transcripts

Several M13 clones that together represented the entire length of P2 template strand were used in separate S1 nuclease protection experiments. Uniformly labelled probes were generated by second-strand synthesis, followed by digestion with the appropriate restriction enzyme. When an EcoRI-HindIII clone (EH3 on Fig. 3 A) was used as a probe for hybridization with PEF 1 mtRNA, followed by S1 nuclease treatment, the predominant protected fragment was 350 nt long (Fig. 3 A). When this probe was digested with PstI instead of EcoRI (reducing the probe length from 847 to 638 nt), a protected fragment of the same size was obtained (data not shown). This result indicates that the RNA-DNA hybrid detected is covering the 5′ end of the transcript.

To locate more precisely the 5′ end point, a smaller EH3 probe was used. It was generated by Klenow extension of a synthetic oligonucleotide corresponding to nucleotides 559 to 572. mtRNAs were annealed to this probe and the hybrids were digested with S1 nuclease. The protected fragments were 86–88 nt long (Fig. 3 B). As expected, no protected fragment was observed with the mtRNA from the fertile line. Comparison with a sequencing ladder indicated that the 5′ boundary of P2 transcripts corresponds to the GGT residues at position 484–486 in Fig. 1.

Determination of 3′ ends

S1 mapping experiments were performed using a probe extending from position 1749 to 903, produced by second-strand synthesis from a 14-nt primer. Three protected fragments were obtained (Fig. 4). Their approximate sizes were found to be 560, 155, and 103 nt, suggesting three 3′ ends of P2 transcripts at positions 1470, 1060, and 1005, respectively. Since only one 5′ end was detected, at position 485, estimated sizes of the transcripts are 980, 570, and 520 nt. These values are in agreement with the transcript sizes detected on Northern blots (Fig. 2).

Nuclear homology to P2

No sequence homology was found between P2 and the main mtDNA from PEF 1 (Fig. 5 A). On the contrary, hybridization of a P2 probe to nuclear DNA from fertile of PEF1 sunflower (nuclear background HA89) showed a high-molecular-weight signal (Fig. 5 B). When samples

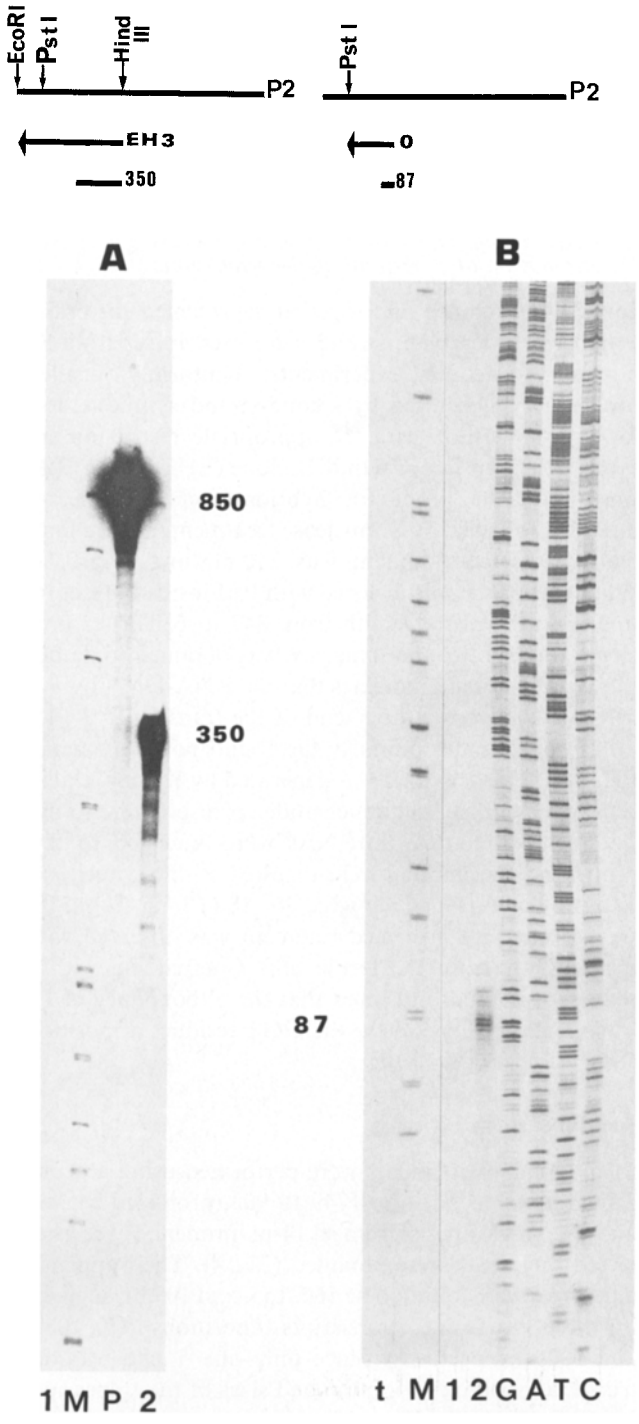


Fig. 3 A and B. S1 protection analysis to determine the 5′ end of P2 transcripts. The DNA probes used to map the transcripts and the protected fragments obtained are diagrammed at the top (EcoRI, PstI, and HindIII sites are localized at positions 1, 209, and 830 respectively). **A** Probe EH3 of 847 nt; **B** probe O of 363 nt. Probes were hybridized to *1:* mtRNA from fertile sunflower; *2:* mtDNA from sterile PEF 1; *t:* tRNA as a control. *P* indicates migration of the probe and *M* is a MW marker. *G, A, T, C:* sequencing reactions of probe O. S1 nuclease-resistant hybrids were electrophoresed in 5% sequencing gels and autoradiographed. The apparent size of the protected fragments and probes is marked

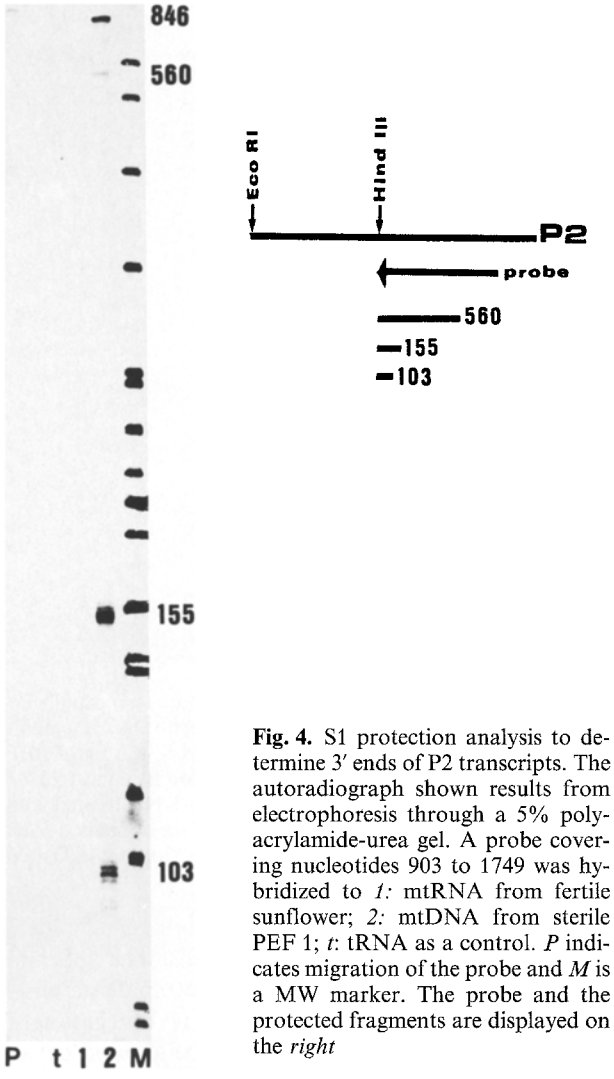


Fig. 4. S1 protection analysis to determine 3′ ends of P2 transcripts. The autoradiograph shown results from electrophoresis through a 5% polyacrylamide-urea gel. A probe covering nucleotides 903 to 1749 was hybridized to *1:* mtRNA from fertile sunflower; *2:* mtDNA from sterile PEF 1; *t:* tRNA as a control. *P* indicates migration of the probe and *M* is a MW marker. The probe and the protected fragments are displayed on the *right*

were digested with EcoRI, both lines displayed the same radioactive bands, in accordance with their isogenicity.

Sequences homologous to mt-plasmid P1 were also found in HA89 nuclei (Crouzillat et al. 1989). To avoid a possible cross-hybridization with P1 sequences, a P2 probe extending from residue 209 to 990 was used in other experiments. When this probe was hybridized to Southern blots, the signals obtained on nuclear DNA were the same as in Fig. 5 (results not shown). Hence, the nuclear genome HA89 contains sequences homologous to mt-plasmid P2, in addition to other sequences homologous to P1.

Discussion

The nucleotide sequence of mt-plasmid P2 described here enables comparison with another mt-plasmid from sun-

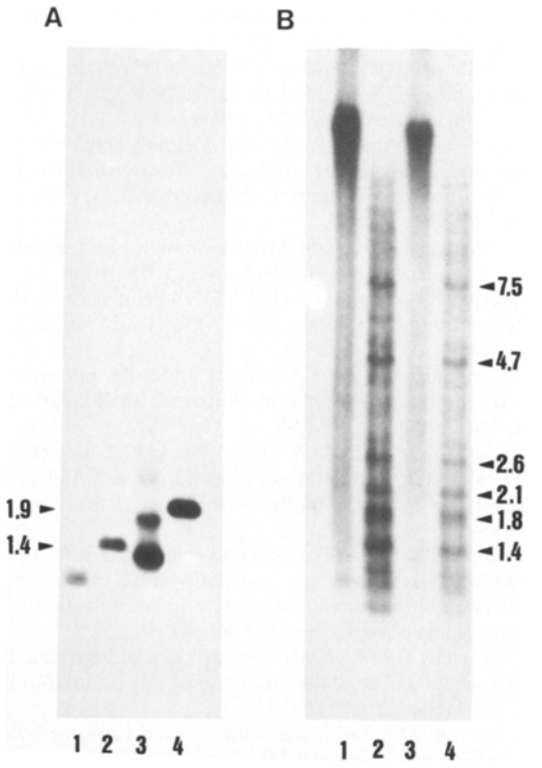


Fig. 5 A and B. Hybridization of mt-plasmid P2 to Southern blots containing 0.5 μg of mtDNA (**A**) or 10 μg of nuclear DNA (**B**). *Lanes 1:* native male-fertile DNA; *2:* male-fertile DNA digested with EcoRI; *3:* native PEF 1 DNA; *4:* PEF 1 DNA digested with EcoRI. Sizes (kb) of the fragments are indicated

flower (P1). These minicircles have a homologous region of 437 nt, suggesting that they are derived either from a common ancestor or from intramolecular recombination mediated by a direct repeat, as was reported for sugar beet (Thomas 1986).

Sequence analysis of mt-plasmids from broad bean (Wahleithner and Wolstenholme 1987), maize (Smith and Pring 1987; Ludwig et al. 1985), and sugar beet (Hansen and Marcker 1984) revealed a large direct repeat for each of them. A similar feature was found in mt-plasmids from sunflower. Furthermore, the same tandem repeat was observed in P1 and P2, within their homologous region.

No sequence homology was observed between P2 and the main mtDNA, but a P2 probe was shown to hybridize to a nuclear DNA. The same features have been reported for mt-plasmids from maize (Abbot et al. 1985; Kemble et al. 1983; Smith and Pring 1987), rice (Shikanai et al. 1989), and sunflower (Crouzillat et al. 1989). It has been suggested that mt-plasmids were originally sequestered from the nucleus (Pring and Lonsdale 1985), but transfer from mitochondria to the nuclear genome cannot be excluded.

Data resulting from Northern blots and S1 mapping indicate the presence of three transcripts for mt-plasmid P2. Only one 5′ boundary and three 3′ termini were detected. In a recent report (Maloney et al. 1989, it was proposed that the nucleotide sequence AAAC (or AAUC), found at the 5′ end of several plant mt-rRNA and mt-mRNA, may be a transcription or processing signal. This sequence is also present on the 5′ boundary of the major transcript from mt-plasmid S2 of maize. Immediatly upstream of the 5′ end determined for P2 transcripts, an AAAC sequence was observed. In addition, the sequence surrounding the 5′ terminus of P2 transcripts is similar to a consensus sequence for plant mitochondrial promoters (Young et al. 1986) and complementary to a 9-nt sequence at the 5′ end of a mt-plasmid transcript from broad bean (Wahleithner and Wolstenholme 1987).

1.7-kb broad bean	3′	TTTGGTGGA	5′
1.9-kb sunflower (P2)	5′	AAACCACCAAAGTGTG	3′
consensus	5′	AAATYTCNTAAGTGAA	3′

Concerning the 3′ termini of the transcripts, no special feature was observed on the template strand, except for a putative hairpin loop begining at residue 1475. The 3′ end of the major P2 transcript was determined at this position.

It is interesting to note that the transcribed region of P2 is specific to this plasmid, as no sequence homology with mt-plasmid P1 from sunflower was found between residues 485 and 1480. The largest open reading frame (ORF) present in P2 transcripts could encode a polypeptide of only 76 amino acids (228 bp, from residue 752 to 979). No striking homology to mitochondrial or eukaryotic RBS was found upstream of the AUG codon, but two interesting features were seen in the ORF. First, AUG codon is followed by an A residue, as in most higher plant mitochondrial genes. Second, codon usage shows a 35.5% of T in the third position (21% A) as observed in mt-genes (Dawson et al. 1984). Long ORFs were never found in mt-plasmids, except in the case of linear DNAs from maize (Levings and Sederoff 1983; Paillard et al. 1985). No evidence has been provided showing that the products of small ORFs exist. Even if no peptide product is synthesized, the transcripts themselves could play a role in the autonomy of mt-plasmids.

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