PLASTID DNA REPLICATION AND PLASTID DIVISION IN THE GARDEN PEA

John BENNETT and Catherine RADCLIFFE
Department of Biological Sciences, University of Warnick, Coventry CV4 7AL, UK

Received 6 June 1975

1. Introduction

In serveral species of alga and higher plant, chloroplast DNA (cDNA) exists as a circular duplex about 40 μm in contour length [1–3]. The mass of a circle is approx. 10^8 daltons which is also the mass of the unique base sequences of cDNA as estimated from the rate of renaturation [1,2]. These results suggest that one circle constitutes the entire chloroplast genome. As the mass of DNA contained within a chloroplast is generally 20–60 times greater than the mass of a circle [1,4], each organelle may contain many copies of the genome. Independent evidence for the existence of many DNA molecules per organelle comes from electron microscope studies on the distribution of DNA within the chloroplast [5].

In the physical properties of its cDNA, the garden pea (*Pisum sativum*) is typical of the species so far examined [2]. The DNA has a buoyant density in neutral CsCl gradients of 1.698 g cm^{-3}, it has the renaturation kinetics of a 41 ± 2 μm genome, and under the electron microscope it has the form of a circle of contour length 39 ± 2 μm. However, as the mass of DNA per pea chloroplast has not been determined, the number of circles per organelle is unkown for this species; nor is it known whether this number changes during the light-induced development of chloroplasts from etioplasts.

We now report the DNA content of plastids from pea buds. In the 7–10 day-old seedlings examined in this study, developing chloroplasts were about 2 μm in diameter and contained approx. 14 copies of the circular genome. In contrast, etioplasts were about 1 μm in diameter and could contain in excess of 50 DNA molecules. We suggest that the DNA content of a plastid is determined by the relative rates of plastid DNA replication and plastid division and that the main effect of light in this context is to stimulate the rate of plastid division.

2. Materials and methods

Pea seedlings (*Pisum sativum* L. var. Feltham First) were grown in the dark or in the light as described previously [6]. Plastids were prepared from bud tissue (15 g) as described [6], except that two bursts of homogenization (2 sec followed by 6 sec) were used. These and all subsequent operations were performed at 0–4°C. The pellet of crude plastids was carefully resuspended in 1 ml of mannitol buffer (330 mM mannitol, 50 mM 2[*N*-morpholino] ethane sulphonic acid, 2.5 mM magnesium acetate, pH 7.0) and incubated with deoxyribonuclease I (Worthington, 100 μg, 20 min) and then snake venom phosphodiesterase (Boehringer, 30 μg, 10 min) to digest contaminating nuclear DNA [3]. Digested DNA fragments were removed by diluting the plastids with 9 ml of 'sucrose isolation medium' [6] and spinning out the plastids at 2500 *g* for 1 min. The pellet was gently resuspended in 2 ml of 'KCl resuspension medium' [6] to give the purified plastid preparation (40–60% refractile under phase contrast).

An aliquot (1 ml) of the preparation was used for the extraction of total plastid nucleic acids [7]. DNA was separated from RNA by electrophoresis in the cold through 2.4% polyacrylamide gels [8] and absorbance profiles were recorded at 260 nm with a Gilford gel scanner. Chloroplast DNA purified by the procedure of Wong and Wildmann [9] was used as electrophoretic mobility marker and absorbance standard in the range 0–4 μg DNA per gel. In this range Beer's law was obeyed.

North-Holland Publishing Company – Amsterdam

The numbers of refractile and non-refractile plastids in the preparations were determined using a hemacytometer on a phase contrast microscope.

Chlorophyll [10] and carotenoid [11] assays were performed on the plastid preparations and on exhaustive acetone : water (4 : 1, v/v) extracts of buds. Chlorophyll was measured in the illuminated tissue; carotenoid was measured in etiolated tissue.

Purified plastid preparations were also examined for contamination by non-plastid DNA. Total nucleic acids were extracted as described above and RNA was removed by digestion at 37°C with pancreatic ribonuclease followed by pronase [12]. After phenol extraction and ethanol precipitation, the DNA was resuspended in standard saline citrate (SSC) and either examined immediately by analytical ultracentrifugation (Beckman Model E) or after a cycle of denaturation (100°C for 10 min) and renaturation (60°C for 240 min) [2]. DNA of *Micrococcus luteus* was used as density marker. The buoyant densities of native, denatured and renatured cDNA were calculated by the standard procedure [13] using densitometer tracings obtained from UV-sensitive film.

Each result in tables 1, 2 and 3 represents the mean and standard deviation of duplicate experiments.

Table 1
DNA content of pea plastids

Plastid	Age of Plant (days)	DNA content per plastid ($g \times 10^{15}$)	(genome copies)
Etioplasts	7	3.4 ± 0.9	25 ± 6
	10	8.8 ± 0.9	54 ± 6
Chloroplasts	7	2.0 ± 0.3	14 ± 2
	10	2.1 ± 0.8	14 ± 5

3. Results

Fractionation of the nucleic acids extracted from purified plastids was achieved by gel electrophoresis (fig.1A). DNA was the most slowly migrating species on the gel. The RNA species included transfer RNA, the 1.05×10^6 dalton and the 0.56×10^6 dalton ribosomal RNA species and a cold-induced aggregate which closely preceded DNA. When purified plastids were lysed by exposure to 1% (w/v) Triton X-100 and then subjected to a second round of digestion with DNAase I and snake venom phosphodiesterase,

Table 2
Number of plastids in pea buds

Bud (pigment)	Age of plant (days)	Pigment per plastid ($g \times 10^{15}$)	Pigment per bud ($g \times 10^6$)	Plastids per bud ($\times 10^9$)	Number of doublings (in three days)
Etiolated (carotenoid)	7	4.4 ± 0.7	1.6 ± 0.1	0.4 ± 0.1	0.5 to 1
	10	4.8 ± 0.2	3.2 ± 0.2	0.7 ± 0.1	
Illuminated (chlorophyll)	7	22 ± 2	22 ± 1	1.0 ± 0.2	2 to 2.5
	10	43 ± 4	190 ± 10	4.5 ± 0.9	

Table 3
Chloroplast DNA content of pea buds

Buds	Age of plant (days)	cDNA per bud ($g \times 10^6$)	Average rate of cDNA accumulation (g per bud per day, $\times 10^6$)
Etiolated	7	1.2 ± 0.2	1.6 ± 0.1
	10	6.0 ± 0.2	
Illuminated	7	2.1 ± 0.3	2.4 ± 0.4
	10	9.4 ± 0.9	

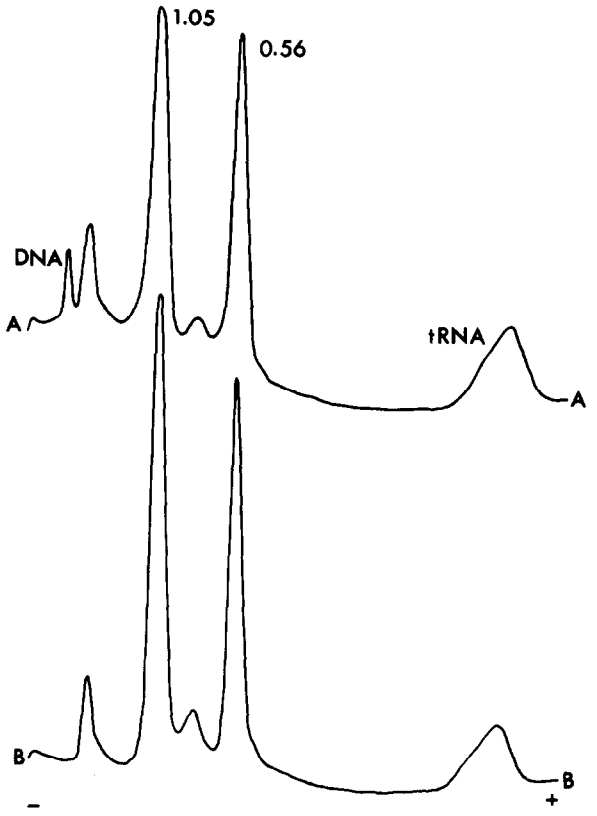

Fig.1. Gel-electrophoretic fractionation of total chloroplast nucleic acids. (A) Nucleic acids extracted from purified, enveloped chloroplasts. (B) Nucleic acids extracted from purified chloroplasts subjected to lysis with Triton X-100 and digestion with DNAase I and snake venom phosphodiesterase. The peaks are identified in the text. Conditions of electrophoresis: 50 V, 3 mA per gel, run for 4 hr at 4°C.

the DNA peak was abolished (fig.1B), indicating that only DNA protected by a membrane (presumably the plastid envelope) was detected in the assay procedure used in the present study.

Further indication of the chloroplast origin of the DNA is provided in fig.2. This shows the buoyant density of native, denatured and renatured cDNA in CsCl gradients. The buoyant density of the native DNA (1.698 g cm^{-3}) distinguishes it from mitochondrial DNA (1.706 g cm^{-3}, [2]), while the ability of the denatured DNA to renature rapidly and completely after heat denaturation distinguishes it from slowly and incompletely renaturing nuclear DNA [1,2].

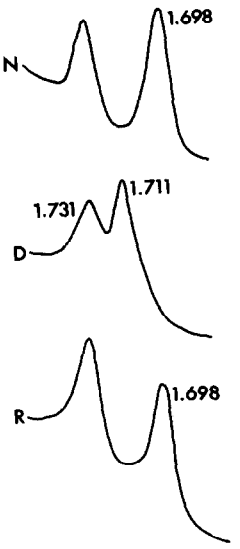

Fig.2. Banding of pea chloroplast DNA in CsCl density gradients. The densitometer traces show the positions in the gradients of native cDNA (N), denatured cDNA (D), renatured cDNA (R) and *M. luteus* marker DNA (ρ = 1.731 g cm^{-3}). Conditions of centrifugation: 44 000 rev/min, 20 hr, 25°C.

The DNA content of etioplasts and chloroplasts is presented in table 1. The results were calculated from two experimentally determined parameters: (i) the mass of DNA per gel, and (ii) the number of refractile plastids per ml of purified plastid preparation. Each gel was loaded with the nucleic acid extracted from 0.5 ml of plastid preparation, and the refractile plastids were of interest because they are the organelles bounded by an intact envelope.

The DNA content per plastid is expressed in two ways: firstly, as the mass of DNA, and, secondly, as the number of genome copies, assuming that each circle has a mass of 1.6×10^6 g. The etioplasts contained more DNA than chloroplasts but even the latter were markedly polyploid in the sense defined by Kirk [4]. An additional aspect of these results is that the DNA content per organelle increased rapidly in the dark but remained approximately constant in the light.

These results provided no information about the cDNA content per bud. To calculate such a parameter it was necessary to determine the number of plastids per bud. Table 2 shows that this was done directly from the pigment contents of plastids and buds. In

the three day period of the experiment there was less than one doubling of the etioplast number but more than two doublings of the chloroplast number (assuming all plastids in a bud are equally capable of division). The figures reveal a marked stimulation of division by light.

The consequences of this effect for plastid DNA synthesis are indicated in table 3. Both etiolated and greening buds synthesized plastid DNA. The rate of DNA accumulation (we have not investigated the possibility of DNA turnover) was about 50% greater in the case of the illuminated apices.

4. Discussion

The plastid DNA content of peas may be expressed per organelle or per bud. However, when this is done two different assessments of the effect of light on plastid DNA replication are obtained. When a population of plastids is examined, it appears that DNA synthesis occurs only in the dark. In contrast, when the whole bud is considered, DNA replication is seen to occur in both light and dark, with the rate in the light being 50% higher than that in the dark.

The resolution of this apparent paradox is provided by the effect of light on plastid division. Although light stimulates plastid DNA replication in buds, the DNA so synthesized is distributed among a rapidly dividing plastid population. As a result the DNA content per chloroplast remains approximately constant at 14 genome copies, while that per etioplast increases with time. It is clear that if a mechanism exists to maintain a constant DNA content per plastid, the mechanism does not operate successfully in the dark. The same conclusion has recently been reached by Rose et al. [14] from autoradiographic studies on plastid DNA in spinach leaf disks. However, it is not possible to compare the rates of DNA synthesis in two tissues from silver grain counts unless it can be shown that precursor pool sizes are identical in the two tissues. Rose et al. [14] did not investigate this point. The problem of pool sizes is avoided in our approach which assays extracted plastid DNA by a spectrophotometric procedure. The major problem with such an approach is to eliminate contaminating nDNA. By application of analytical CsCl density gradient centrifugation to native, denatured and renatured DNA extracted from purified plastids, we showed that this problem was successfully overcome.

It is not clear why developing pea chloroplasts should contain about 14 copies of the plastid genome. The phenomenon may be related in some way to transcription. Thus, DNA replication may provide additional template for RNA synthesis, or it may provide the template in a structural form which facilitates transcription of specific genes. These two possibilities are currently under investigation in this laboratory.

Acknowledgements

This work was supported by the Science Research Council through a grant to Dr R. J. Ellis. We thank Mrs J. Gardiner for assistance, Dr C. S. Dow for the gift of *Micrococcus* DNA and Dr Ellis for encouragement and critical reading of the manuscript.

References

[1] Ellis, R. J. and Hartley, M. R. (1974) in: Biochemistry of Nucleic Acids (Kornberg, H. L., Phillips, D. C. and Burton, K. eds.), pp. 323–348. M. T. P. International Reviews of Science, Butterworth, London.
[2] Kolodner, R. and Tewari, K. K. (1972) J. Biol. Chem. 247, 6355–6364.
[3] Herrmann, R. G., Bohnert, H. -J., Kowallik, K. V. and Schmitt, J. M. (1975) Biochim. Biophys. Acta 378, 305–317.
[4] Kirk, J. T. O. (1972) Sub-Cell. Biochem. 1, 333–361.
[5] Kowallik, K. V. and Herrmann, R. G. (1972) J. Cell. Sci. 11, 357-367.
[6] Siddell, S. G. and Ellis, R. J. (1975) Biochem. J. 146, 675–685.
[7] Hartley, M. R. and Ellis, R. J. (1973) Biochem. J. 134, 249–262.
[8] Leaver, C. J. (1973) Biochem. J. 135, 237–240.
[9] Wong, F. Y. and Wildman, S. G. (1971) Biochim. Biophys. Acta 259, 5–12.
[10] Arnon, D. I. (1949) Plant Physiol. 24, 1–15.
[11] Bottomley, W. (1970) Plant Physiol. 45, 608–611.
[12] Gillespie, D. and Spiegelman, S. (1965) J. Mol. Biol. 12, 829–842.
[13] Schildkraut, C. L., Marmur, J. and Doty, P. (1962) J. Mol. Biol. 4, 430–443.
[14] Rose, R. J., Cran, D. G. and Possingham, J. V. (1975) J. Cell. Sci. 17, 27–41.