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# Restriction endonuclease cleavage site map of safflower (*Carthamus tinctorius* L.) chloroplast DNA

M. A. Smith [1] and C. Ma [2]

[1] Graduate Section of Biochemistry, Brigham Young University, Provo, UT 84602, USA
[2] Institute of Botany, Academia Sinica, Peking, China

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**Summary.** The restriction endonucleases SalI, PstI, KpnI and HindIII have been used to construct a physical map of safflower (*Carthamus tinctorius* L.) chloroplast DNA. This was accomplished by hybridizing Southern blots of single and double digested chloroplast DNA with $^{32}$P-dCTP nick-translated SalI, KpnI and HindIII probes which were individually isolated from agarose gels. The chloroplast DNA was found to be circular and to contain approximately 151 kbp. In common with many other higher plant chloroplast DNAs a sequence of about 25 kbp is repeated in an inverted orientation. The small and large single copy regions separating the two repeated segments contain about 20 kbp and 81 kbp, respectively. The rRNA structural genes were also mapped by Southern blot hybridization and are co-linear with several other plant species.

**Key words:** Chloroplast – DNA – Physical map – Safflower – Restriction

## Introduction

Chloroplast biogenesis requires the cooperation of nuclear and chloroplast genetic systems (Ellis 1977; Herrmann and Possingham 1980). However, the coding capacity of the plastid genome is rather modest by comparison to that of the nuclear genome. Chloroplast DNA (ct-DNA) is contained in a single circular molecule ranging in size from 85 to more than 190 kilobase pairs (kbp) (Whitfeld and Bottomley 1983). Most higher plants so far examined have about a 150 kbp chloroplast genome (Bohnert et al. 1982). A 20–28 kbp sequence of DNA that includes ribosomal RNA genes has been shown in several species to be repeated and arranged in an inverted orientation, however at least three species of legumes differ from the general pattern in that they have smaller genomes, lack the inverted repeat and contain only one set of ribosomal RNA genes (Mubumbila et al. 1983). Genetic mapping data shows the chloroplast genome to be involved in coding, at least in part, both the energy transducing and $CO_2$ fixing systems of plants. In addition, ct-DNAs of tobacco, spinach, *Atriplex,* petunia, and cucumber are essentially colinear (Whitfeld and Bottomley 1983).

In this study we have physically mapped the chloroplast genome of safflower (*Carthamus tinctorius* L.) which four restriction endonucleases and have mapped genes for the ribosomal RNA(rRNA).

## Materials and methods

### *DNA isolation*

The procedure was modified from that described by Kolodner and Tewari (1975). In a typical isolation two-week-old plants were placed in the dark for 2–3 days and leaves and coleoptiles removed and homogenized in ice cold Buffer A (4 ml/g fresh weight) containing 0.3 M mannitol, 0.05 M Tris, 0.003 M EDTA, 0.001 M mercaptoethanol, 0.1% bovine serum albumin, with two 5 s bursts in a Waring blender at medium setting. Homogenates were filtered through two layers of cheesecloth and then successively through Nitex nylon mesh (Tetko, Inc., NY) of 74, 44 then 20 μ. Fibers retained by the cheesecloth were again homogenized in one half of the original volume of Buffer A, filtered as above, combined with the first filtrate and centrifuged for 10 min at 40×g at 4 °C. The supernatant was decanted, then centrifuged at 1,020×g for 15 min at 4 °C and the resulting crude chloroplast pellet suspended in Buffer A (0.2 ml/g fresh weight). $MgCl_2$ (0.01 M) and DNaseI (50 ug/ml) were added and the suspension incubated for 1 h at 4 °C. Following this incubation, 3 volumes of Buffer B (0.6 ml/g fresh weight) containing 0.3 M sucrose, 0.05 M Tris, 0.02 M

EDTA, pH 8.0 were added and the suspension centrifuged at 1,500×g for 15 min at 4 °C. The pellet was washed twice by suspending it in the same volume of Buffer B and recentrifuging as above. The final pellet was suspended in Buffer C (0.048 ml/g fresh weight) containing 0.05 M Tris, 0.02 M EDTA, pH 8.0, pronase (200 ug/ml) and 0.012 ml (per g fresh weight) of Buffer C containing 10% sodium sarkosyl were added, then incubated for half an hour at 37 °C and then extracted with one volume of neutralized phenol (pH 8) : chloroform (1 : 0.5 v/v). The aqueous phase was precipitated with 2.5 volumes of ethanol (–20 °C) and stored overnight at –20 °C. Nucleic acid was collected by centrifugation at 6,000×g, and the pellet suspended in TE Buffer (~ 2 ul/g leaves) containing 0.01 M Tris, 0.001 M EDTA, pH 7.4. The yield of chloroplast nucleic acid is about 12 µg per 1 g original leaf wet weight. This preparation is of sufficient purity for restriction endonuclease cleavage and subsequent agarose gel electrophoresis, or for ligation and generation of a recombinant DNA library. RNA is easily degraded by treatment with RNase and removed if necessary by Sepharose 6B gel filtration. Chloroplast rRNA: Chloroplast RNA was isolated by the cetyltrimethylammonium bromide technique (Murray and Thompson 1980) as adapted by Taylor and Powell (1982) and radiolabelled in an AMV polymerase reaction with random thymus oligomer primers as described by Robertson and Varmus (1981).

*Restriction with endonuclease*

Chloroplast DNA (ct-DNA) (about 2 ug) was digested with 5–10 units endonuclease in TA Buffer containing 33 mM Tris-Acetate, 66 mM KAcetate, 10 mM MgAcetate, 0.05 mM dithiothreitol, 100 ug/ml BSA (nuclease free), pH 7.9 at 37 °C for 2–6 h. The resulting fragments were separated by horizontal electrophoresis in 0.8% Agarose gel (Bethesda Research Laboratories, Inc.) in TAE buffer containing 0.04 M Tris-Acetate, 0.002 M EDTA, pH 8.0, 0.5 ug/ml ethidium bromide. HindIII fragments of lambda DNA were used as molecular size standards. Electrophoresis was carried out at about 2 volts/cm at room temperature.

*Southern blots*

Bidirectional transfers were prepared as described by Smith and Summers 1980) using either nitrocellulose filters (Schleicher and Schuell) or GeneScreenPlus (New England Nuclear).

*Isolation of restriction fragments from agarose gels*

Restriction fragments were separated as above except in 1% LMP agarose gels (Bethesda Research Laboratories). Gels were cast at 4 °C and run at room temperature for about 25 h. Individual fragments were recovered as described by Maniatis et al. (1982) or by the freeze thaw method of Vedel and Mathieu (1983).

*$^{32}$P-radiolabeling*

DNA was labelled by nick translation using the method of Maniatis et al. (1982) with 1–2 uCi of $^{32}$P-dCTP (1 µCi/1.26 umoles) and 0.01–0.1 ug of ct-DNA per reaction. Unincorporated $^{32}$P-dCTP was removed by Sephadex G-50 using either column or centrifugation techniques (Maniatis et al. 1982).

*Hybridization with nick-translated probes*

Hybridizations were carried out in sealed freezer bags at 41 °C for 48 h in the presence of 50% formamide as described by Maniatis et al. (1982). Post hybridization washes were carried out in 0.1×SSPE Buffer containing (for 1×) 0.18 M NaCl, 10 mM $NaH_2PO_5$, 1 mM EDTA, pH 7.4 and 0.1% SDS; three times at 53 °C with gentle aggitation each time for 20 min. GeneScreenPlus was washed as described by the manufacturer with comparable results. Autoradiograms were generated using Kodak XAR-5 X-ray film in cassettes containing X-Omatic Regular intensifying screens at –70 °C from a few to several hours depending on observed intensities.

## Results and discussion

*Isolation and characterization of ct-DNA*

Details of the isolation and characterization of safflower chloroplast DNA will be published elsewhere. At present it is sufficient to mention that this DNA is similar with regard to cesium chloride isopycnic centrifugation, and renaturation kinetics to other higher plant chloroplast DNAs, having a density of 1.700 g/cc and a kinetic complexity of about $10^8$ daltons (Ma et al. 1985).

*Cleavage of chloroplast DNA with restriction endonucleases*

Chloroplast DNA isolated from safflower was digested with four restriction endonucleases for physical mapping studies. These enzymes are listed in Table 1 along with the sequence recognized and the approximate number of cleavage sites in safflower ct-DNA. Restriction fragments obtained from PstI, SalI, and KpnI are comparatively large and few in number, while those from HindIII (and BamHI and EcoRI, not shown) are smaller and more numerous. Single and double digestion products using combinations of these enzymes were separated by agarose gel electrophoresis in buffers containing ethidium bromide and photographed over short UV light (Fig. 1). Restriction fragment sizes were estimated, using HindIII digested lambda DNA as molecular size markers and compiled in Table 2. The

**Table 1.** Number of cleavage sites recognized by various restriction endonucleases of DNA from safflower chloroplasts

| Enzyme | Sequence recognized[a] | No. of cleavage sites[b] |
|---|---|---|
| PstI | CTGCA↓G | 15 |
| SalI | G↓TCGAC | 9 |
| KpnI | GGTAA↓C | 14 |
| HindIII | A↓AGCCT | 30 |

[a] Recognition sequences are 5′ to 3′ (Roberts 1978)
[b] Based on a limiting resolution of about 1 kbp

**Table 2.** Summary of safflower chloroplast DNA fragments (kbp) produced by single and double digestion with various restriction endonucleases. Numbers in brackets refer to multiple copies

| Band no. | PstI | PstI/ SalI | SalI | Kpn/ SalI | KpnI | PstI/ KpnI | PstI | PstI/ HindIII | HindIII | KpnI/ HindIII | KpnI |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 24 | 24 | 46 | 24 | 44 | 18.5 | 24 | 14 | 14 | 14 | 44 |
| 2 | 18.5 (3×) | 18.5 | 24 | 15 | 27 | 15 (2×) | 18.5 (3×) | 8.7 | 1 (3×) | 11 (2×) | 27 |
| 3 | 14.2 | 12.0 (2×) | 16.5 (2×) | 14.2 | 15 (2×) | 12 | 14.2 | 8.0 (2×) | 9.2 (3×) | 9.2 (2×) | 15 (2×) |
| 4 | 12 | 11.5 (2×) | 14.2 (2×) | 10 | 10.0 (2×) | 10 (2×) | 12 | 7.7 (2×) | 7.4 | 8.2 | 10 (2×) |
| 5 | 10 | 10 | 11.5 | 9.7 (3×) | 6.8 | 7.7 (2×) | 10 | 6.8 | 6.8 | 6.8 (2×) | 6.8 |
| 6 | 9.2 | 9.2 | 7.1 | 8.1 | 6.3 | 6.8 | 9.2 | 6.5 | 6.5 (2×) | 6.5 | 6.3 |
| 7 | 6.0 | 7.1 | 1.8 | 7.1 | 4.7 (2×) | 4.7 | 6.0 | 5.7 | 6.3 | 6.2 | 4.7 (2×) |
| 8 | 5.0 | 6.4 | | 6.8 | 3.5 | 4.5 (3×) | 5.0 | 5.1 | 4.8 | 6.0 | 3.5 |
| 9 | 4.5 (3×) | 6.0 | | 6.3 | 3.0 | 3.5 (3×) | 4.5 (3×) | 4.1 (3×) | 4.3 | 4.8 (2×) | 3.0 |
| 10 | 1.5 | 5.0 | | 5.6 | 0.6 (2×) | 3.3 (2×) | 1.5 | 3.9 (2×) | 4.1 (2×) | 4.5 | 0.6 (2×) |
| 11 | 1.0 | 3.5 | | 4.7 | | 3.0 | 1.0 | 3.5 | 3.7 | 4.1 (2 or 3×) | |
| 12 | | 2.4 (2×) | | 3.5 | | 2.6 | | 3.4 (2×) | 3.3 | 3.8 | |
| 13 | | 1.8 (2×) | | 3.2 | | 1.6 | | 3.3 (2×) | 2.7 | 3.5 | |
| 14 | | 1.6 | | 3.0 | | 1.5 | | 2.9 (2×) | 2.5 | 3.3 | |
| 15 | | 1.1 | | 1.9 (2×) | | 1.3 (2×) | | 2.7 | 2.2 (2×) | 2.7 | |
| 16 | | 1.0 | | 1.85 | | 1.0 | | 2.5 (2×) | 1.6 | 2.3 (2×) | |
| 17 | | 0.8 | | 1.8 | | 0.6 (2×) | | 2.2 (2×) | 1.1 (4×) | 2.2 (2×) | |
| 18 | | | | 1.7 | | | | 1.5 (2×) | 1.0 | 1.9 | |
| 19 | | | | 1.4 | | | | 1.45 | 0.9 | 1.8 | |
| 20 | | | | 0.6 (2×) | | | | 1.4 (2×) | | 1.6 | |
| 21 | | | | | | | | 1.1 (4×) | | 1.5 | |
| 22 | | | | | | | | 1.0 | | 1.0 (2×) | |
| 23 | | | | | | | | 0.9 | | 0.9 | |
| 24 | | | | | | | | | | | |
| Sum | 152 | 150 | 152 | 152 | 151 | 152 | 152 | 147 | 151 | 149 | 151 |

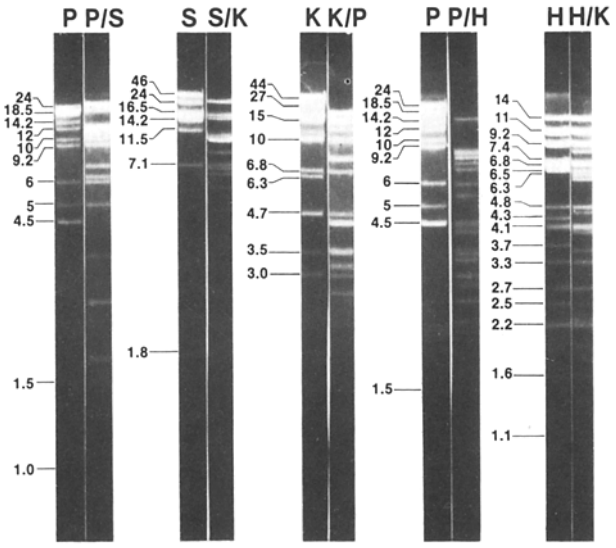


**Fig. 1.** Agarose gel electrophoresis fractionation of restriction endonuclease digests of safflower chloroplast DNA. DNA samples were digested with PstI, SalI, KpnI, HindIII and combinations of these enzymes. Sizes of fragments obtained upon digestion with single enzymes are given in kbp as determined by comparison with HindIII digests of lambda DNA

molecular size of intact ct-DNA was estimated by summing fragment sizes in both single and double digestions, and found to be about 151 kbp. Although Pst, Sal and Kpn gave relatively few fragments each restriction profile contained ethidium bromide stained bands with two or more DNA fragments of approximately the same size. For example PstI 18.5 (×3) and 4.5 (×3) kbp; SalI 16.5 (×2) and 14.2 (×2) kbp and KpnI 15 (×2), 10 (×2) and 4.7 (×2) kbp. In most cases these proved not to be identical sequences and complicated interpretation of physical mapping data, especially in light of the expected inverted repeat.

*Mapping strategy*

The serial order or physical mapping of the ct-DNA restriction fragments was determined largely by cutting individual ethidium bromide stained bands from agarose gels, labeling by nick translation, and hybridizing with Southern blots of single and double digested ct-DNA separated by agarose gel electrophoresis. Gel electrophoresis of radiolabeled restriction fragments digested with a second endonuclease also proved useful

in mapping smaller fragments. Cross contamination of isolated fragments with neighboring fragments of similar size was a source of irritation, especially while attempting to distinguish between cross contamination and regions of short overlapping sequences. However when Southern blots were compared in order of probe fragment size, true hybridization signals generally stood out and were recognizable above the background of artifactual bands.

*Construction of the physical map*

Autoradiograms obtained from SalI probes were aligned with photographs of corresponding ethidium bromide stained gels. This facilitated the identification in most cases of restriction fragments having regions of base sequence homology with $^{32}$P-SalI probes (Fig. 2). A summary of this data is shown in Table 3, where fragments hybridizing to specific probes are identified by their characteristic sizes in kilobase pairs (kbp). Autoradiograms with double digestions were especially valuable and contributed greatly in mapping fragments and in identifying regions of overlap.

The striking feature of Sal 46 kbp hybridizations with Southern blots of single and double digested ct-DNA is the large number of radioactive bands that exist as multiple copies, Table 3. Many of these are duplicates and come from repeated regions of the genome. For example, Pst 4.5 (2×) is not further split by Kpn (see Pst/Kpn 4.5 (2×)) but disappears upon further digestion with Sal or Hind with the generation of two double bands Pst/Sal 1.8 (2×) and Pst/Hind 1.4 (2×), respectively; and Hind 11 (2×) and 9.2 (2×) yield Pst/Hind 2.9 (2×), and Pst/Hind 7.7 (2×) and 1.4 (2×), respectively. Other examples of double bands are Hind and Pst/Hind 2.2 (2×) and 1.1 (2×). Matching of single and double digestion fragments which hybridize with Sal 46 leads to the restriction map shown in Fig. 3. All of these fragments were mapped directly except Hind and Hind/Pst fragments 2.2 (2×) and 1.1 (2×), and Kpn and Kpn/Pst 0.6 (2×), which were mapped from rRNA hybridization data (see below). Taken together the SalI hybridization mapping data provides convincing evidence of a typical inverted repeat in safflower chloroplast DNA, with a small single copy region of about 20 kbp (i.e., HindIII 4.1–9.2–6.5) (Fig. 3).

This is consistent with hybridization maps of Sal 24 and 14.2 kbp. Both of these probes hybridize to Pst 4.5 (2×), Pst/Sal 2.4 (2×), Kpn 44 and 27, Pst/Kpn 4.5 (2×), Hind 11 (2×) and Pst/Hind 8.0 (2×) and 2.9 (2×) (Table 3). Except for the two large Kpn fragments (44, 27) all of these are duplicates as shown and therefore very likely map within the inverted repeat. Inasmuch as Pst 4.5 was shown to map on each end of the Sal 46 and also to hybridize to Sal probes 24 and 14.2, the latter probes most likely map on each end of Sal 46 and contain the remaining portions of the inverted repeats. If this is correct, the repeated portions in Sal 24 and 14.2 must be less than 14.2 kbp and Sal 24 must extend into the large single copy region. Likewise Kpn 44 and 27 must also overlap the inverted repeat and large single copy regions. Since Sal 24 is not further digested by Kpn (see Table 2) it must map entirely within Kpn 44 or 27, other fragments being too small. Only the larger of these Kpn fragments can accomodate this Sal fragment and approximately 9.5 kbp (Pst/Kpn 7.7 + Pst/Sal 1.8) that overlap Sal 46. Therefore Sal 24 maps with Kpn 44 and Sal 14.2 with Kpn 27. This taken in consideration with other fragments hybridizing to Sal 24 leads to restriction maps shown in Fig. 4. Since Sal 14.2 is a double band (but not a duplicate), identification of restriction fragments which hybridize to one of these bands, which is part of the inverted repeat, will be most helpful in identifying those associated with the other.

That Sal 16.5 is linked to Sal 24 is suggested by the hybridization of both probes to Kpn 44, Pst 18.5 and Hind 14, all of which were tentatively mapped as described above (Fig. 4). However, there are two 16.5 kbp SalI fragments which together constitute nearly 25% of the chloroplast genome, and mapping of all the restriction fragments which hybridize to these probes is rather complex. Comparison of Pst and Pst/Sal fragments which hybridize suggests the Pst/Sal sequences 9.2–6.4 and 12–1.0–3.5, both of which total about 16 kbp, where neither Pst 9.2 or 1.0 kbp fragments contain Sal restriction sites. The unexpected finding that these Sal 16.5 fragments are linked is suggested by the observed hybridization of Kpn 10 (2×) kbp probes with only Sal 46 and 16.5, Table 4. Since one of these 10 kbp fragments does not contain any Sal sites and maps entirely within Sal 46 (Fig. 3), the remaining fragment must map with one or both Sal 16.5 fragments. Inasmuch as ethidium bromide stained agarose electrophoresis gels suggest a Sal site in one Kpn 10 kbp fragment (Table 2), it is assumed that this fragment must overlap both Sal 16.5 kbp fragments. This is consistent with single and double digested restriction fragments found to hybridize to these probes and leads directly to the map shown in Fig. 5. HindIII restriction site mapping was difficult because of the number of small fragments of similar size, however with information obtained from Hind digests of Pst fragments, Table 5, the mapping of Hind fragments was possible in this and other difficult regions.

The involvement of one of the Sal 14.2 fragments in the inverted repeat has already been shown, because of similarities with Sal 24 hybridizing fragments (Fig. 4). If one considers only those restriction fragments which

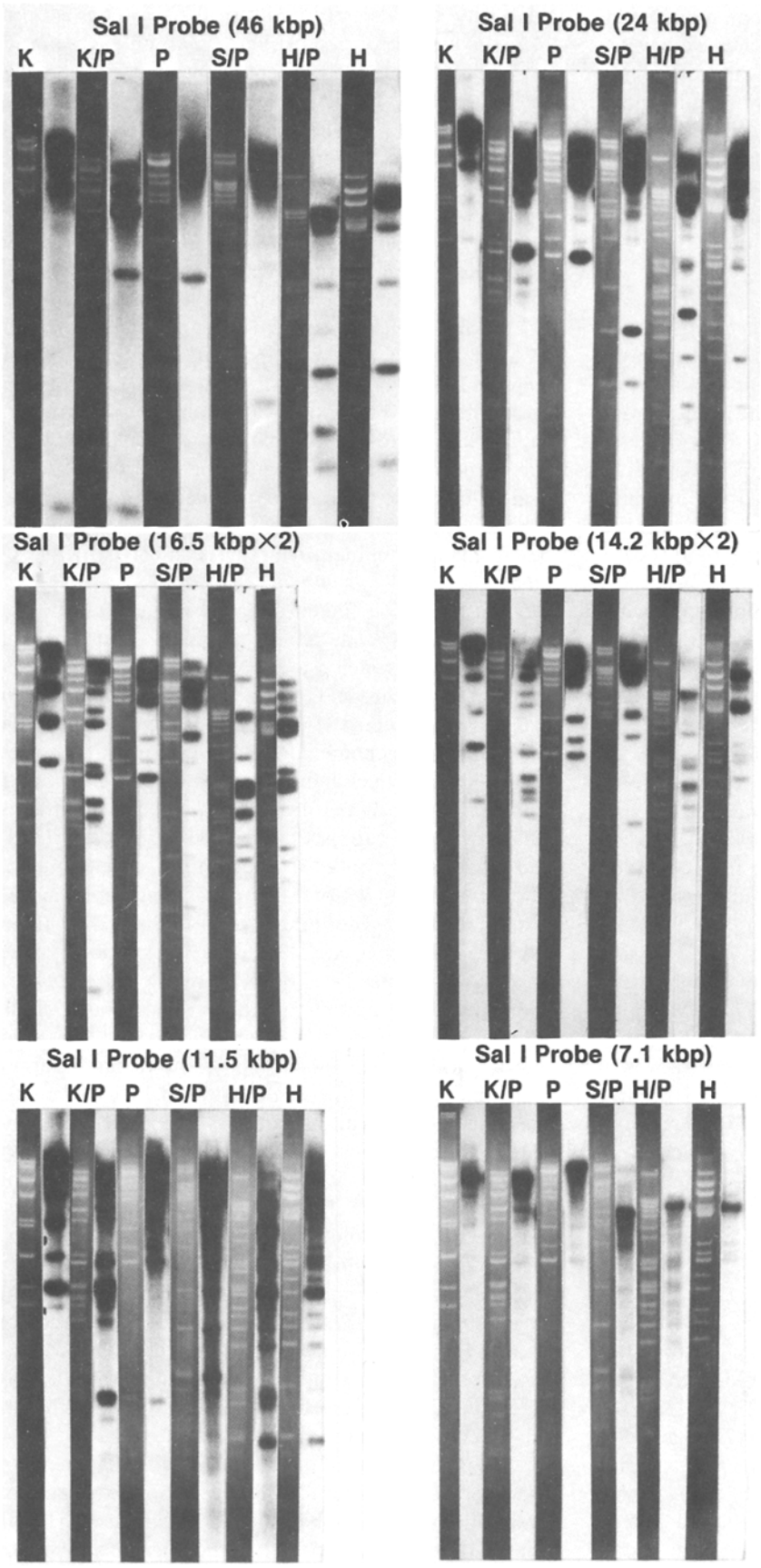


**Fig. 2.** Hybridization of safflower ct-DNA SalI probes to Southern blots of fragments generated by other restriction endonucleases. KpnI, KpnI/PstI, PstI, SalI/PstI, HindIII/PstI, and HindIII digests of safflower ct-DNA were separated on 0.8% agarose gels and transferred to nitrocellulose or GeneScreenPlus filters. On the *left* are the respective ethidium bromide stained gel patterns, and on the *right* the corresponding autoradiographs obtained with each $^{32}$P-dCDP-SalI probe

**Table 3.** Summary[a] of hybridizations of filter-bound safflower chloroplast DNA restriction fragments to SalI probes (see autoradiograms, Fig. 2). Numbers in brackets refer to multiple copies

| Probe | SalI | 46 kpb | |
|---|---|---|---|
| | PstI | 24, 18.5, 4.5 (2×) | |
| | PstI/SalI | 24, 18.5, 1.8 (2×) | |
| | KpnI | 44, 27, 15, 10, 0.6 (2×) | |
| | PstI/KpnI | 15, 10, 7.7 (2×), 4.5 (2×), 0.6 (2×) | |
| | PstI/HindIII | 11, 7.7 (2×), 6.5, 4.1, 2.9 (2×), 2.2 (2×), 1.4 (2×), 1.0 (2×) | |
| | HindIII | 11 (2×), 9.2 (3×), 6.5, 4.1, 2.2 (2×), 1.1 (2×) | |
| Probe | SalI | 24 kbp | |
| | PstI | 18.5, 12, 10, 4.5 (2×) | |
| | PstI/SalI | 12, 11.5, 10, 2.4 (2×) | |
| | KpnI | 44, 27 | |
| | PstI/KpnI | 18.5, 12, 10, 4.5 (2×) | |
| | PstI/HindIII | 14, 8.0 (2×), 2.9 (2×), 1.5 | |
| | HindIII | 14, 11 (3×), 1.6 | |
| Probe | SalI | 16.5 kbp | |
| | PstI | 18.5, 14.2, 9.2, 4.5, 1.0 | |
| | PstI/SalI | 12, 9.2, 6.4, 3.5, 1.0 | |
| | KpnI | 44, 10, 6.8, 4.7 (2×) | |
| | PstI/KpnI | 18.5, 6.8, 4.7, 4.5, 3.5, 3.3 (2×), 1.3, 1.0 | |
| | PstI/HindIII | 14, 5.1, 4.1 (2×), 3.9 (2×), 3.5, 2.7, 2.5, 1.1 | |
| | HindIII | 14, 7.4, 6.5, 4.8, 4.3, 4.1, 2.7, 2.5, 1.1 | |
| Probe | SalI | 14.2 kbp | |
| | PstI | 18.5, 14.2, 12, 10, 6.0, 5.0, 4.5 (2×) | |
| | PstI/SalI | 12, 10, 6.0, 5.0, 2.4 (2×), 1.6 | |
| | KpnI | 44, 27, 15.0, 6.3, 4.7, 3.0 | |
| | PstI/KpnI | 12, 10, 4.5 (2×), 3.5, 3.3, 3.0, 2.6, 1.3 | |
| | PstI/HindIII | 8.0 (2×), 5.1, 3.4, 3.3, 2.9 (2×), 2.5, 1.5, 1.0 | |
| | HindIII | 11 (3×), 6.5, 6.3, 3.7 | |
| Probe | SalI | 11.5 kbp | 7.1 kbp |
| | PstI | 18.5, 1.5 | 18.5 |
| | PstI/SalI | 11.5 | 7.1 |
| | KpnI | 27, 15, 3.5 | 15 |
| | PstI/KpnI | 15, 3.5, 1.6, 1.5 | 15 |
| | PstI/HindIII | 5.7, 3.3, 1.1 | 6.8 |
| | HindIII | 11, 3.3, 1.1 | 6.8 |

[a] Hybridizing fragments arising from cross contamination have been omitted to avoid confusion

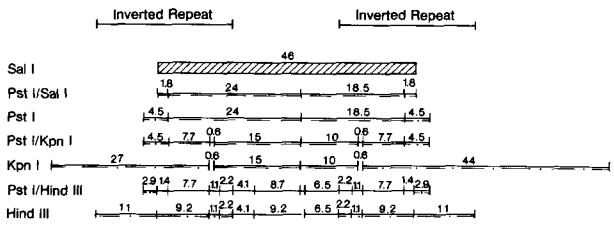


**Fig. 3.** Restriction map of the portion of safflower ct-DNA which hybridizes with SalI 46 kbp probe. Shaded horizontal bars represent $^{32}$P-dCTP-SalI probes, and numbers represent sizes in kbp. Lines under each shaded bar represent PstI/SalI, PstI, PstI/KpnI, KpnI, PstI/HindIII and HindIII fragments hybridizing to each SalI probe as summarized in Table 3. Double lines ═══ represent fragments which hybridize with two or more probes. A solid line and a dash-dot line ═·─·═ represent fragments which hybridize two or more probes and in addition are part of the inverted repeat. A solid line and dashed lines ═─═ represent fragments which hybridize to only one probe but are also part of inverted repeat. Dotted lines · · · · represent fragments which hybridize to specific probes but required additional data for mapping

**Table 4.** Summary[a] of hybridizations of filter-bound safflower chloroplast DNA restriction fragments to KpnI probes (autoradiograms not shown). Numbers in brackets refer to multiple copies

| Probe | KpnI | 44 kbp | | |
|---|---|---|---|---|
| | PstI | 24, 18.5 (2×), 12, 10, 9.2, 4.5 (2×) | | |
| | PstI/KpnI | 18.5, 12, 10, 7.7 (2×), 4.5 (2×), 3.3 | | |
| | PstI/SalI | 24, 18.5, 12, 11.5, 10, 9.2, 6.4, 2.4 (2×), 1.8 (2×) | | |
| | SalI | 46, 24, 16.5, 14.2 | | |
| | PstI/HindIII | 14, 8.0 (2×), 7.7 (2×), 3.9, 3.5, 2.9 (2×), 1.5, 1.4 (2×) | | |
| | HindIII | 14, 11 (2×), 9.2 (2×), 7.4, 1.6 | | |
| Probe | KpnI | 27 kbp | | |
| | PstI | 24, 18.5 (2×), 12, 10, 4.5 (2×), 1.6 | | |
| | PstI/KpnI | 12, 10, 7.7 (2×), 4.5 (2×), 1.6, 1.5 | | |
| | PstI/SalI | 24, 18.5, 12, 10, 2.4 (2×), 1.8 (2×), 1.1 | | |
| | SalI | 46, 24, 14.2, 11.5, 1.8 | | |
| | PstI/HindIII | 8.0 (2×), 7.7 (2×), 3.4, 2.9 (2×) | | |
| | Hind | 11 (3×), 9.2 (2×) | | |
| Probe | KpnI | 15 kbp (2×) | | |
| | PstI | 24, 18.5 (2×) | | |
| | PstI/KpnI | 15 (2×), 10 | | |
| | KpnI/SalI | 15, 10, 7.1, 5.6 | | |
| | SalI | 46, 14.2, 11.5, 7.1 | | |
| | KpnI/HindIII | 8.2, 6.8, 6.5, 4.1, 3.3, 2.2 (2×), 1.1 | | |
| | HindIII | 11, 9.2, 6.8, 6.5, 3.7, 3.3, 2.2 (2×), 1.1 | | |
| Probe | KpnI | 10 kbp (2×) | | |
| | PstI | 24, 18.5, 14.2, 9.2, 4.5, 1.0 | | |
| | PstI/KpnI | 15, 10, 4.5, 3.5 | | |
| | KpnI/SalI | 15, 10, 8.1, 1.9 | | |
| | SalI | 46, 16.5 (2×) | | |
| | Kpn/HindIII | 6.5, 4.8, 3.8, 2.2 (2×), 1.7 (2×) | | |
| | HindIII | 6.5, 4.8, 4.3, 2.2 (2×), 1.7 (2×) | | |
| Probe | KpnI | 6.8 kbp | 6.3 kbp | 4.7 kbp (2×) |
| | PstI | 14.2 | 6, 5 | 14.2, 9.2, 5 |
| | PstI/KpnI | 6.8 | 3.5, 2.6 | 4.7, 3.3, 1.3 |
| | KpnI/SalI | 6.8 | 6.3 | 4.7, 3.0, 1.5 |
| | SalI | 16.5 | 14.2 | 16.5 (2×), 14.2 |
| | KpnI/HindIII | 4.1 | 6.0 | 4.7, 2.7, 1.8 |
| | HindIII | 6.5, 4.1, | 6.3 | 6.5, 2.5, 2.7 |

[a] Hybridizing fragments arising from cross contamination have been omitted to avoid confusion

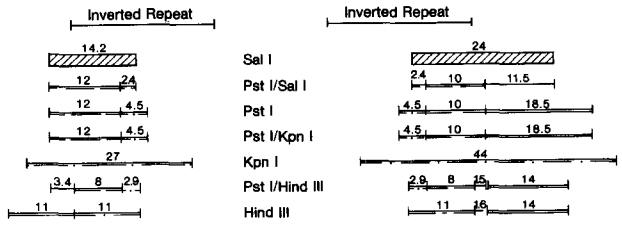


**Fig. 4.** Restriction map of the portion of safflower ct-DNA which hybridizes with SalI 24 kbp probe, and which includes part of inverted repeat which maps with one of the Sal 14.2 kbp probes. Symbols are as described in Fig. 3

are not implicated in this inverted repeat, but hybridize to Sal 14.2, the Pst sequence 14.2–(5, 6)–18.5 and the Kpn sequence 4.7–6.3–3.0 become apparent. Note that the first fragment of each of these sequences has already been mapped with Sal 16.5 (Fig. 5). Therefore Sal 14.2 and 16.5 are linked. Matching of restriction fragments which hybridize to Sal 14.2 (Table 3) and Kpn probes 15, 6.3, and 4.7 kbp (Table 4) leads to the map of this region of the chloroplast genome as shown in Fig. 6. The observation that Sal probes 11.5 and 7.1, in addition to the above mentioned Sal 14.2 probe, also

**Table 5.** Summary of restrictions of PstI ct-DNA fragments with SalI, KpnI and HindIII (autoradiograms not shown)

| PstI fragment digested | Products obtained upon second digestion | | |
|---|---|---|---|
| | SalI | KpnI | HindIII |
| 9.2 kbp | 9.2 | 4.7, 3.3, 1.3 | 3.5, 2.7, 2.5 |
| 10 kpb | 10 | 10 | 8.0, 1.5 |
| 12 kbp | 12 | 12 | 8.0, 3.4 |
| 14.2 kbp | 12, 1.6 | 6.8, 3.5, 3.3 | 5.1, 4.1, 3.9 |
| 18.5 kbp (3×)[a] | 11.5 (2×), 7.1, 6.4, 3.5, 1.6 | 18.5, 15, 10, 7.7, | 14, 7.7, 6.8, 5.7, 3.9, 3.3, 2.2 |
| 24 kbp[a] | 24 | 15, 7.7 | 8.7, 7.7, 4.1, 2.2 |

[a] PstI 24 and 18.5 kbp were not well resolved and therefore had some cross contamination. The major hybridizations regions were, however, as shown

**Table 6.** Summary of hybridizations of filter-bound safflower chloroplast DNA restriction fragments to HindIII probes (autoradiograms not shown)

| Probe | HindIII 14 kbp | HindIII 11 kbp (3×) | HindIII 9.2 kbp (2×) |
|---|---|---|---|
| KpnI | 44 | 44, 27, 3.5 | 44, 27, 15 |
| KpnI/PstI | 18.5 | 12, 10, 4.5 (2×), 3.5 | 15, 7.7 (2×), 4.5 (2×) |
| SalI | 24 | 24, 14.2, 11.5 | 46 |
| SalI/PstI | 11.5, 6.4 | 12, 11.5, 10, 1.3 (2×), 1.1 | 24, 18.5, 1.8 (2×) |
| PstI | 18.5, | 18.5, 12, 10, 4.5, (2×) | 24, 18.5, 4.5 (2×) |
| HindIII/PstI | 14 | 8.0 (2×), 5.7, 3.3, 2.9 (2×) | 8.7, 7.7 (2×) |

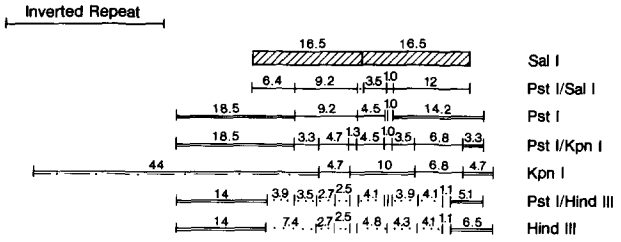


**Fig. 5.** Restriction map of the portion of safflower ct-DNA which hybridizes with Sal 16.5 kbp probes. Symbols are as described in Fig. 3

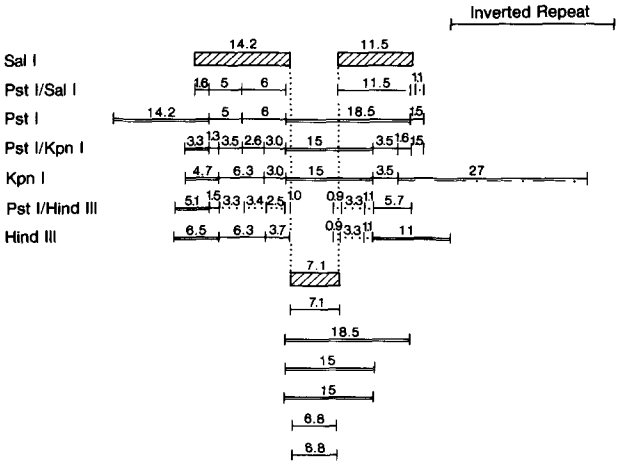


**Fig. 6.** Restriction map of the portion of safflower ct-DNA which hybridizes with Sal 14.2, 7.1, and 11.5 kbp probes. Symbols are as described in Fig. 3

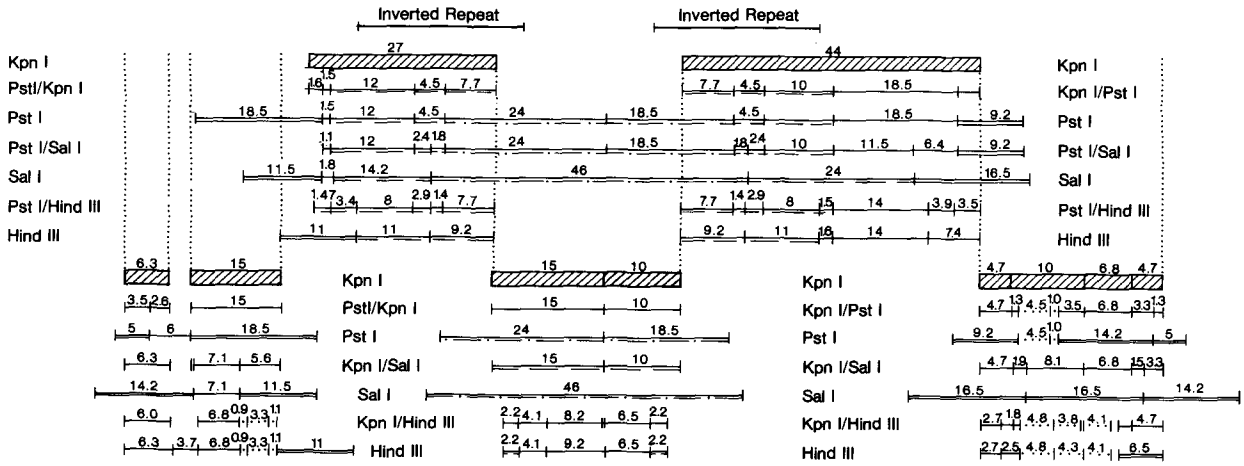


**Fig. 7.** Restriction map of safflower ct-DNA KpnI fragments to other restriction endonuclease fragments. Shaded horizontal bars represent each $^{32}$P-dCTP-KpnI probe and numbers represent sizes in kbp. Lines under each bar represent PstI/KpnI, PstI, PstI/SalI or KpnI/SalI, SalI, PstI/HindIII or KpnI/HindIII and HindIII fragments hybridizing to each probe as summarized in Table 4. Symbols are otherwise as stated in Fig. 3

**Table 7.** Summary of hybridizations of filter-bound safflower chloroplast DNA restriction fragments to ct-rRNA probes (autoradiograms not shown)

| Probe | Band I | Band II | Band III |
|---|---|---|---|
| KpnI | 0.6 | 0.6 | 15, 10, 0.6 |
| PstI | 24, 18.5 | 24, 18.5 | 24, 18.5 |
| SalI | 46 | 46 | 46 |
| PstI/HindIII | 7.7, 2.2 < 1.1[a] | 7.7, 1.1 | 7.7, 2.2 > 1.1[a] |
| SalI/HindIII | 9.2, 2.2 < 1.1[a] | 9.2, 1.1 | 9.2, 2.2 > 1.1[a] |

[a] Refers to relative hybridization intensities of 2.2 and 1.1 kbp fragments

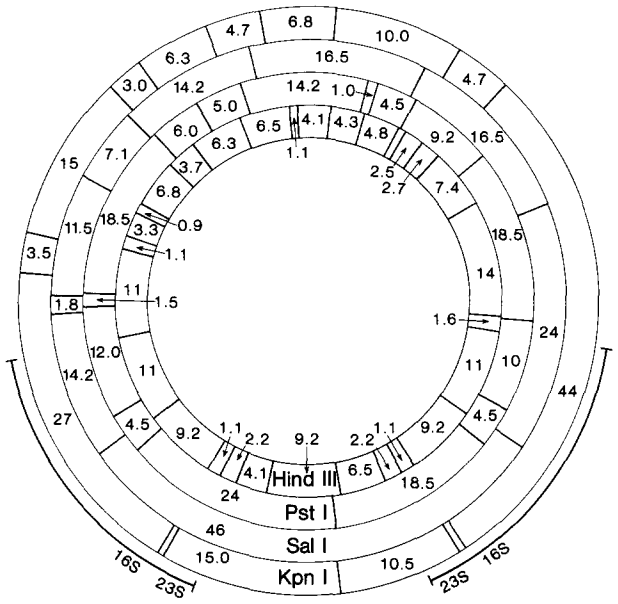


**Fig. 8.** Restriction endonuclease map of safflower chloroplast DNA. Fragment sizes of KpnI, SalI, PstI and HindIII are in kbp. The upper part of the figure represents the large single copy region. The inverted repeat regions and the structural genes for rRNA are as indicated on the periphery

hybridize to Pst 18.5 and Kpn 15 suggests that these three probes map together. Since Sal 7.1 only hybridizes to Kpn 15, while Sal probes 14.2 and 11.5 hybridize to numerous Kpn restriction fragments, Sal 7.1 maps between Sal 14.2 and 11.5. This leads to the mapping of the remainder of fragments associated with these probes, also shown in Fig. 6. It also provides evidence for the circular nature of the safflower chloroplast genome, because Kpn 27 not only maps with Sal 11.5 but is also part of the inverted repeat and is already partially mapped with Sal probes 14.2 and 46 as shown in Figs. 4 and 3, respectively.

Confirming results, of the restriction maps shown in Figs. 3–6, were obtained from hybridizations carried out with probes isolated from KpnI digests as summarized in Table 4 and mapped in Fig. 7. In restriction maps generated by SalI and KpnI probes (Figs. 3–7); Sal, Kpn and Hind digests of selected Pst fragments (Table 5), and Southern blot hybridizations to selected HindIII probes (Table 6), were very useful in selecting among mapping alternatives. This was particularly true of certain small Hind fragments which map closely together as already mentioned above.

A summary of the KpnI, SalI, PstI and HindIII restriction sites of the circular safflower chloroplast genome is shown in Fig. 8. Also shown are the binding sites of rRNAs. Such RNAs were prepared by the method of Taylor and Powell (1982). Three ethidium bromide staining bands were obtained just as they observed with *Nicotiana glauca* callus culture. The hybridization of these rRNA bands to single and double restriction digests of ct-DNA is summarized in Table 7. Restriction fragments near the small single copy region of the inverted repeats were involved in the hybridization. On the basis of relative hybridization intensities of the Pst/Hind or Sal/Hind (or Hind) fragments 2.2 and 1.1, it would appear that the three rRNA bands map in sequence III–I–II. This is consistent with Leaver and Ingle (1971) who found that the large rRNA subunit ($1.1 \times 10^6$) of radish cotyledons breaks into components with molecular weights $0.7 \times 10^6$ and $0.4 \times 10^6$, whereas the small rRNA subunit ($0.56 \times 10^6$) is stable and has intermediate electrophoretic mobility; and with results of several other workers who have found that the large rRNA subunit in many higher plants maps on the small single copy side of the small rRNA subunit (Bohnert et al. 1982).

From these data we conclude that safflower chloroplasts have a circular genome of approximately 151 kbp with a single inverted repeat of approximately 25 kbp containing the structural genes of 16 S and 24 S rRNAs, and small and large single copy regions of approximately 20 and 81 kbp, respectively.

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