

# Unique features of the mitochondrial rolling circle-plasmid mp1 from the higher plant *Chenopodium album* (L.)

Steffen Backert<sup>†</sup>, Karsten Meißner<sup>§</sup> and Thomas Börner<sup>\*</sup>

Institut für Biologie, Humboldt-Universität zu Berlin, Chausseestraße 117, D-10115 Berlin, Germany

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## ABSTRACT

We analyzed the structure and replication of the mitochondrial (mt) circular DNA plasmid mp1 (1309 bp) from the higher plant *Chenopodium album* (L.). Two dimensional gel electrophoresis (2DE) revealed the existence of oligomers of up to a decamer in addition to the prevailing monomeric form. The migration behavior of cut replication intermediates during 2DE was consistent with a rolling circle (RC) type of replication. We detected entirely single-stranded (ss) plasmid copies hybridizing only with one of the two DNA strands. This result indicates the occurrence of an asymmetric RC replication mechanism. mp1 has, with respect to its replication, some unique features compared with bacterial RC plasmids. We identified and localized a strand-specific nicking site (origin of RC replication) on the plasmid by primer extension studies. Nicks in the plasmid were found to occur at any one of six nucleotides (TAAG/GG) around position 735 of the leading strand. This sequence shows no homology to origin motifs from known bacterial RC replicons. mp1 is the first described RC plasmid in a higher plant.

## INTRODUCTION

The mitochondria of numerous groups of eukaryotic organisms, such as fungi and plants, harbor several extrachromosomal elements in addition to the genomic DNA (reviewed in 1). By far the largest number and strongest diversity among these mitochondrial (mt) plasmids have been described for higher plants (reviewed in 2,3). These plasmids were classified into three categories: circular DNA plasmids, linear DNA plasmids and RNA plasmids. The circular DNA plasmids are very small and lack homology to known genes. Their origin remains a matter of debate. A few mt plasmids were reported to share homology with sequences in the nucleus (4) or with parts of the chloroplast genome (5). Plasmids can be lost without phenotypic consequences to the plant, possibly with one exception, a 2.3 kb DNA

molecule from maize was reported to bear a tRNA gene (6). Almost nothing is known so far about replication of these molecules.

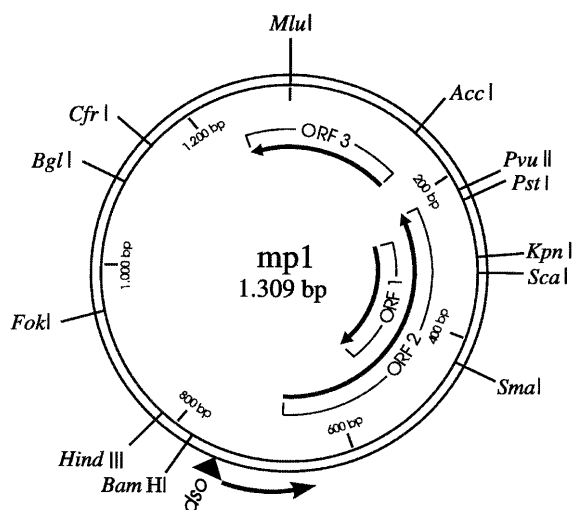
Most of our knowledge about replication of circular plasmids was obtained from bacteria (7,8). Two modes of replication have been described for these molecules. According to the characteristic structures of replication intermediates, these modes were conventionally named  $\theta$  and  $\sigma$ . During the  $\theta$  mode, which is used by most of the plasmids, the sites of priming of leading and lagging strand synthesis are located close to one another within the origin of replication (7–11). Elongation of DNA synthesis can proceed either unidirectionally or bidirectionally to dimers of the replicon.

In the case of the  $\sigma$  or rolling circle (RC) mode of replication, priming events for replication of the two strands are unlinked, occurring at different origins (reviewed in 12–16). In the first step, the plasmid-encoded nicking/closing protein introduces a strand- and site-specific nick in the so-called double-stranded (ds) replication origin (*dso*) (17–19). The free 3'-OH end generated is then utilized as a primer for leading strand replication. Usually, after one round of replication the nicking/closing enzyme terminates strand displacement at its recognition sequence. Two full-sized products, a ds and a single-stranded (ss) circular molecule, are generated. However, the production of long linear plasmid concatemers is well known from phage  $\lambda$  replication (20) and has also been described for bacterial plasmids (21–24). In the latter case,  $\sigma$ -type replication was found to be recombination dependent. The second RC replication step is synthesis of the lagging strand. It is initiated via oligonucleotide priming in a different plasmid region, the ss origin (*sso*) and is discontinuous.

To date, plasmid replication in plant mitochondria has only been described for two circular DNA molecules from *Vicia faba* (25) and one DNA circle from *Chenopodium album* (26–28). Electron microscopic analyses of linearized replicative intermediates of the *V.faba* plasmids indicated that replication originates at a specific origin and proceeds in a unidirectional manner around the molecules via  $\theta$ -shaped intermediates. More recently, we reported on the unusual migratory behavior of the circular 1.3 kb mt plasmid mp1 from *C.album* (see map in Fig. 1) during pulsed-field gel electrophoresis, showing additional linear

\* To whom correspondence should be addressed. Tel: +49 30 2093 8140; Fax: +49 30 2093 8141; Email: thomas=boerner@rz.hu-berlin.de

Present addresses: <sup>†</sup>Department of Botany and Microbiology, Auburn University, Auburn, AL 36849, USA and <sup>§</sup>Institut für Pflanzenwissenschaften, Eidgenössische Technische Hochschule Zürich, Universitätsstrasse 2, CH-8092 Zürich, Switzerland



**Figure 1.** Restriction map of the mt plasmid mp1 from *C. album*. The positions of recognition sites of endonucleases with single restriction sites in the sequence are shown. The *MluI* site was chosen as the position of the first nucleotide. Putative open reading frames are indicated by arrows according to their size and direction: ORF 1, length 243 bp, position 268–510; ORF 2, length 465 bp, position 674–209; ORF 3 length 208 bp, position 150–1252. ORF 1 and ORF 2 would have a GUG initiation codon, which has been shown to serve as a start codon in only one instance, in sunflower mitochondria (53). Limiting to AUG for the start codon, which is most common for plant mitochondria, only smaller ORFs are possible, with up to 153 bases, except for ORF3. The arrowhead indicates the location of the ds origin *dso* and the arrow shows the direction of leading strand replication (28).

molecules and signals retained in the well (26). This pattern was very similar to that observed for several mt plasmids from fungi, for which an RC mechanism of replication was proposed (29,30). During further EM studies we detected  $\sigma$ -shaped molecules of mp1 and other subgenomic circles (27). The structure of these molecules suggested that they could indeed represent intermediates of an RC type of replication. An RC type of replication was also indicated by the observation of ss copies of mp1. We have localized a *dso* around position 730 of the mp1 sequence (Fig. 1) (28).

The mechanism(s) and the biochemical basis of DNA replication in plant mitochondria are not known, although some enzyme components such as the  $\gamma$ -type DNA polymerase are well characterized and a type I topoisomerase has been described (31–33). The aim of the present study was to further elucidate the nature of the  $\sigma$ -like mp1 molecules. The understanding of the replication of this plasmid may help in understanding the mechanism(s) of replication of chromosomal mtDNA in higher plants. By a combination of 2DE and primer extension studies, we confirmed that the previously observed  $\sigma$  structures are indeed replication intermediates of an RC type of replication. We determined the sequence of a replication origin (*dso*) and found that entirely ss molecules are represented by only one of the plasmid strands, the leading strand. Replication of mp1 shows features which are unique among RC replicons.

## MATERIALS AND METHODS

### Plant material and preparation of mtDNA

Mitochondria were isolated from suspension culture C.9.1. of *C. album*. Conditions of cultivation have been described previously (34). Cells were usually harvested 6 days after transfer into new

medium during the logarithmic growth phase. Mitochondria were isolated and lysed as described recently (27,28). Total mtDNA, including the plasmid mp1 (1309 bp; Fig. 1; EMBL accession no. X58911) was purified by RNase digestion, phenol/chloroform extraction and ethanol precipitation (35).

### Bacterial plasmid preparation

The mt plasmid mp1 was cut with *Bam*HI, ligated into the *Bam*HI site of vector pGEM3zf(+) (Promega, Madison, WI) and cloned in *Escherichia coli* cells (28). Transformants were grown at 37°C for 3–5 h in LB medium supplemented with ampicillin (50  $\mu$ g/ml). The cells were harvested and recombinant plasmid DNA was isolated according to a standard protocol (35).

### Two-dimensional (neutral/neutral) gel electrophoresis (2DE)

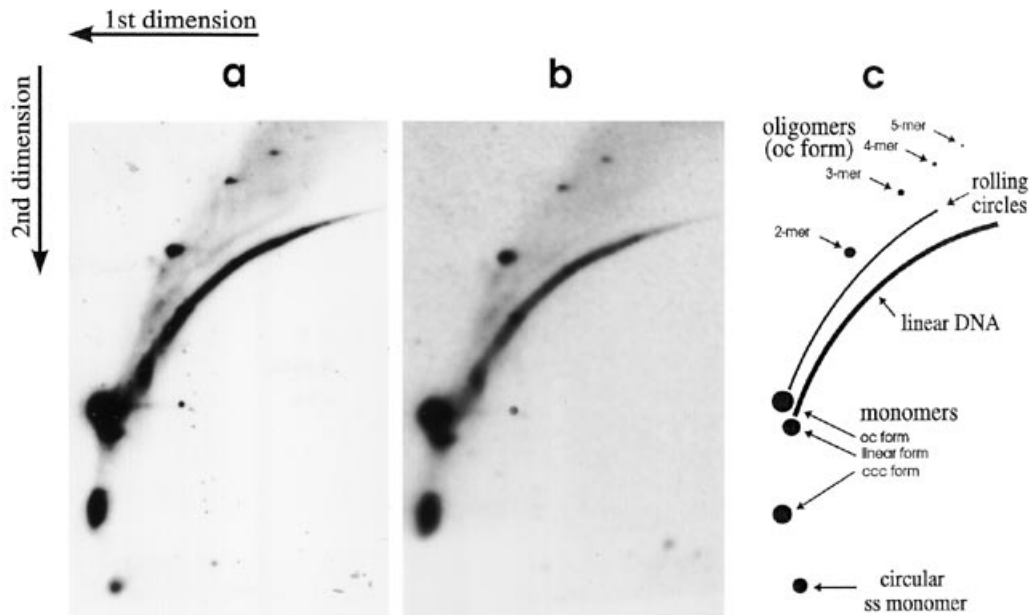
This method was performed according to Brewer and Fangman (36–38) and used for replication studies with plasmid mp1. For this purpose, ~3  $\mu$ g mtDNA was cut with restriction endonucleases. To digest mp1, enzymes were selected which had only one cutting site in the plasmid (see map of the plasmid in Fig. 1). Restriction enzymes were purchased from Amersham-Buchler (Braunschweig, Germany). Cut and uncut samples were separated for 25 h at 1 V/cm in the first dimension in 0.4% agarose gels in 1 $\times$  Tris–borate–EDTA (TBE) electrophoresis buffer without ethidium bromide in a large electrophoresis chamber (model HRH; IBI, New Haven, CT). The lanes were cut out (in the absence of UV light) and stained by ethidium bromide. Separation in the second dimension was done in 1.5% agarose gels in 1 $\times$  TBE with 0.3  $\mu$ g/ml ethidium bromide at 5 V/cm for 4 h at 90° orientation to the first dimension. All electrophoresis steps were performed at 4°C.

### Blotting and hybridization

After electrophoresis, the DNA was blotted by alkaline transfer to Zeta Probe GT membranes according to the instructions of the supplier (BioRad, Richmond, VA). The cloned plasmid mp1 (cut out of the vector) was used as a probe for hybridization. Radioactive labeling of the plasmid DNA was performed with the Rediprime kit and 1.85 MBq [ $\alpha$ -<sup>32</sup>P]dCTP, provided by DuPont (Bad Homburg, Germany). For identification of ssDNA forms of mp1, we prepared ss-specific RNA probes of the plasmid integrated in vector pGEM3zf(+) using the MAXIScript *in vitro* transcription kit (Ambion Inc., Austin, TX). Filters were hybridized overnight in 6–8 ml 7% SDS, 250 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.2, at 65°C in hybridization tubes from Schott (Mainz, Germany) and then washed under stringent conditions according to standard protocols (35). Quantification of hybridization signals was done with a GS-363 phosphorimager (BioRad).

### Primer extension assay and DNA cycle sequencing

For primer extension studies the sequence-specific primers 1 (5'-GCCATCTAAAACGAGCGACG-3'), 2 (5'-CCTTGTAACATCCCCCGA-3') and 3 (5'-GGGAGCACAAACCGAGTACCG-3') were 5'-end-labeled using the Ready-To-Go T4 polynucleotide kinase kit (Pharmacia Biotech, Uppsala, Sweden) and [ $\gamma$ -<sup>32</sup>P]ATP (0.37 MBq; DuPont). Asymmetric PCR reactions with one of the primers were performed in a 50  $\mu$ l volume including 0.2 mM dNTPs, 2 mM MgCl<sub>2</sub> in 1 $\times$  PCR buffer in the presence of 0.5  $\mu$ g total mtDNA (harvested 1 or 6 days after transfer into new medium), 0.5  $\mu$ g open circular and  $\sigma$ -like mp1



**Figure 2.** Autoradiographs of 2DE gels of uncut plasmid mp1 from *C. albim*. Hybridization was done with *in vitro* transcribed RNA which was specific for the leading (a) and lagging strand (b) of mp1. The signals obtained are explained (c) as described by Brewer and Fangman (36–38). Arrows indicate the positions of ds plasmid DNA such as circular covalently closed (ccc), open circular (oc) and linear forms, as well as ss mp1 copies and the expected rolling circles.

molecules (electroeluted from the respective zones of an agarose gel as depicted in Fig. 2c) or 0.5 µg cloned mp1 in vector pGEM3zf(+). Thermostable Goldstar DNA polymerase was purchased from Eurogentec (Seraing, Belgium). Cycling was done at 95°C for 30 s, 58°C for 30 s and 72°C for 1 min for 40 cycles. Extension products were resolved in denaturing polyacrylamide gels (4.5 and 6% polyacrylamide, 7 M urea) in 1× TBE buffer. As a size marker, a 5'-end-labeled 10 bp ladder was used (Gibco BRL, MD). Additionally, a sequencing reaction was done by the method of 3-dNTP internal label cycle sequencing according to the instructions of the manufacturer (Amersham-Buchler) using [ $\alpha$ -<sup>35</sup>S]dATP (1.85 MBq; DuPont) and primer 1. Template DNA was plasmid mp1, cloned in the vector pGEM3zf(+). After electrophoresis the DNA in the gel was fixed by incubation in 5% acetic acid. The gels were dried on a glass plate and exposed to X-ray films (Amersham-Buchler).

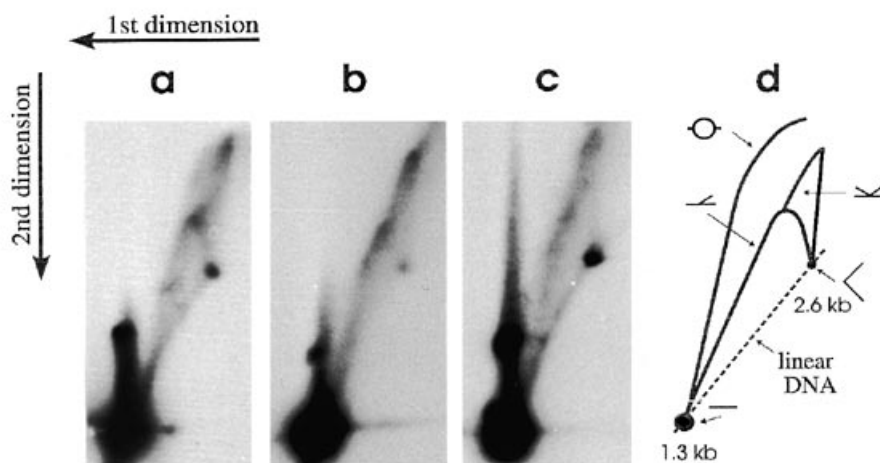
## RESULTS

### Identification of ds oligomers and ss copies of plasmid mp1

In the last few years 2DE of DNA molecules has been developed into a powerful tool for the detection of replication intermediates and for determining the replication type (36–47). This technique takes advantage of the fact that DNA molecules are separated according to their molecular mass in the first dimension and that a non-linear DNA molecule does not migrate at the same rate as a linear molecule of equal mass in the second dimension, i.e. migration is additionally dependent on the structure (36,46). Replicative DNA forms can be unequivocally distinguished from recombination intermediates. Therefore, it should be possible to determine whether the  $\sigma$ -like

structures of plasmid mp1 recently observed by EM (27,28) represent replication or recombination intermediates.

In a first experiment, uncut mtDNA was separated in two dimensions as described above, blotted by denaturing transfer and then hybridized with a leading strand-specific radioactively labeled RNA probe obtained by *in vitro* transcription of mp1 (Fig. 2a). After exposure, the filter was stripped and reprobed with a lagging strand-specific RNA probe (Fig. 2b). The patterns of hybridization signals obtained were completely identical with probes for both strands, except a faint spot in the lower part of the gel. The signals are explained schematically (Fig. 2c) according to Brewer and Fangman (36–38). The strongest signals were always located at the position of the open circular, linear and supercoiled forms of the monomer as well as at a curve representing linear molecules starting from 1.3 up to 10–12 kb, which should represent oligomeric plasmid forms. At the position of linear multimers, signals appeared over a strong background. This smear stops exactly at the position of the monomer, i.e. there was no breakage of the monomers during preparation. Open circular forms up to a 5mer could be observed. Moreover, we detected a curve between the linear and circular molecules which extended past the linear dimer. This signal most probably represents plasmid molecules with a growing tail of up to 2–3 contour lengths of the corresponding circle, since this arc looks very similar to that obtained from rolling circles on analysis of *in vitro* (43,44) and *in vivo* (45) replication in other systems. Therefore, this curve could represent the  $\sigma$ -like mp1 molecules observed by EM studies (27,28). The observation that an arc only originates from the open circular monomer spot suggests that monomeric forms are the predominant templates for plasmid replication. Bubble-like structures as known for  $\theta$  replication were



**Figure 3.** Autoradiographs of 2DE gels of plasmid mp1 after digestion with *Hind*III (a), *Pst*I (b) and *Bgl*I (c). The hybridization probe was cloned mp1 DNA. (d) The resulting hybridization signals of (a)–(c) according to Brewer and Fangman (36–38). Each arrow indicates the respective pattern of restricted plasmid replication intermediates, which are shown schematically.

not found. Such molecules would form an arc between the open circular forms of the monomer and dimer (36). Furthermore, in the lower part of the 2DE gel a faint spot appeared which migrated in the second dimension faster than the supercoiled monomer. Hybridization showed only signals with the leading strand-specific plasmid probe (Fig. 2a) and not for the lagging strand (Fig. 2b). Position and hybridization behavior are in agreement with the ss nature of this molecule (12–16). Hence, this spot represents the ss circular form, more precisely the leading strand, of the mp1 monomer. In addition to the ss monomer, a much weaker signal appeared on an imaginary line of ss molecules which should represent the ss circular dimer of mp1 (data not shown). Quantitative analysis of the plasmid hybridization signals in Figure 2a revealed the following distribution: 48% linear molecules, 42% circles, 6%  $\sigma$ -like replication intermediates and 4% ssDNA molecules. In the total fraction of plasmid DNA, monomers comprised only ~43% of the sequences.

#### Analysis of cut replication intermediates by 2DE

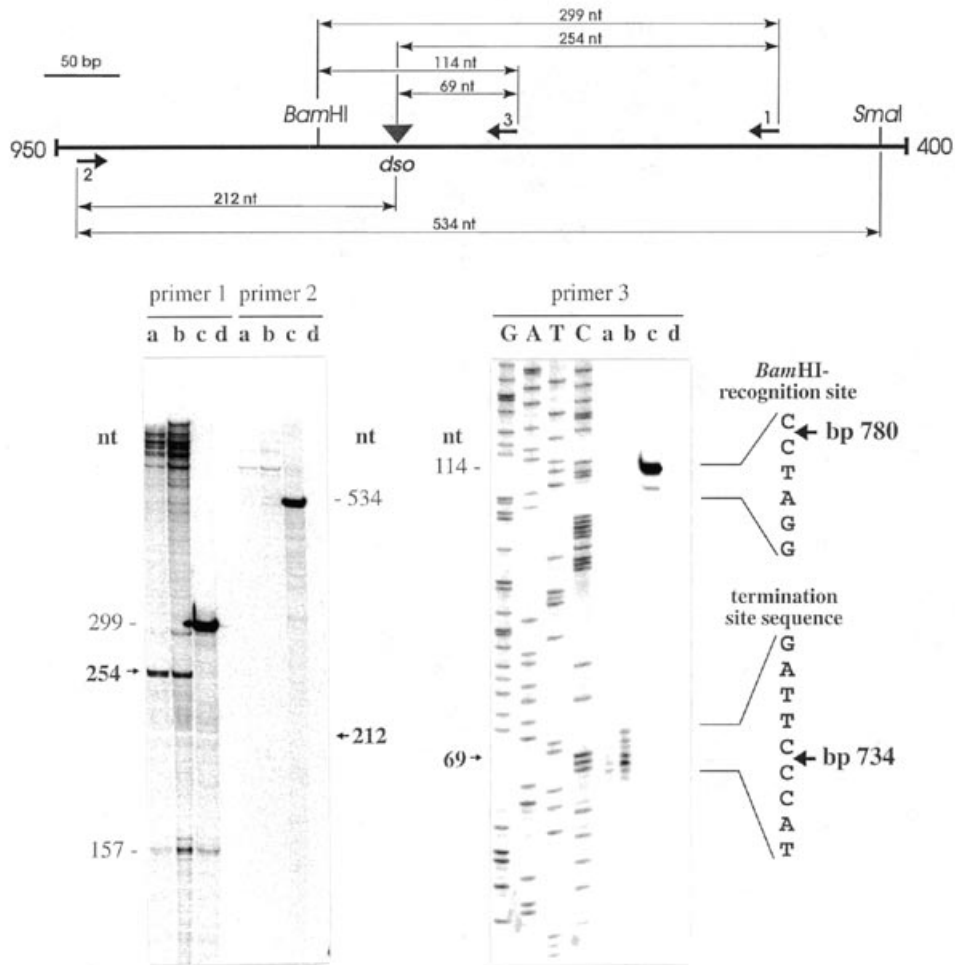
In further experiments mtDNA was digested with restriction endonucleases that linearize the circular plasmid mp1. The digested DNA samples were separated in 2DE gels, transferred to Nylon membranes and hybridized with the plasmid mp1 DNA as a probe (Fig. 3a–c). The patterns of hybridization signals revealed different types of DNA molecules, including replicative forms. The interpretation of these patterns according to Brewer and Fangman (36–38) is depicted in Figure 3d. A very strong signal was observed at the position of linearized monomers. A much weaker but clearly visible spot occurred at the position of linearized dimers. Other linear molecules were expected to migrate on a straight line between these two spots. All hybridization signals above the line of linear molecules represent forms of mp1 other than linear molecules. A continuous arc of growing Y-shaped replication intermediates expanding from the linear monomer to dimer was observed. A complete arc was seen only with DNAs digested by *Hind*III (Fig. 3a) and *Bam*HI (data not

shown). In the case of all other enzymes used (*Acc*I, *Bgl*II, *Cfr*I, *Fok*I, *Kpn*I, *Mlu*I, *Pst*I, *Pvu*II, *Sca*I and *Sma*I), this arc of simple Y molecules ended before reaching the dimer (shown for *Pst*I and *Bgl*II digests in Fig. 3b and c). The patterns are not compatible with those obtained from intermediates of  $\theta$ -type replication, which would migrate in a much higher position (36–38). We never detected intermediates which could result from digestion of molecules with replication bubbles (compare with the scheme in Fig. 3d). The observed arcs of simple Y structures are best explained by  $\sigma$ -like intermediates of replication initiating at a position near the cutting sites of *Bam*HI and *Hind*III, but distant from the sites of those enzymes which did not lead to patterns with complete arcs. In addition, we found weak signals at the position of so-called double Ys. The presence of such structures could be attributed either to recombination intermediates (36–38,46,47), to fragments with replication bubbles at both ends cut by the enzyme (36–38), to circles with a tail exceeding the contour length of the circle (designated as extended 'E' arcs by Han and Stachow; 42) which were not cut (e.g. because of stretches of ssDNA at the cutting site or entirely ss tails respectively) and to circles with two tails, as detected in EM analyses (27). Circles with two tails could be generated, for example, by a second initiation of replication at the origin before completion of the first round of replication or by the simultaneous initiation of replication at more than one origin (28).

#### Mapping of a double-stranded replication origin (*dso*)

The existence of ss mp1 copies (see above) of only one of the DNA double strands is not compatible with a  $\theta$  type of replication. Such intermediates should occur, however, during RC replication, where the introduction of a site-specific nick in only one strand of the supercoiled template DNA characterizes the initial event of replication (12–16). We identified such an origin of RC replication of mp1 by mapping short ds fragments produced by cleaving the tails of  $\sigma$ -like replication intermediates with restriction endonucleases (28). This is not a very precise



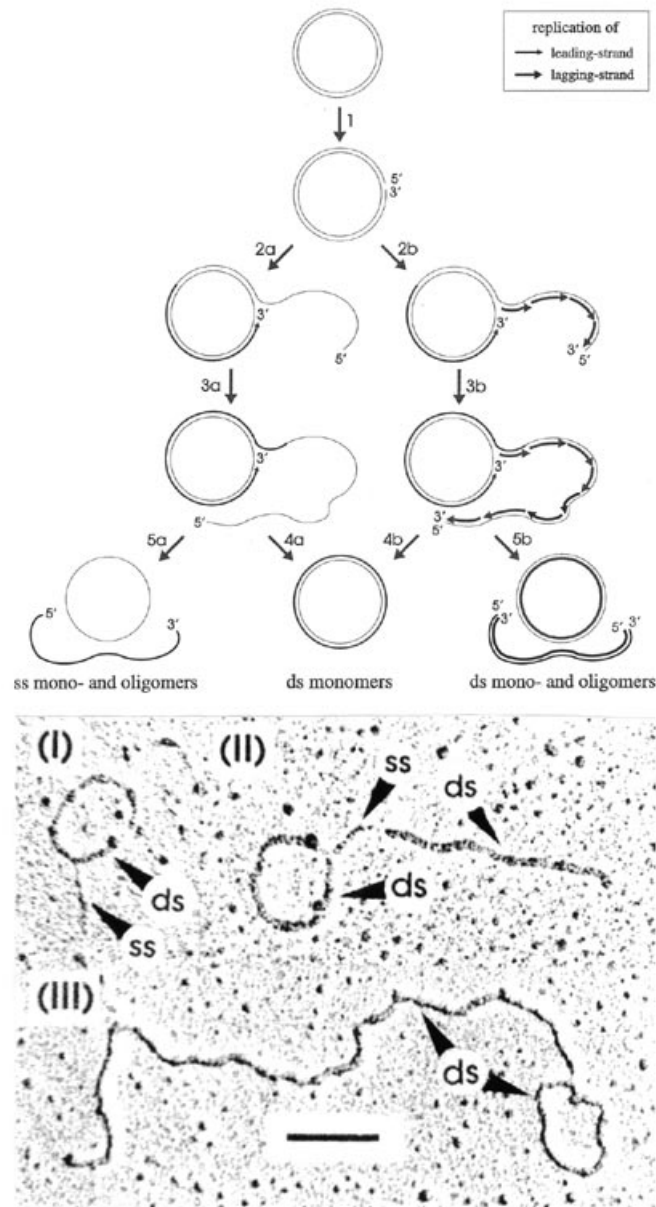


**Figure 4.** Fine mapping of the replication origin *dso* of plasmid mp1 by primer extension assay. The position and direction of three primers in the map are depicted at the top. The resulting products were resolved in sequencing gels. Lanes G, A, T and C represent the sequence ladder of mp1 using primer 3. The blank reaction without DNA is shown in lanes d. The template for asymmetric PCR reactions was mtDNA from suspension cultured cells of *C.album* harvested 1 (lanes a) or 6 days (lanes b) after transfer into new medium. As a control, we used cloned mp1 partially digested with *Bam*HI (primers 1 and 3, lanes c) or *Sma*I (primer 2, lane c). The products were separated in a 4.5 (for reactions with primer 1 and 2) or 6% (for primer 3 reactions) polyacrylamide gel respectively.

approach, but showed that the origin is located around position 730 of the physical map of mp1 (cf. Fig. 1).

To verify the existence of this nicking site, to localize it precisely and to determine its sequence, primer extension studies were done. For this purpose, we designed sequence-specific primers for both strands of the plasmid and performed asymmetric PCR assays. The position of a nick in one strand as well as of breakage and cleavage products can be determined by denaturing polyacrylamide gels combined with a DNA sequencing reaction (48–50). By using this method the origin of mp1 replication was mapped and sequenced (Fig. 4). The position of a site- and strand-specific nick could only be identified when primer extensions were carried out for multiple cycles (40), reflecting the scarcity of replication intermediates of mp1 in mtDNA. The effectiveness and precision of the applied method was controlled by the identification of cleavage products of recombinant plasmid

DNA, as shown for *Bam*HI (primers 1 and 3, lanes c) and *Sma*I (primer 2, lane c). Extension of primer 1 indicated the origin of RC replication: using the leading strand-specific primer 1 and uncut plasmid DNA from mitochondria, we identified a strong band of ~250 nt (lanes a and b). mp1 cloned in vector pGEM3zf(+) and transformed in *E.coli* served as a negative control, since this plasmid is not replicated via the RC mode in bacterial cells. The band at a size of 250 nt was absent in lane c, as expected. When using primer 2, which is specific for the lagging strand and mtDNA, we could not detect products of the corresponding size of 212 nt (arrow, lanes a and b). These results demonstrate that the extension product obtained with primer 1 was specific for the leading strand, i.e. termination was caused by the nicking site expected to serve as an origin of RC replication (*dso*). To localize the exact position of the *dso*, we designed primer 3 situated closer to the nicking site and compared the size



**Figure 5.** Scheme of plasmid mp1 replication via an RC mechanism. This model, including asymmetric (pathway a) and symmetric (pathway b) replication, was proposed on the basis of results from 2DE, hybridization studies and primer extension assays and is supported by the detection of replication intermediates by EM (27,28). **(I)** A circle with a short ss tail (see arrows). **(II)** This circle has a tail greater than the unit length containing both ds and ss regions. **(III)** This circle has a tail much longer than its circumference. Bar represents 0.5 kb.

of its extension products with a ladder obtained from the sequencing reaction of recombinant plasmid DNA using the same primer. The resolution of these products at the nucleotide level showed several bands corresponding to positions 733–738 of the plasmid (position 1 is the *Mlu*I site in Fig. 1). Strongest signals were obtained at positions 734 and 735. Identical results were obtained when we utilized open circular forms of mp1 or  $\sigma$ -like molecules (isolated from the respective regions of agarose gels, cf. Fig. 2c) as templates instead of total mtDNA. Minimal variability of signal intensities was seen between template

mtDNAs isolated at different times of growth (Fig. 4, lanes a and b). Additional specific products could be amplified from the plasmid DNA with both primers 1 and 2 (Fig. 4, lanes c) which map according to their size to regions different from the *dso*. These products indicate termination sites of *in vitro* DNA synthesis which may be caused by short inverted repeats found near the *dso*. Such sequences would allow the formation of secondary structures by intramolecular base pairing. Putative stem-loop structures with a low free energy are located in the region between 937 and 913 (–21.2, –14.0 and –12.0 kcal/mol) as

well as at positions 875–823 (–18.6 kcal/mol) and 908–881 (–3.8 kcal/mol). The stem–loop structures may function *in vivo* as a recognition motif during the initiation of RC replication.

## DISCUSSION

We have presented here several lines of evidence for replication of the mitochondrial plasmid mp1 according to a rolling circle mechanism. This includes detection of uncut  $\sigma$ -shaped and cut Y-shaped replication intermediates by 2DE, observation of ss copies of only one strand of the plasmid and identification of a nicking site in the leading strand, a characteristic feature of RC replication. The observation of a strand-specific nicking site and of circular ss forms of the same mp1 strand support the idea of the activity of a nicking/closing enzyme (17–19) in the mitochondria of *C. album*. The localization of the *dso* on mp1 around nucleotide 735 by primer extension is in good agreement with recent mapping of the origin around position 730 by a less precise approach (28) and is also compatible with the 2DE data. When mtDNA was cut by restriction endonucleases which linearized mp1 and separated electrophoretically by 2DE, we could detect continuous arcs of hybridization signals only for *Bam*HI and *Hind*III. These cutting sites are clustered together around positions 750–800, i.e. not far from the *dso*. In the case of all other enzymes the arcs stopped before reaching the dimer, indicating the absence of an origin close to these endonuclease recognition sites. The circular DNA of mp1 is the first RC plasmid detected in a higher plant. A model of the replication cycle of mp1 based on the data outlined here and in recent reports (27,28) is depicted in Figure 5. This plasmid shows some unique features in comparison with bacterial RC plasmids.

(i) The replication of RC plasmids has been studied very extensively in bacteria (12–16). These replicons replicate in a similar manner to a mechanism described for ssDNA phages and accumulate ss plasmid copies during this process. In an early study on *Bacillus subtilis* and *Staphylococcus aureus* it was shown that the ss plasmid DNA exists as a circular molecule of the same size as the parental monomer and corresponds to only one of the two DNA strands (51). It represented ~20% of the total hybridization signal. The *in vivo* occurrence of one of the two possible ssDNA circles of mp1 in mtDNA preparations from *C. album* is a strong indication for RC replication, the only known process producing ss copies of ds molecules. In the case of asymmetric RC replication, ss copies of only one of the DNA strands are to be expected. This is by definition the leading strand of replication (12–16). The percentage of single-stranded plasmid mp1 copies (~4% of the hybridization signal) is lower than normally observed for the bacterial RC plasmids described above. Notable exceptions, for example, are plasmid pUB110 (*S. aureus*) and pBC16 (*Bacillus cereus*), which generated amounts of ssDNA in the same range as mp1 (51).

(ii) The organization of bacterial RC plasmids is highly conserved (12–16). The *dso* region is placed immediately upstream of a gene which encodes a nicking/closing enzyme involved in the initiation and termination of leading strand synthesis. This replication initiator protein binds to and introduces a strand- and site-specific nick in the leading strand of supercoiled DNA, providing a free 3-OH end for elongation. The function of this protein can be substituted, however, by nucleases which create random nicks in both plasmid strands, leading to recombination-dependent replication (21–24). Like most of the

described circular plasmids in plant mitochondria, mp1 does not bear genetic information necessary for the function of the organelle (52). The sequence of mp1 contains small putative ORFs (Fig. 1). Database alignments exhibited insignificant sequence homology of ORF2 to DNA and RNA polymerase genes. However, this ORF would not be large enough to encode a complete polymerase. No homolog of a gene encoding a complete replication protein, including the nicking/closing enzyme on plasmid mp1, was found. Even shorter conserved motifs, including tyrosine or serine residues, which are part of the active center of the latter proteins, could not be detected. These facts do not rule out the possibility that such a replication protein is encoded in the nucleus or in chromosomal mtDNA.

(iii) DNA sequences proximal to the *dso* of mp1 (TAAGGG) show no homology to consensus motifs of bacterial *dsos*. A cluster of G residues (GGG) at the nicking site was not found in any RC origin of bacterial plasmids or phages. An AT-rich sequence of 5–8 nt upstream of the nicking site, which is common to many RC systems (12–16), is absent in the case of mp1. In comparison with bacterial RC plasmids, which have a relatively low GC content (16), mp1 contains ~47.5% GC. Moreover, unlike the situation in bacteria, the nicking site of mp1 is not represented by a single nucleotide. Our data from primer extension experiments demonstrated that there is a nicking region of ~5 nt. The most prominent extension product occurs at position 734, which indicates the most common nicking site to be TAAG/GG (behind position 735). Degradation of DNA nicked at only one position would be an alternative explanation for the observed multiple bands. However, since the cleavage sites of restriction endonucleases were exactly determined by single bands (cf. Fig. 4, lanes c), degradation seems not to occur under the applied conditions. In previous mapping studies we obtained data suggesting the existence of an additional, less often used origin on mp1 located between positions 510 and 560 (28). Interestingly, the *dso* sequence TAAGGG is also found at position 540. During the present study the introduction of nicks at this position could not be observed, which is likely due to its rare usage. In addition, we have found no evidence for termination of plasmid mp1 replication at a second *dso* with subsequent re-initiation, which would result in greater size diversity of ss and ds plasmid copies.

(iv) Many of the  $\sigma$ -like mtDNA molecules of *C. album* were found to have tails several times longer than the circumference of the corresponding circle, suggesting the synthesis of concatemeric replication products (27,28), as known from classical phage  $\lambda$  replication (20). The products of replication of several classes of RC plasmids from Gram-positive and Gram-negative bacteria are monomers (12–16), whereas long linear concatemers are produced during recombination-dependent RC replication (21–24). In contrast to the situation with mp1, where replication is initiated at one or two distinct origins, recombination-dependent replication in bacteria is mostly not initiated at specific origins (reviewed in 22,24). In the case of mp1, monomers represented ~50% of the total plasmid DNA. mp1 also exists in linear and circular oligomeric forms which may be products of recombination events and/or represent concatemeric products of RC replication (Fig. 5, pathway b).

In conclusion, our data revealed new features of an RC plasmid. This is the first report of an organellar plasmid in plants replicating via an RC mechanism. Its high copy number could make mp1 an interesting and promising model system for further studies of the replication and structural organization of chromoso-

mal mtDNA in higher plants, because  $\sigma$ -like molecules and entirely ss circles were also found for the chromosomal mtDNA in *C. album* (28). This study may also provide clues for the explanation of a common phenomenon of plant mitochondria, the occurrence of a heterogenous population of linear molecules (26,27), which could also arise by a rolling circle mechanism of replication in these organelles.

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