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ORIGINAL PAPER

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Structural features and expression analysis of a linear mitochondrial plasmid in rapeseed (*Brassica napus* L.)

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Abstract A linear plasmid molecule about 11 kb in length is present in the mitochondria of some varieties of rapeseed (*Brassica napus* L.). This plasmid can be inherited from the male parent, through the pollen, as well as by the usual maternal route, although the main mitochondrial genome is maternally inherited in rapeseed. We determined the complete nucleotide sequence of this plasmid DNA and clarified its genetic organization. The length of the linear plasmid is 11,640 bp. At the termini of the plasmid molecule are inverted repeats of 327 bp. The GC content of the plasmid DNA is 30.9%; thus, the plasmid is quite AT-rich compared to the main mitochondrial genome in higher plants. The plasmid has six ORFs, two of which encode a phage-type DNA polymerase and a phage-type RNA polymerase, respectively. RT-PCR analyses revealed that all six ORFs are transcribed, and all four ORFs on the minus strand are probably cotranscribed from a single promoter located in the terminal inverted repeat. We also show here that at least three of the six ORFs are translated into proteins in rapeseed mitochondria, and expressed at relatively high levels in flowers, as shown by Western analysis. These results suggest that this linear DNA molecule is able to replicate as an autonomous replicon and to express the genes it carries in rapeseed mitochondria.

Keywords Linear plasmid · Mitochondria · *Brassica napus* L. · Paternal transmission · Gene expression

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Introduction

In addition to a large and complex main mitochondrial genome, the mitochondria of many species of higher plants contain a variety of smaller DNA molecules (reviewed in Brown and Zhang 1995). These molecules can be regarded as extrachromosomal replicons or plasmids which can replicate autonomously in mitochondria, because they are present in high copy number relative to the main genome. These smaller DNA molecules can be either circular or linear in form.

Linear mitochondrial plasmids are quite frequently found in fungi and less often in higher plants, but they seem to be absent from most animal cells (for a review, see Meinhardt et al. 1990). They usually contain terminal inverted repeats and have proteins covalently attached to their 5′ termini. They share this DNA structure with some DNA viruses, e.g. adenovirus or *Bacillus subtilis* bacteriophage Φ29, and various transposable elements such as *Ac* and *Ds* of maize and *Tam* from *Antirrhinum majus* (reviewed in Sakaguchi 1990).

It has been reported that the mitochondria of some *Brassica* species contain a linear DNA molecule of approximately 11.3 kb in length, and that the presence of this molecule is associated with cytoplasmic male sterility (cms; Palmer et al. 1983). However, an extensive analysis using more than 100 different *Brassica* nucleocytoplasmic combinations later revealed that there was no close association between the presence of this plasmid DNA and the cms trait (Kemble et al. 1986). There have been no further reports regarding the biological role of this linear molecule in rapeseed mitochondria to date. Therefore, it is still not known what the plasmid molecule actually does in mitochondria. Although our knowledge of this linear DNA is very limited, its most striking known feature is that it can be transmitted to progeny plants through the pollen (Erickson et al. 1989). In many higher plants, mitochondria are transmitted uniparentally from the maternal plant to the progeny, and thus the main genomic DNAs and smaller DNAs in

mitochondria are also transmitted maternally. However, in crosses of plasmid-less females and plasmid-bearing males, the *Brassica* mitochondrial plasmid was found at very high rates in F_1 progeny, regardless of the cytoplasmic and nuclear genotypes of the male or female plants involved.

To date we know nothing about the molecular structure of the linear plasmid DNA molecule or the biochemical mechanism of pollen transmission, with the exception of one report that the plasmid has terminal inverted repeats of 325 bp at both ends (Turpen et al. 1987). In order to elucidate the structure of this linear plasmid and obtain clues to its biological function, we determined the complete nucleotide sequence and genetic organization of this DNA molecule, and analyzed the patterns of transcription and translation of the ORFs encoded in the plasmid DNA.

Materials and methods

Analysis of mitochondrial DNA and RNAs

Mitochondria were isolated from green leaves of 8-week-old rapeseed plants (cv. Isuzu-natane, which has the plasmid in its mitochondria, and cv. Murasaki-natane, which is a plasmid-less cultivar). Mitochondrial DNA (mtDNA) and mitochondrial RNA (mtRNA) were prepared using procedures described previously (Itani and Handa 1998). Southern and Northern hybridization analyses were carried out under standard conditions (Sambrook et al. 1989). Nucleotide sequences were determined using a sequencing kit (Thermo-sequenase fluorescent labeled primer cycle sequencing kit; Amersham) using a LI-COR 4200 autosequencer. Nucleotide and amino acid sequence analyses were performed with GENETYX-MAC software version 10.1 (Software Development). The computer program package Clustal W (Thompson et al. 1994) was used for the alignment of protein sequences and the construction of phylogenetic trees.

PCR amplification and cloning of plasmid DNA

To amplify most of the plasmid DNA sequence, long-range PCR was carried out using a single primer (R1, see Table 1), which anneals to the terminal inverted repeats (Turpen et al. 1994). The reaction was performed according to the following protocol: denaturation for 80 s at 98°C, followed by 30 cycles of incubation for 20 s at 98°C and 10 min at 68°C using ExTaq DNA polymerase (TaKaRa).

We employed the 5′RACE PCR technique to obtain both ends of the plasmid DNA. Homopolymeric dT tailing of mtDNA was carried out with terminal deoxynucleotidyl transferase (Toyobo). PCR was conducted with the primer pair T1 and T3 for the left end, and T2 and T3 for the right end (Table 1). PCRs designed to amplify the regions including each end were performed according to the following protocol: denaturation for 5 min at 94°C; 35 cycles of 1 min at 94°C, 2 min at 50°C, and 3 min at 72°C; followed by extension for 5 min at 72°C. In all PCR experiments, a Gene Amp PCR System 2400 thermal cycler (Perkin-Elmer) was used.

Amplified PCR fragments were cloned into the pCR2.1 vector using a TOPO TA cloning kit (Invitrogen).

RT-PCR amplification

DNase I-treated mtRNA (5 μg) was mixed with 2 pmol of oligonucleotide primer in a solution containing 1× reverse transcriptase buffer (supplied by the enzyme manufacturer), each dNTP at 0.5 mM, 10 mM DTT, 50 U of SuperscriptII reverse transcriptase (GIBCO-BRL) and 36 U of RNAguard (Pharmacia). The reaction mixture was incubated for 50 min at 42°C, and the reaction was stopped by heating at 70°C for 15 min. The resulting cDNAs were then used for PCR. For PCR products up to 3 kb long, we used each dNTP at 0.2 mM, oligonucleotide primers at 1 μM, 2.5 U of Taq DNA polymerase (Promega) and the buffer recommended by the enzyme manufacturer, and carried out amplification for 25 cycles of denaturation, annealing and polymerization (94°C for 1 min, 50°C for 2 min, and 72°C for 3 min), followed by a final incubation at 72°C for 7 min. For products longer than 3 kb, PCR was performed according to the following protocol: initial denaturation for 80 s at 98°C, and 30 cycles of incubation for 20 s at 98°C and 10 min at 68°C using ExTaq DNA polymerase (TaKaRa).

Table 1. Primer sequences used in this study

Primer	Sequence 5′→3′ (position)
R1	CGGCCTGTGTCCTTCTACAGTATTG[a]
T1	ATCAGTCCATCATATGGGTTCAC (558–536)
T2	CCTTTTTGTCTTCGACCAGATCA (10666–10688)
T3	GCGAGCTCAAGCAAAAAAAAAAAAAAAAAA
P1F	CTACATCAGAGAGAATCTGGGGAAC (6829–6853)
P1R	GTATGGATCTGTAGCTGACGCAACT (7133–7109)
P2F	GCCTGTTCACACATATGTTGTGTGG (4695–4671)
P2R	GATCTGAGTTCACAAGAGGTGCTAC (3891–3915)
P3F	GGAGATCTCTCCTGAAGCTCTTTTG (5442–5418)
P3R	TTTTCCTCCTAACCATACCGGGTGT (5147–5171)
P4F	GTTCCCCAGATTCTCTCTGATGTAG (6853–6829)
P4R	AATTTCAGTGCCTCCAGTCTTCTCC (6449–6473)
P5F	GAGTTCTCCCCCGATAGTTCAAAGT (991–1015)
P5R	CGTCGATGTAATCTTTACCAACGCC (2780–2756)
P6F	CTATGATCAGCCCACCTGTTGATTG (10459–10435)
P6R	CACGCTCGTGAAAATGTAACACACC (9887–9911
PE-L	CTCAGCAAATATTTCAGAATAACTC (445–421)
PE-R	CACAATCCTTCCAGAATTTCTTC (11204–11226)

[a]Primer R1 anneals to the terminal repeats at positions 103–127 and 11538–11514

Primer extension

The primers were labeled at their 5′ ends with the fluorescent dye IRD800 (Aloka). Each primer was annealed with mtRNA and then extended with AMV reverse transcriptase (TaKaRa) for 90 min. Template RNA was digested with RNase A and the sample was analyzed under standard electrophoresis conditions in a LI-COR 4200 autosequencer (Li-Cor).

Expression of plasmid-encoded proteins in *E. coli* and preparation of antisera

DNA fragments containing parts of *orf2* and *orf5* were amplified by PCR using appropriate primer pairs, cloned into the pCRT7/CT-TOPO vector (Invitrogen), and expressed in *Escherichia coli* strain BL21(D3)pLysS. *E. coli* cultures were grown to an OD_{600} value of 0.7 and protein expression was induced by adding 1 mM isopropyl thio-β-D-galactoside (IPTG). After cells had been cultured at 37°C for an additional two hours, they were harvested by centrifugation, resuspended in 20 mM sodium phosphate (pH 7.8), 0.5 M NaCl, 6 M guanidine hydrochloride, and lysed by several cycles of sonication. Fusion proteins bearing a 6×His tag were purified by metal chelate affinity chromatography on ProBond resin (Invitrogen). After adsorption of the overexpressed protein, the resin was washed repeatedly, and bound protein was eluted in a buffer containing 20 mM sodium phosphate (pH 4.0), 0.5 M NaCl, 8 M urea. The proteins expressed in *E. coli* were injected into mice to raise specific antisera. An oligopeptide corresponding to a C-terminal

sequence (NH_2-KHQIESYNPNYSLHY-COOH) of ORF6 was also synthesized and injected into rabbits to raise a specific antiserum.

Preparation and immunodetection of mitochondrial proteins

Mitochondria were isolated from flower buds, stems, and roots of mature rapeseed plants by the method described previously (Itani and Handa 1998). Mitochondria were lysed and then fractionated by SDS-PAGE. The amount of protein loaded in each lane was normalized by probing with an antibody raised against wheat NAD9 (Lamattina et al. 1993). After electrophoresis, proteins were electroblotted onto PVDF membranes (Immobilon-P; Millipore). Anti-ORF2 (1/5000 dilution), anti-ORF5 (1/5000 dilution), or anti-ORF6 (1/1000 dilution) antibodies described above were used as primary antibodies. The signals were developed using anti-mouse or anti-rabbit IgG conjugated to horseradish peroxidase and an ECL Western detection kit (Amersham) according to manufacturer's instructions.

Oligonucleotide primers

Primer sequences and nucleotide positions used this study are listed in Table 1. Primer locations are shown in Fig. 1B.

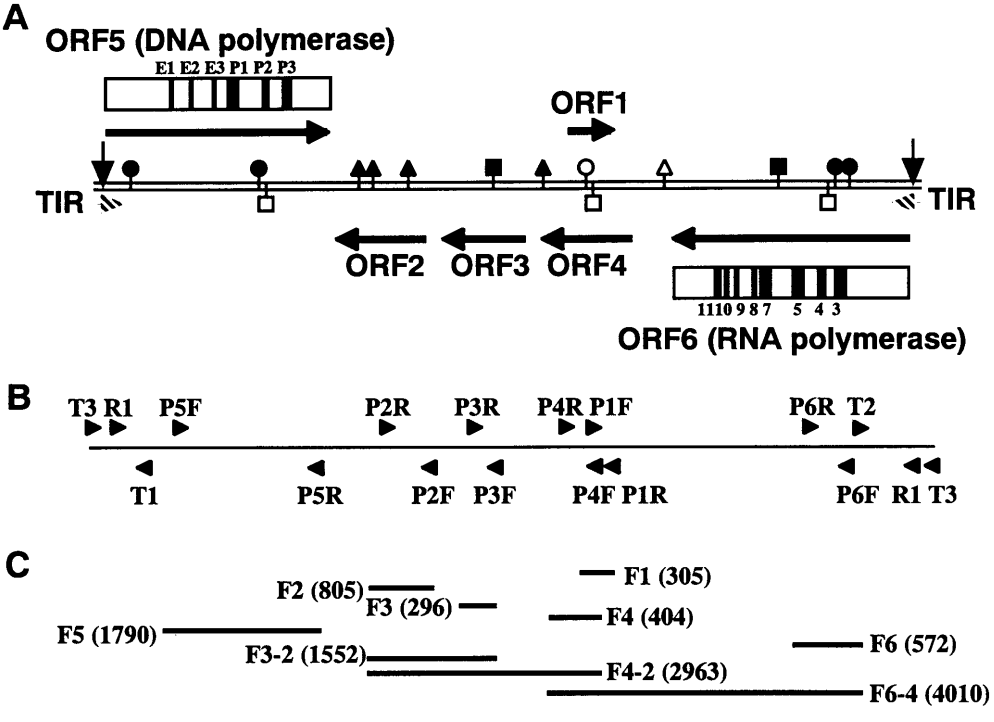
Fig. 1A–C. Overall structure of the linear plasmid in rapeseed mitochondria. The entire nucleotide sequence has been deposited in the databases under the Accession No. AB073400. **A** Restriction sites and terminal inverted repeats (TIRs) are shown: *open circles*, *Bam*HI; *open squares*, *Cla*I; *filled circles*, *Eco*RI; *filled squares*, *Spe*I; *filled triangles*, *Xba*I; *open triangles*, *Xho*I. Predicted ORFs are indicated by *horizontal arrows*. The predicted gene products ORF5 and ORF6 are indicated schematically, with conserved motifs (*filled boxes*) characteristic of DNA and RNA polymerases, respectively. The 5′-ends of RNAs were localized by primer extension analyses, and are indicated by *vertical arrows*. **B** Positions of oligonucleotides used for sequencing and PCR amplifications are indicated by the *arrowheads*. **C** Positions of DNA segments used for RT-PCR analyses. Calculated sizes (in bp) are shown in *parentheses*

Results

Nucleotide sequence and coding capacity of the mitochondrial plasmid

To clone the mitochondrial plasmid DNA, we carried out a long-range PCR based on the sequence information available for the inverted terminal repeats of this molecule (Turpen et al. 1987), and succeeded in amplifying almost the entire molecule (data not shown). To clone the ends, we added poly-dT to the ends of the plasmid molecule using terminal deoxyribonucleotide transferase, and then amplified each end separately by PCR (data not shown). To determine the entire sequence of the rapeseed mitochondrial plasmid DNA, we sequenced these three amplified products.

The length of the mitochondrial plasmid DNA from rapeseed is 11,640 bp (the nucleotide sequence data reported in this paper have been deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases under the Accession No. AB073400). As reported earlier, the plasmid contains perfect terminal inverted repeats (TIRs) (Turpen et al. 1987), the length of which was determined to be 327 bp, 2 bp longer than previously reported. However, at present we cannot exclude the possibility that some terminal nucleotides may have been lost during the cloning procedures due to the nature of the termini of the plasmid molecule which have a protein attached to their 5′ ends (Turpen et al. 1987). Overall, the plasmid has a GC content of 30.9%, which is quite AT-rich compared with the main mitochondrial genomes that have been completely sequenced so far (44.8% and 43.9% for *Arabidopsis* and sugar beet, respectively; Unseld et al. 1997; Kubo et al. 2000).



To identify potential protein-coding regions, the nucleotide sequence of the plasmid DNA was analyzed using the universal genetic code in order to determine the positions of stop codons in all possible reading frames. As shown in Fig. 1A, the plasmid DNA contains six ORFs that have potential methionine initiation codons and encode more than 100 amino acids. They include the 2970-bp *orf5* (990 amino acids) starting at nt 230 and terminating at nt 3199, and the 408-bp *orf1* (136 amino acids) starting at nt 6765 and terminating at nt 7172, on the plus strand (Fig. 1A). Four ORFs are encoded on the opposite (minus) strand. The order of the ORFs on the minus strand is *orf6* (nt 11411–8178, 1078 amino acids), *orf4* (nt 7462–6377, 362 amino acids), *orf3* (nt 5993–5043, 317 amino acids), and *orf2* (nt 4611–3538, 358 amino acids) (Fig. 1A).

orf5 and *orf6* encode a DNA polymerase and an RNA polymerase, respectively

Using protein databases, the amino acid sequences deduced from the potential coding regions of the plasmid DNA were compared with those of known proteins.

The deduced amino acid sequence of ORF5 shows significant similarity to several group B DNA polymerases, a class of enzymes which are characteristic of bacteriophages, viruses and plasmids (Jung et al. 1987). The deduced ORF5 protein sequence also showed similarity to two putative DNA polymerases encoded by the linear mitochondrial plasmids of other higher plants (41% identity to ORF1 of the sugar beet 10.4-kb plasmid; Accession No. Y10854; 36% identity to ORF3 of the maize S1 plasmid; Paillard et al. 1985). In addition to its overall similarity to known DNA polymerases, ORF5 contains highly conserved motifs which are characteristic of two catalytic domains (Fig. 2). One is the proofreading domain with 3′→5′ exonuclease activity, characterized by three N-terminally located motifs, Exo I, Exo II and Exo III (Bernad et al. 1989). The other one is the C-terminal polymerization domain involved in the binding of deoxynucleotide triphosphates and pyrophosphates via three conserved motifs (Pol I, Pol II and Pol III) (Bernad et al. 1987; Jung et al. 1987). Thus, it is likely that *orf5* encodes a family B-like DNA polymerase.

The comparison of the *orf6*-derived amino acid sequence with the sequences in the databases revealed that the deduced ORF6 protein shows similarity to single-subunit bacteriophage-type RNA polymerases from various organisms, especially from mitochondrial plasmids of fungi and higher plants. The overall sequence of ORF6 revealed 32% and 28% identity, and 49% and 46% similarity, to putative RNA polymerases encoded by two mitochondrial plasmids from higher plants, the 10.4-kb plasmid from sugar beet and the maize S2 plasmid (Levings and Sederoff 1983), respectively. Within this group of RNA polymerases, a number of conserved sequence motifs have been identified (Oeser and Tudzynski 1989; Chan et al. 1991). Eight such conserved motifs are located in the correct spatial arrangement in

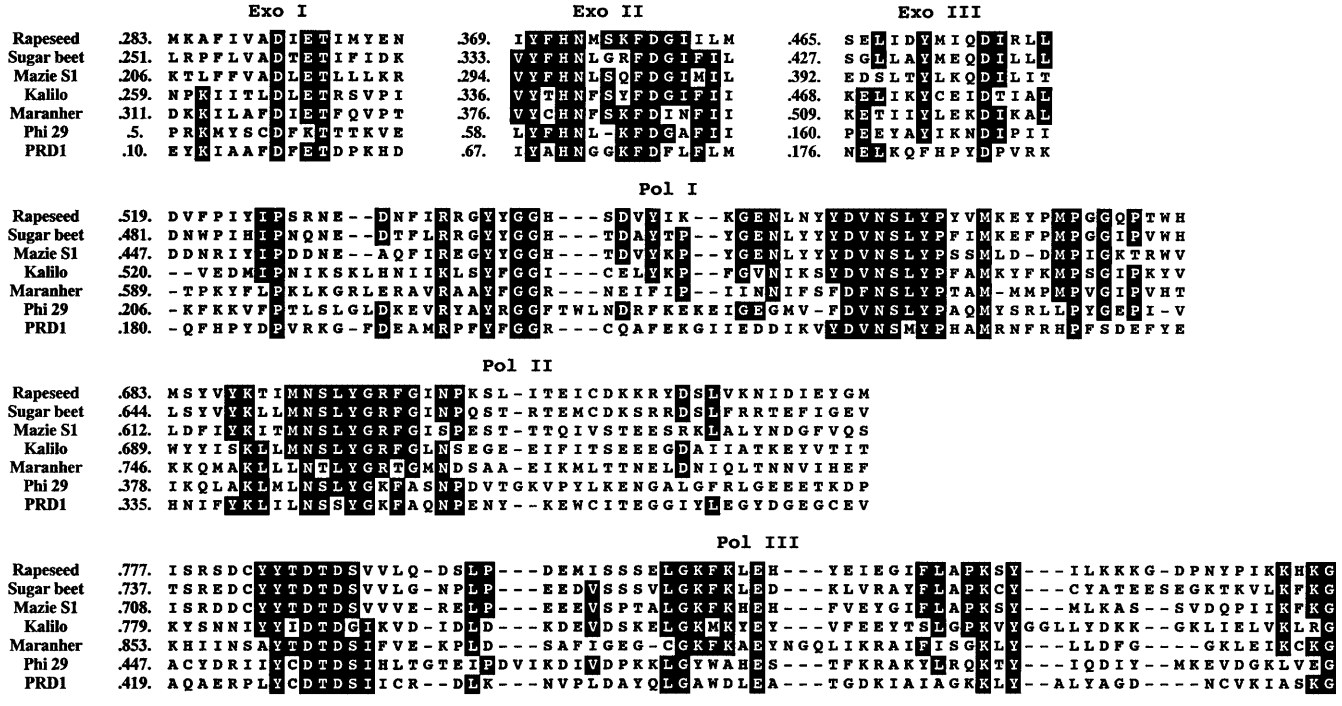


Fig. 2. Alignment of protein sequences deduced from *orf5* of the rapeseed linear plasmid with conserved motifs of group B DNA polymerases from several mitochondrial plasmids and viruses. Rapeseed, the linear plasmid investigated in this study; Sugar beet, the 10.4-kb plasmid (Accession No. Y10854); Maize S1, the S1 plasmid (Accession No. X02451); Kalilo, the *kalilo* plasmid from *Neurospora crassa* (Accession No. X52106); Maranhar, the *maranhar* plasmid from *N. crassa* (Accession No. X55361); Phi 29, *Bacillus subtilis* bacteriophage Φ29 (Accession No. P03680); PRD1, bacteriophage PRD1 (Accession No. M69077). Amino acids that are conserved in at least four sequences are *highlighted*

the rapeseed ORF6 protein (Fig. 3). These data indicate that ORF6 may encode a single-subunit phage-type RNA polymerase. Phylogenetic analysis of single-subunit RNA polymerases showed that the polymerases encoded by plant linear plasmids, including that from rapeseed, were clearly separated from nucleus-encoded polymerases used for the transcription of mitochondrially encoded genes in higher plants such as *Arabidopsis* (Hedtke et al. 1997, 2000) and wheat (Ikeda and Gray 1999) (data not shown).

The amino acid sequences deduced from the other four ORFs (*orf1*, *orf2*, *orf3* and *orf4*) show no significant homology to sequences in the protein databases.

All ORFs are transcribed

When Northern blots of mitochondrial RNA from rapeseed leaves were probed with PCR fragments corresponding to all six ORFs, no specific transcripts were detectable (data not shown).

To check for the presence of plasmid transcripts in mitochondria, we therefore performed RT-PCR using the appropriate primer combinations for each ORF (primers and expected amplified fragments are shown in Fig. 1B and C; for primer sequences, see Table 1). As shown in Fig. 4, specific amplified products with the expected sizes were obtained by RT-PCR from all of six ORFs only when mtRNA from the plasmid-bearing variety (cv. Isuzu-natane) was used as the template. Moreover, no amplified products were generated from control samples incubated without reverse transcriptase. These results clearly demonstrate that all six ORFs encoded by the plasmid are transcriptionally active in rapeseed mitochondria.

We also carried out other RT-PCR experiments to explore the mode of transcription of the ORFs of the plasmid molecule. The ORFs in the linear mitochondrial plasmid mF of the true slime mold *Physarum polycephalum* are cotranscribed from a single promoter region (Takano et al. 1994). To test for a similar mode of transcription in the rapeseed plasmid, several primer combinations were used to detect possible cotranscripts. The results of this experiment are shown in Fig. 5. The primer pair P6F + P4R was able to amplify a 4.0-kb fragment (F6–4 in Fig. 5), which is in good agreement with the estimated size of a cotranscript extending from *orf6* to *orf4*. On the other hand, 3.0-kb and 1.5-kb fragments (F4–2 and F3–2 of Fig. 5) were amplified using primer pairs P2R + P4F, and P2R + P3F, which correspond to the possible cotranscripts from *orf4* to *orf2* and from *orf3* to *orf2*, respectively. These RT-PCR results suggest that the four ORFs on the minus strand, from *orf6* to *orf2*, are cotranscribed into a single RNA

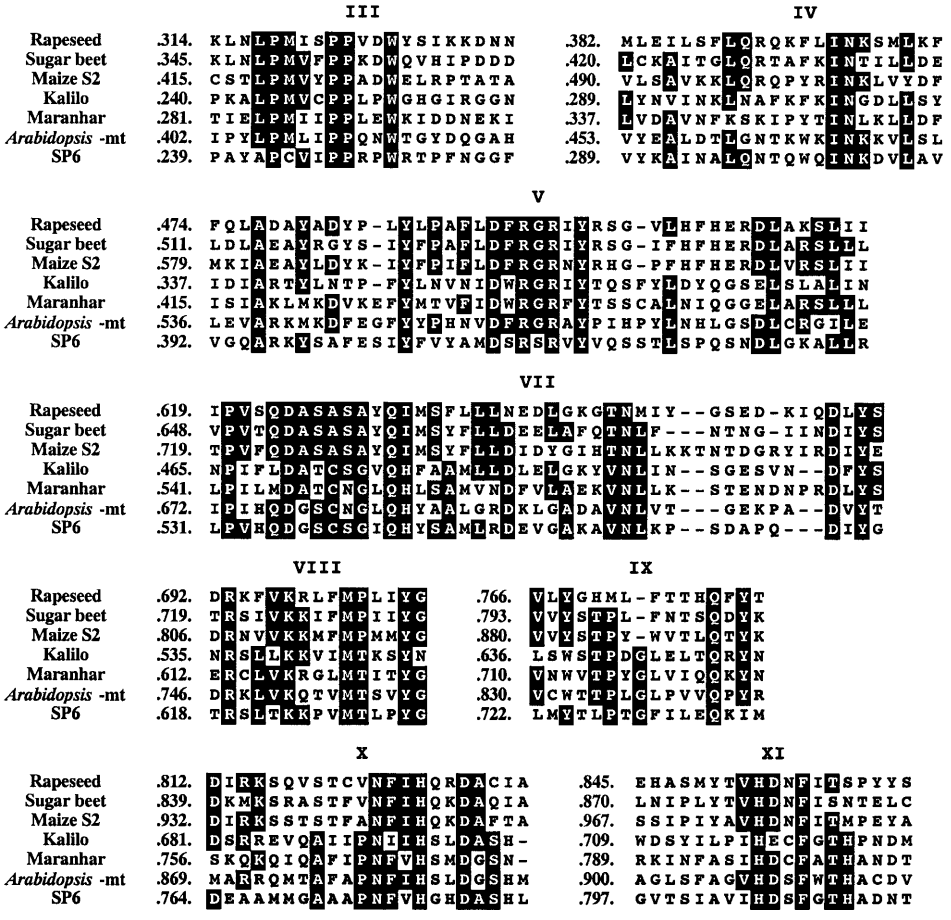


Fig. 3. Comparison of the putative ORF6 protein with conserved motifs of single-subunit RNA polymerases encoded by several mitochondrial plasmid, phage and plant nuclear genomes. Rapeseed, the rapeseed linear plasmid (this study); Sugar beet, the sugar beet 10.4-kb plasmid (Accession No. Y10854); Maize S2, the maize S2 plasmid (Accession No. J01426); Kalilo, the *Neurospora crassa kalilo* plasmid (Accession No. X52106); Maranhar, the *N. crassa maranhar* plasmid (Accession No. X55361); *Arabidopsis*-mt, *A. thaliana* nucleus-encoded mitochondrial RNA polymerase (Accession No. Y09006); SP6, bacteriophage SP6 (Accession No. CAA68288). Amino acids that are conserved in at least four sequences are *highlighted*

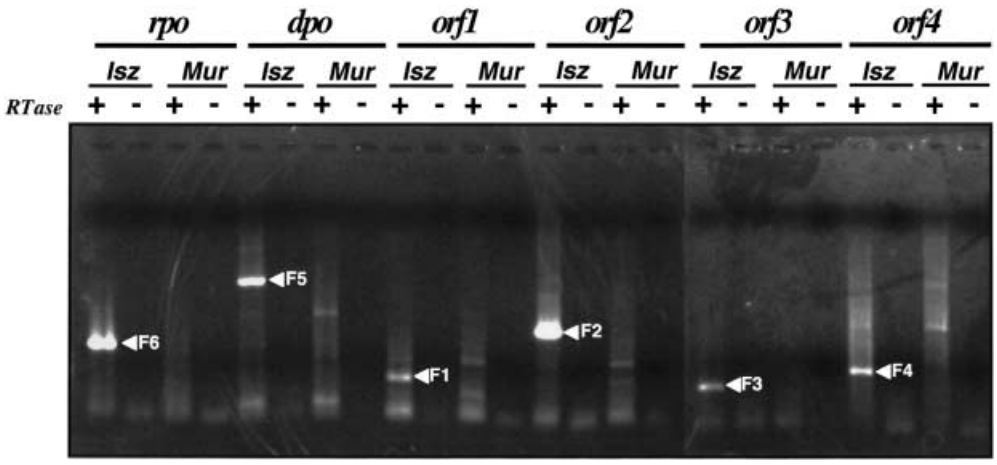


Fig. 4. Agarose gel electrophoresis of RT-PCR amplification products derived from each ORF. The names of the ORFs are shown *above* the gel. Isz, cv. Isuzu-natane (plasmid-bearing); Mur, cv. Murasaki-natane (plasmid-less). +, PCR products obtained with reverse transcriptase; –, PCR products without reverse transcriptase. Amplified bands are indicated by *arrowheads* and named as in Fig. 1C

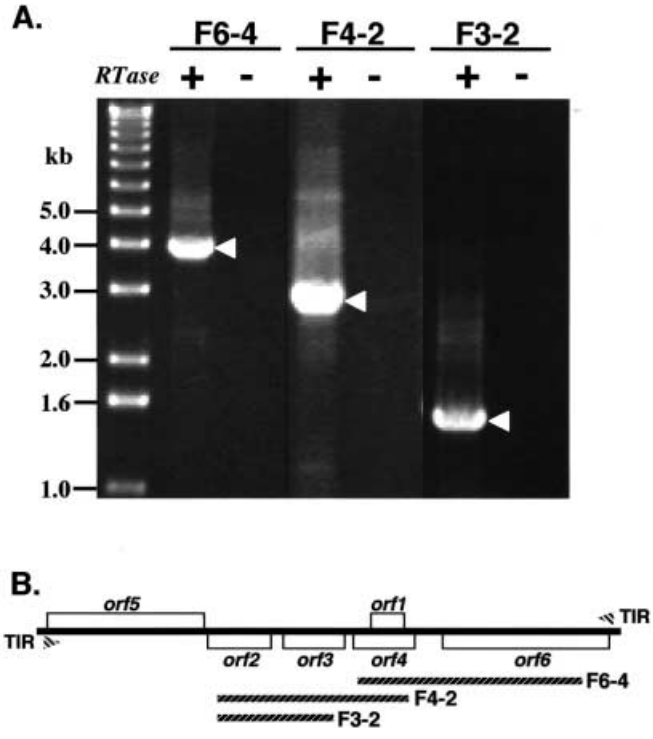


Fig. 5. **A** Agarose gel electrophoresis of RT-PCR amplification products derived from possible cotranscripts. Amplified bands are indicated by *arrowheads*, and named as in Fig. 1C *above* the gel. +, PCR products obtained with reverse transcriptase; –, PCR products without reverse transcriptase. Size markers (bp) are indicated on the *left*. **B** Positions of RT-PCR fragments expected for possible cotranscripts. The *boxes* indicate predicted ORFs

molecule. However we failed to amplify an RT-PCR fragment corresponding to the cotranscript (6.6 kb in size) from *orf6* to *orf2*. This was probably due to our inability to synthesize a first-strand cDNA of adequate length under the conditions used for reverse transcription.

In order to obtain preliminary information on the location of potential transcription initiation sites, a primer-extension experiment was performed using primers located just outside of each TIR – PE-L and PE-R for the left end and right end, respectively. With primer PE-R, the 5′ end of the RNA was mapped 200 bp upstream of *orf6* (data not shown). This corresponds to a position in the TIR, just 30 bp from the right end of the plasmid (Fig. 1A). Primer PE-L gave a signal in precisely same position in the left TIR. The nucleotide sequence surrounding the initiation sites did not show any homology to the consensus CRTA motif for mitochondrial promoters of dicot plants.

At least three ORFs are translated and their products accumulate to relatively high levels in flower tissues

In order to demonstrate that the ORFs in the rapeseed linear plasmid are functional rather than cryptic genes, we used immunological methods to detect their gene products. We raised antibodies against parts of three predicted proteins, ORF2, ORF5 and ORF6, and analyzed protein expression by Western blotting.

Nearly the entire coding region (346 of 358 amino acids, corresponding to the region from codon 10 to codon 355) of ORF2, and the segment encoding the N-terminal part (263 of 990 residues, corresponding to the region from codon 1 to codon 263) of ORF5 were amplified and inserted into the expression vector pCR T7/CT. The proteins were overexpressed in *E. coli* and injected into mice to raise specific antisera. Each antiserum specifically recognized the fusion protein used to raise the antibody (data not shown). A synthetic oligopeptide corresponding to a C-terminal sequence (NH_2-KHQIESYNPNYSLHY-COOH) of ORF6 was also injected into rabbits to raise a specific antiserum.

To investigate the pattern of protein expression, we isolated total mitochondrial proteins from flower buds, stems and roots, and used them for Western analysis. Anti-ORF2 antiserum detected a polypeptide of about 42 kDa, which coincides precisely with the predicted mass (42.1 kDa) of the *orf2* gene product (Fig. 6). In stems, we detected a strong signal at about 50 kDa, in addition to the ORF2 signal (Fig. 6). At present, we have no data that

can explain the origin of this second signal. The ORF2 protein might be modified specifically in stems, or rapeseed could produce a protein that is immunologically related to ORF2. Antibodies raised against the ORF5 fusion protein and the ORF6 synthetic oligopeptide also produced signals at 105 kDa and 130 kDa in the total mitochondrial fraction (Fig. 7). These signals were not detected in any tissues when preimmune serum was used (data not shown). The sizes of 105 kDa and 130 kDa were similar to the predicted sizes of the ORF5 and ORF6 proteins, respectively. These data clearly indicate that the linear plasmid in rapeseed indeed expresses at least three proteins, ORF2, ORF5 and ORF6.

Western analyses using these three antisera also revealed that the expression of the plasmid-encoded proteins was tissue-specific. The levels of these three proteins were relatively higher in flower buds than in stems and roots.

Discussion

The linear mitochondrial plasmid in rapeseed is 11,630 bp long, which makes it the longest of all mitochondrial plasmids discovered in higher plants to date. Its nucleotide sequence, together with other characteristics reported by other groups (Palmer et al. 1983; Turpen et al. 1987), show that this plasmid belongs to a group of replicons, the so-called "invertrons", consisting of linear double-stranded DNAs that have relatively long inverted terminal repeats with proteins covalently bound to their 5′ ends, which includes prokaryotic and eukaryotic linear DNA plasmids, viruses and transposons (Sakaguchi 1990). Although the invertron structure was previously found in other sequenced linear mitochondrial plasmids from higher plants – namely, the sugar beet 10.4-kb plasmid (accession no. Y10854, Saumitou-Laprade et al. 1989), and the maize S1 (Paillard et al. 1985), S2 (Levings and Sederoff 1983), and 2.3-kb plasmids (Leon et al. 1989) – the rapeseed linear plasmid has the largest number of coding sequences, two long ORFs and four shorter ORFs, among the linear mitochondrial plasmids so far sequenced in higher plants.

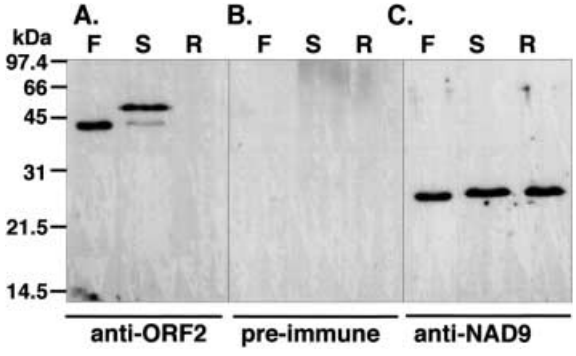


Fig. 6A–C. Immunoblot analysis of mitochondrial proteins from several rapeseed tissues: flower buds (F), stems (S) and roots (R). **A** Western blot probed with anti-ORF2 antiserum. **B** Western blot probed with preimmune serum. **C** Western blot probed with anti-NAD9 antiserum

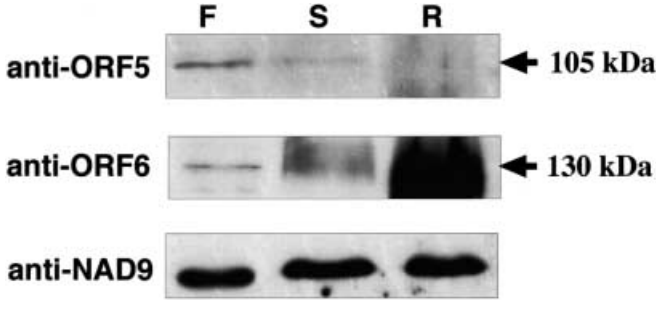


Fig. 7. Immunoblots of mitochondrial proteins from several tissues rapeseed tissues – flower buds (F), stems (S), and roots (R) using anti-ORF5 and anti-ORF6 antibodies. The sizes of the putative ORF5 and ORF6 proteins are indicated by the *arrows* on the right. A control experiment with wheat NAD9 antibody was shown in the panel at the *bottom*

Linear mitochondrial plasmids in fungi and plants often contain sequences encoding putative DNA polymerases and/or RNA polymerases, which may be used for replication and/or expression of genes encoded by the plasmids themselves, respectively. The rapeseed plasmid also contains ORFs encoding a putative DNA polymerase and RNA polymerase, *orf5* and *orf6*, respectively, and these two ORFs are expressed at the protein level. These results indicate that the rapeseed plasmid might function as an autonomous replicon in the mitochondrion. *orf5* and *orf6* are located on opposite strands, and extend from within the terminal inverted repeats toward the center of the plasmid. This genomic organization shows the typical features of the linear mitochondrial plasmids from fungi, such as the plasmids *kalilo* and *maranhar* in *Neurospora* (Chan et al. 1991; Court and Bertrand 1992), which replicate in mitochondria. Maize S1 and S2 plasmids also have similar structures, but contain only one polymerase-encoding sequence, either a DNA polymerase or an RNA polymerase, respectively (Levings and Sederoff 1983; Paillard et al. 1985). The gene structure of the sugar beet 10.4-kb plasmid also differs from that of the rapeseed plasmid. The ORFs for the DNA and RNA polymerases are tandemly located on the same strand, although the plasmid size, 10.4 kb, is close to that of the rapeseed plasmid. Therefore, it is clear that, among the mitochondrial plasmids of higher plants sequenced so far, the rapeseed linear plasmid most closely resembles the fungal mitochondrial plasmids in terms of its genomic organization.

There is a limited amount of information available regarding transcription initiation in linear mitochondrial plasmids. In this study, we found that the 5′ ends of the transcripts from the two strands were located at identical positions relative to the ends of the two TIRs. However, in the maize 2.3-kb plasmid (Leon et al. 1992) and the mF plasmid from slime molds (Takano et al. 1994), the transcripts do not start in the TIRs. On the other hand, the ORFs of the fungal mitochondrial plasmids pC1K1

and *kalilo* are transcribed as two major RNAs whose 5′-ends have been mapped within the TIRs (Gessner-Ulrich and Tudzynski 1992; Vickery and Griffiths 1993). The sequences around the transcription initiation sites show no similarity in the rapeseed and fungi mitochondrial plasmids. However, it is interesting to note that transcription starts inside the TIR regions in both plasmid systems. This similarity in the mode of transcription suggests that the mechanisms of expression of the genes in the rapeseed and fungal linear mitochondrial plasmids depend on the structure of plasmid molecule, especially the TIR structure.

One of our interests in the linear mitochondrial plasmid in rapeseed is the origin of this molecule. Our sequence analysis revealed that the GC content of the plasmid is only 30%, which is quite low compared to those of the completely sequenced mitochondrial genomes in higher plants, such as *A. thaliana* (44.8%; Unseld et al. 1997) and *B. vulgaris* (43.9%; Kubo et al. 2000). However, low GC content is a common feature of linear plasmids in higher-plant mitochondria; the 10.4-kb sugar beet plasmid has a GC content of 38.9%, and the maize plasmids are 37–39% GC. Low GC content is also one of the major characteristics of fungal mitochondrial genomes as compared with higher-plant mitochondrial genomes. In fact, the base composition of fungal mtDNAs is generally around 30% GC (for example, 29.9% in *Podospora anserina*), which is similar to the values for the plant linear plasmids. Taken together, the data on genome structure, base composition and transcript initiation suggest that linear mitochondrial plasmids of higher plants, especially the rapeseed plasmid, may have originated in fungi, and been transferred to higher plant species. However, we have no experimental data concerning the mode of transfer of mitochondrial plasmids from fungi to plant species.

Our primary interest in the rapeseed mitochondrial plasmid concerns its unique property of high-level transmission to progeny plants through the pollen, in addition to maternal transmission through the egg. There have been no reports of such transmission via pollen for other plant mitochondrial plasmids (maize S1, S2 and sugar beet 10.4 kb plasmid). In higher plants, maternal inheritance of mitochondrial DNA is virtually axiomatic. Rapeseed plants also show maternal inheritance of the mitochondrial genome, although quite limited paternal inheritance in an unusual genetic context has been reported (Erickson and Kemble 1990). What mechanisms are involved in the transmission of linear plasmids through the pollen? One or some of the four shorter ORFs, ORF1–ORF4, could be responsible for the maintenance and paternal transmission of the linear plasmid, because these ORFs are novel ones with no homology to known amino acid sequences in the protein databases. We found that ORF5 and ORF6 proteins, possible DNA and RNA polymerases, respectively, accumulated at relatively higher levels in flower tissues than in other tissues of rapeseed. This fact suggests that replication of, and gene expression from, the linear plasmid might be active in flowers, although we have no data for the polymerase activities of ORF5 and ORF6 proteins yet. We also detected higher accumulation of ORF2 protein in flowers than in other tissues, which supports the hypothesis that the linear plasmid is more active in rapeseed flowers, although we cannot rule out the possibility that whole mitochondrial system, and not just the linear plasmid, may be activated in flowers. ORF2 protein might be involved in causing the pollen-mediated transmission of the linear plasmid, although we have no information about the expression of the other three ORFs.

In this report, we provide basic but important information that clarifies the molecular structure of the linear mitochondrial plasmid in rapeseed. Further biochemical characterization of plasmid-encoded proteins and histochemical analysis of the linear plasmid molecule during pollen development in rapeseed are needed to elucidate the biological function and the mechanism of pollen transmission of this unique plasmid.

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