Phytochemistry 1972, Vol. 11, pp. 943 to 947. Pergamon Press. Printed in England.

RIBOSOMAL-RNA DISTRIBUTION DURING LEAF DEVELOPMENT IN SPINACH

P. DETCHON and J. V. POSSINGHAM

CSIRO Division of Horticultural Research, Box 350, Adelaide, South Australia 5001

(*Received* 8 *July* 1971, *in revised form* 15 *September* 1971)

Abstract—The numbers of cells and chloroplasts, and the amounts of DNA, RNA and chlorophyll in emerged spinach leaves of different age, have been measured. In addition the ribosomal-RNAs have been fractionated by polyacrylamide gel electrophoresis. Cell size, the number of chloroplasts and the quantities of chlorophyll, DNA and ribosomal-RNAs per cell all increased with age. Despite a 3-fold increase in cytoplasmic ribosomal-RNA, and a 15-fold increase in chloroplast numbers per cell, chloroplast ribosomal-RNA per chloroplast remained unchanged as the leaves expanded.

INTRODUCTION

THE GROWTH of spinach leaves and leaf cells both *in vivo*[1,2] and more recently *in vitro*,[3] has been examined in this laboratory. In both cases the number of plastids per cell in the basal region of newly-emerged leaves (i.e. approx. 2 cm in length) was low (approx. 20), but increased 10-fold or more as the cells enlarged during further growth over a period of about 14 days. This increase in plastid number can be measured cytologically and so expanding spinach leaves provide a good system for studying the characteristics and control of plastid replication.

The mechanism of, and factors affecting, chloroplast ribosomal-RNA synthesis in this system are now being studied. This paper provides data of the amounts and the normal distribution of ribosomal-RNAs between chloroplast and cytoplasm in leaf cells in which chloroplast replication is occurring.

RESULTS

Cell and Chloroplast Numbers

Table 1 shows that cell numbers per gram fresh weight decreased with increasing leaf size, whilst the size of the cells and the number of chloroplasts per cell increased. Chloroplast numbers per cell in 2 cm leaf tips were significantly greater than those of 2 cm leaf bases.

TABLE 1. CELL AND CHLOROPLAST NUMBERS IN LEAVES OF 24-DAY-OLD SPINACH PLANTS

Tissue	Cell No. ($\times 10^6$/g fresh wt.)	Cell area ($mm^2 \times 10^{-4}$)	Chloroplast No. (per cell)
Base of 2 cm leaf	280 ± 25	7·3 ± 1·4	19 ± 2
Tip of 2 cm leaf	112 ± 9	19·9 ± 3·3	62 ± 5
Tip of 4 cm leaf	43 ± 2	63·6 ± 10·7	135 ± 10
Tip of 7 cm leaf	9 ± 0·5	77·6 ± 12·3	275 ± 18

[1] W. SAURER and J. V. POSSINGHAM, *J. Exptl Bot.* **21**, 151 (1970).
[2] J. V. POSSINGHAM and W. SAURER, *Planta* **86**, 186 (1969).
[3] J. V. POSSINGHAM and J. W. SMITH, Unpublished observations (1971).

DNA, RNA and Chlorophyll Contents

Expressed on a fresh weight basis, the RNA and DNA contents of the leaf decreased with age, although chlorophyll remained constant (Table 2). However, when expressed on a per cell basis, DNA, RNA and chlorophyll all increased with age. The increases between the cells of 2 cm leaf bases and 7 cm leaf tips were approximately 2-fold for DNA, 5-fold for RNA and 29-fold for chlorophyll.

TABLE 2. NUCLEIC ACID AND CHLOROPHYLL CONTENTS IN LEAVES OF 24-DAY-OLD SPINACH PLANTS

Tissue	Total nucleic acids (mg/g fresh wt.)	Total nucleic acids ($g \times 10^{-11}$/ cell)	DNA (mg/g fresh wt.)	DNA ($g \times 10^{-12}$/ cell)	RNA (mg/g fresh wt.)	RNA ($g \times 10^{-11}$/ cell)	Chlorophyll (mg/g fresh wt.)	Chlorophyll ($g \times 10^{-12}$/ cell)
Base of 2 cm leaf	7·9 ± 0·9	2·8	1·3 ± 0·12	4·6	6·6	2·4	0·27 ± 0·04	1·0
Tip of 2 cm leaf	4·4 ± 0·1	3·9	0·7 ± 0·03	6·3	3·7	3·3	0·26 ± 0·02	2·3
Tip of 4 cm leaf	2·2 ± 0·03	5·1	0·3 ± 0·04	7·0	1·9	4·4	0·27 ± 0·02	6·3
Tip of 7 cm leaf	1·1 ± 0·06	12·2	0·1 ± 0·05	11·1	1·0	11·1	0·26 ± 0·02	28·9

Ribosomal–RNA Distribution

Nucleic acid extracts prepared from spinach leaves of different age were electrophoresed on polyacrylamide gels and a typical E_{265} absorption scan of the gels is shown in Fig. 1. With increasing tissue age the amount of chloroplast specific ribosomal–RNAs (1·1 and 0·56 × 10^6 daltons mol. wt. species) increased progressively relative to the cytoplasmic specific ribosomal–RNAs (1·3 and 0·7 × 10^6 daltons mol. wt. species).

From each gel scan, the ratio of chloroplast to cytoplasmic ribosomal–RNA was calculated (see Experimental), and from these ratios and the data presented in Table 2, values for cytoplasmic and chloroplast ribosomal–RNA per cell were obtained (Table 3). Cytoplasmic ribosomal–RNA per cell increased approximately 3-fold, whilst chloroplast ribosomal–RNA per cell increased approximately 9-fold over the age range studied.

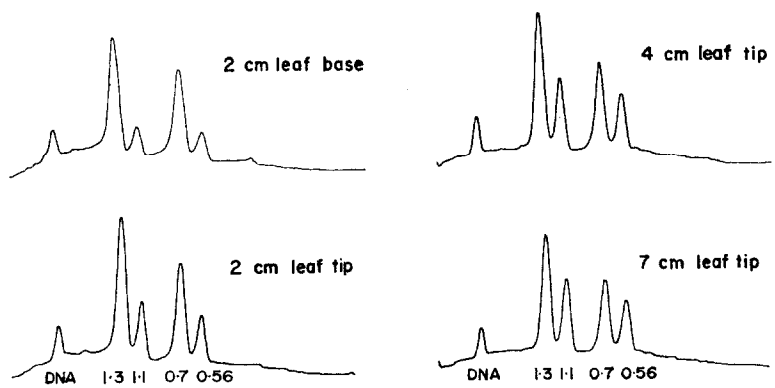

FIG. 1. TYPICAL E_{265} ABSORPTION SCANS OF POLYACRYLAMIDE GELS FOLLOWING ELECTROPHORESIS OF NUCLEIC ACIDS FROM SPINACH LEAVES OF DIFFERENT SIZE. RIBOSOMAL–RNA PEAKS ARE IDENTIFIED ACCORDING TO THEIR MOL. WTS. (×10^6 daltons).

TABLE 3. DISTRIBUTION OF RIBOSOMAL-RNAS AND CHLOROPHYLL IN LEAVES OF 24-DAY-OLD SPINACH PLANTS

Tissue	Cytoplasmic ribosomal-RNA ($g \times 10^{-11}$/ cell)	Chloroplast ribosomal-RNA ($g \times 10^{-11}$/ cell)	Chloroplast ribosomal-RNA ($g \times 10^{-13}$/ chloroplast)	Chlorophyll ($g \times 10^{-14}$/ chloroplast)
Base of 2 cm leaf	1·8	0·6	3·2	5·3
Tip of 2 cm leaf	2·1	1·2	1·9	3·7
Tip of 4 cm leaf	2·5	1·9	1·4	4·7
Tip of 7 cm leaf	5·9	5·2	1·9	10·5

Ribosomal-RNA and Chlorophyll Content of Chloroplasts

From the present data it was possible to calculate the content of chlorophyll and chloroplast-specific ribosomal-RNA per chloroplast (Table 3). Chlorophyll increased approximately 2-fold over the age range studied, whereas ribosomal-RNA remained fairly constant throughout.

DISCUSSION

The increase with age in cytoplasmic ribosomal-RNA per cell (Table 3) was expected in view of the great increase in cell size (Table 1) and membrane complexity that occurs with leaf age. By contrast, the amount of chloroplast-specific ribosomal-RNA per chloroplast (Table 3) did not increase over the same period. Plastids in developing leaves of barley increase in size and complexity with age,[4] and it was observed during the present studies by both light and electron microscopy that chloroplasts in the basal cells of 2 cm leaves were smaller than those in the cells of older leaves containing more chloroplasts per cell. Studies on the incorporation of ^{32}P orthophosphate into the RNAs of wheat, swiss chard and onion[5] and also spinach suggest that chloroplast RNA is only synthesized over a limited time period when the leaves are young and that it undergoes a much lower rate of turnover than cytoplasmic RNA. Thus it would appear that the ribosomal-RNA (and thus ribosome) content of the chloroplast does not increase with increasing age or size of the chloroplasts. This situation is contrary to that which exists in the cell cytoplasm and may in some way be related to situations recently reported where the synthesis of some chloroplast proteins may be in part programmed by the protein synthesizing machinery of the cytoplasm rather than by that of the chloroplasts.[6,7]

The ratios of high molecular weight to low molecular weight ribosomal-RNAs in this study, did not approach the expected values of approximately 2:1. This was presumed to be due to breakdown of the labile $1·1 \times 10^6$ daltons chloroplast 'heavy' ribosomal-RNA into smaller units, including a $0·7 \times 10^6$ daltons molecule.[8]

The DNA measured (Table 2) was contributed by three classes of organelle known to contain DNA: nuclei, plastids and mitochondria; and since endopolyploidy, with consequent increases in mean DNA level per cell, has been shown to occur in a number of higher

[4] M. J. C. RHODES and E. W. YEMM, *Nature, Lond.* **200**, 1077 (1963).
[5] J. INGLE, J. V. POSSINGHAM, R. WELLS, C. S. LEAVER and U. E. LOENING, *Symp. Soc. Exptl Bot.* **xxiv**, 303 (1970).
[6] T. A. DYER, R. H. MILLER, and A. D. GREENWOOD, *J. Exptl Bot.* **22**, 125 (1971).
[7] J. L. CHEN and S. G. WILDMAN, *Biochim. Biophys. Acta* **209**, 207 (1970).
[8] C. J. LEAVER and J. INGLE, *Biochem. J.* **123**, 235 (1971).

plants,[9,10] it is not possible to calculate DNA per plastid from results such as we have obtained. This is indeed unfortunate since this would appear to be a good system in which to test the findings of Woodcock and Bogorad[11] and of Boasson and Laetsch[12] whose results suggest that DNA replication may not be a prerequisite for chloroplast formation.

EXPERIMENTAL

Plant material and culture conditions. American round-seeded spinach (*Spinacea oleracea*) seeds were germinated in moist vermiculite in the dark at 16°. At 10 days seedlings were transplanted into aerated liquid culture medium (Hoagland medium) and grown in a growth cabinet under fluorescent lights (14 hr day; 21,500 lx) with day and night temps of 24° and 22° respectively. Plants were harvested after 24 days further growth.

At harvest, in order to obtain uniform leaf material covering an age progression, leaves were selected on the basis of measured length rather than position, since slight variations occurred between the growth rates of individual plants. Leaves chosen as the youngest tissue were 2 cm long since they were the smallest emerged leaves which could be conveniently used and which also contained cells with low plastid numbers. Leaves 7 cm long were selected since they were the largest and most mature leaves of the plants grown under our conditions. As an intermediate age, leaves 4 cm long were selected. 0·5 cm pieces of the apical or basal tissue were harvested as specified.

Cell counts. Cell counts were made on suspensions prepared from known weights of fresh tissue using the method of Brown and Broadbent.[13] The tissue was incubated in 5% chromic acid at 60° for 2 hr prior to maceration, and the suspensions were diluted with water to give approx. 200 cells in the area of the haemocytometer grid. Counts were made in quintuplicate.

Chloroplast counts and cell area measurements. Chloroplasts were counted in tissue fixed in 3% glutaraldehyde overnight at 4°. After incubating the tissue in 50 mM EDTA pH 9·0 at 60° for 4 hr, a squash was prepared and the chloroplasts per cell were counted as previously described.[2] In each case, 10 counts per tissue sample were made whilst the area of these cells was obtained from the product of length times breadth measured with an eyepiece graticule.

Chlorophyll determination. Chlorophyll was determined by the spectrophotometric method of Arnon[14] using 80% acetone extracts of 50 mg samples of fresh tissue. All determinations were performed in duplicate.

Extraction of nucleic acids for quantitative determination. Samples (100 mg) of fresh tissue were homogenized in 1 ml 5% $HClO_4$ at 0° for 1 min using an all-glass homogenizer at 350 rev/min. After 15 min vigorous extraction, the homogenate was centrifuged at 2000 *g* for 10 min and the supernatant was discarded. Two further extractions were performed with 5% $HClO_4$ at 0° to complete the removal of acid-soluble P. The residue was then extracted with 1 ml vol. of EtOH–Et_2O–$CHCl_3$ (2:2:1) at room temp. four times (15 min extraction periods) to remove phospholipids.[15] The final nucleic acid residues were dried *in vacuo*, and then hydrolysed in 0·5 ml 5% trichloroacetic acid at 90° for 30 min.

Total nucleic acid determination. The absorbancy of the hot trichloroacetic acid digest was measured at 268·5 nm against a suitable blank (5% trichloroacetic acid solution treated in the same manner as the test sample). Total nucleic acid content was then calculated using the relationship that the molar extinction with respect to nucleotide phosphate was $\Sigma(p) = 9850\ M^{-1}\ cm^{-1}$ at 268·5 nm[16] and the assumption that the average mol. wt. of nucleotides was 320.

DNA determination. DNA was determined by the diphenylamine reaction as described by Burton[17] using duplicate 0·2 ml samples of the hot trichloroacetic acid digest adjusted to 0·5 M with respect to $HClO_4$. Highly polymerized calf thymus DNA (Sigma Chemical Co.) was used as a standard.

RNA determination. RNA was calculated as the difference between total nucleic acid and DNA content of the hot trichloroacetic acid digest.

Extraction of undegraded nucleic acids for gel electrophoresis. Nucleic acids were extracted from leaf tissue by a modification of the method of Solymosy *et al.*[18] Tissue (100 mg) was homogenized in 0·5 ml 50 mM tris HCl buffer pH 7·4, containing 10 mM $MgCl_2$, 1% sodium lauryl sulphate and 50 μl of a 50% solution of

[9] R. SHANKS, *Austral. J. Bot.* **13**, 143 (1965).
[10] J. K. HEYES, *Proc. Roy. Soc.* **152B**, 218 (1960).
[11] C. L. F. WOODCOCK and L. BOGORAD, *J. Cell. Biol.* **44**, 361 (1970).
[12] R. BOASSON and W. M. LAETSCH, *Science* **166**, 749 (1969).
[13] R. BROWN and D. BROADBENT, *J. Exptl Bot.* **1**, 249 (1951).
[14] D. J. ARNON, *Plant Physiol.* **24**, 1 (1949).
[15] J. INGLE, *Phytochem.* **2**, 353 (1963).
[16] J. E. LOGAN, W. A. MANNELL and R. J. ROSSITER, *Biochem. J.* **51**, 480 (1952).
[17] K. BURTON, *Biochem. J.* **62**, 315 (1956).
[18] F. SOLYMOSY, I. FEDORCSAK, A. GULYAS, G. L. FARKAS and L. EHRENBERG, *Europ. J. Biochem.* **5**, 520 (1968).

diethyl pyrocarbonate (Calbiochem, Los Angeles, California) in abs. EtOH, in an all-glass homogenizer at 0°, for 20 sec at 350 rev/min. The homogenate was incubated at 37° for 5 min with frequent mixing before centrifuging at room temp. for 10 min at 2000 *g*. The residue was discarded and the supernatant was transferred to a fresh tube containing 50 mg of finely powdered NaCl (A.R. grade) and 10 μl diethylpyrocarbonate. A further incubation was performed at 37° for 5 min with frequent mixing and the mixture was recentrifuged at room temp. for 10 min at 2000 *g*. The residue was discarded, and 1 ml abs. EtOH was added to the supernatant. Precipitation of the nucleic acids was completed by standing at −20° overnight. The residue was collected by centrifugation at 2000 *g* for 5 min and dried *in vacuo*. Further purification and washing of the precipitated nucleic acids as described by Solymosy *et al.*[18] was found to be unnecessary.

Polyacrylamide gel electrophoresis of nucleic acids. Polyacrylamide gels (2·4%) were prepared and the nucleic acid preparations were electrophoresed as described by Loening.[19] The gel cylinders were scanned at 265 nm with a chromoscan recording densitometer (Joyce Loebl Ltd., Gateshead, England). The amounts of individual nucleic acid species were estimated by quintuplicate planimetric measurements of the area under each peak. The contributions of 'polydisperse RNA' and background absorption of the gel to the peak areas was subtracted by projection of a curved baseline through the bases of the ribosomal RNA peaks.

The relative amounts of plastid and cytoplasmic ribosomal-RNAs were calculated as described by Smith[20] using values for the ratio of heavy to light ribosomal-RNAs of 1·85:1 for cytoplasmic ribosomal-RNAs and 1·96:1 for chloroplast ribosomal-RNAs.

Acknowledgement—Skilled technical assistance provided by Miss J. W. Smith is very gratefully acknowledged by the authors.

[19] U. E. Loening, *Biochem. J.* **102**, 251 (1967).
[20] H. Smith, *Phytochem.* **9**, 965 (1970).

Key Word Index—*Spinacia oleracea*; Chenopodiaceae; leaf development; DNA; ribosomal-RNA; chloroplasts; chlorophyll.