Ribosomal RNA metabolism in cucumber leaf mesophyll protoplasts

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ABSTRACT

Aspects of the metabolism of RNA have been studied in enzymatically isolated protoplasts from cotyledon and first leaf mesophyll tissue of two cultivars of cucumber. The first leaf mesophyll protoplasts incorporated ($^3$H)-uridine into ribosomal RNA at a constant rate for up to 25 hr in a simple salts medium and for up to 45 hr in a growth medium. Pulse-chase labelling experiments on such preparations showed a rapid dilution of the intracellular ($^3$H)-uridine pool(s) and a high metabolic rate in the cells in one cultivar but not in another. Gel electrophoretic analysis of the RNA from both cotyledon and first leaf protoplasts showed that both protoplast types incorporated either ($^{14}$C)- or ($^3$H)-uridine into ribosomal RNA species. Incorporation of ($^3$H)-uridine into chloroplast RNA was minimal in cotyledon protoplasts, but significant in leaf protoplasts. Greater incorporation into the chloroplast RNA species could be achieved by longer pulses.

Synthesis of all of the ribosomal RNA species was sensitive to actinomycin D at 10 and 25 µg/ml concentrations in all protoplasts tested.

INTRODUCTION

Protoplasts have been isolated and cultured from numerous plant species (1-4), protoplasts obtained from leaf mesophyll cells providing a system which allows a study of the synchronous multiplication of plant viruses (5-8). Such protoplasts provide a useful tool for biochemical experimentation, since they form a uniform suspension in liquid media with obvious advantages over whole tissue (6,9).

Isolated cells and protoplasts from the leaf mesophyll tissue of several plant species have been used for the study of both ribosomal RNA metabolism and the biosynthesis of virus-specific RNAs. Cells from Nicotiana tabacum cv. Turkish Samsun, infected with tobacco mosaic virus (TMV) (9), cells from Brassicae parachinensis cv. Pet Sai infected with turnip yellow mosaic virus (TYMV) (10), cells and protoplasts from Vicia faba cv. Maxima infected with broad bean mosaic virus (BBMV) (11) and protoplasts from Nicotiana tabacum cv.
Xanthin n.c. infected in vitro with TMV (12) have all been studied in some
detail in this respect.

Recently the RNA metabolism of cucumber leaves has been studied in
relation to the effects of fungal disease and senescence (13), cucumber mosaic
virus (CMV) infection, and actinomycin D treatment (14). Under the correct
conditions of isolation and culture, protoplasts of the first leaf mesophyll
of cucumber will divide and grow (4). This communication reports some studies
on ribosomal RNA metabolism of protoplasts isolated from the cotyledons and
first leaves of two varieties of cucumber, and the effect on ribosomal RNA
metabolism of actinomycin D.

MATERIALS AND METHODS

Plant Material

Cucumber plants (Cucumis sativus L. cv. China and Ashley) were grown
singly in 10cm pots, in a glasshouse under conditions outlined previously (4).
Protoplasts were isolated from the first true leaves of 14-21 day old plants
or from the expanded cotyledons of 10-14 day old plants.

Radiochemicals, enzymes and antibiotics

All radiochemicals were obtained from the Radiochemical Centre, Amersham,
Bucks, U.K. Uridine generally labelled with tritium at 3.3 Ci/m mol. specific
activity, and uridine-2-\textsuperscript{3}H\textsubscript{3} at 60.5 mCi/m mol. specific activity, were used
throughout the study. Cellulase was obtained from the Kinki Yakult Manuf.
Co., Tokyo, Japan, pectinase from Koch-Light Biochemicals Ltd., Colnbrook, U.K.
and potassium dextran sulphate (Molecular weight start dextran 760, sulphur
content 17.3%) from the Meito Sangyo Co. Ltd. Tokyo, Japan. Helicase was ob-
tained from Microbio-Labs. Ltd. U.K. Nystatin was obtained from E.F. Squibb
and Sons, New York, U.S.A., carbencillin from Beecham Research Labs.,
Brentford U.K., and actinomycin D from Merck Sharp and Dohme International,
New Jersey, U.S.A.

Isolation of protoplasts

First leaf protoplasts were isolated according to the technique described
by Coutts and Wood (4). The isolation of cotyledon protoplasts was achieved
by pre-plasmolysis of surface sterilized, peeled cotyledon pieces in an
osmotically stabilized salts medium (4) followed by 16hr enzymatic digestion
in the dark at 25°C in the same medium containing 3.3% cellulase, 3.3%
pectinase, 0.005% helicase and 0.5% potassium dextran sulphate, pH 5.8, centrifuged at 500 x g for 15 min. before millipore sterilization. The isolated protoplasts of both first leaf and cotyledon mesophyll were released by gentle agitation and then poured through a 77 µ nylon mesh, to remove tissue fragments and debris. The protoplasts were collected by centrifugation at 100 x g for 5 min. to remove the enzyme solution, washed 3 times with 11% mannitol plus 0.1 mM CaCl₂, with centrifugation as above, and resuspended in mannitol/CaCl₂.

Incubation and radioactive labelling of cellular RNA

The isolated protoplasts were suspended in either the medium of Otsuki et al. (15), or Harada (16), in which the concentration of 6-benzylaminopurine (BAP) and 2,4 dichlorophenoxyacetic acid (2,4-D) were both reduced to 0.5 mg/litre. The protoplasts were cultured in 5 ml of the medium at a concentration of 1.5 x 10⁵ protoplasts/ml in 100 ml Erlenmeyer flasks. Both media contained antibiotics (Nystatin 25 units/ml; carbenicillin 100 µg/ml) to control possible contamination by micro-organisms (17). The protoplasts were pre-incubated for 2 hr at 25°C in an environmental cabinet (Fisons 140 G2) with 2000 lux illumination provided by warm white fluorescent tubes, before addition of 200 µCi (³H)-uridine to each flask. In pulse-chase experiments, two protoplast samples were pulsed with (³H)-uridine and after 1 hr incorporation, unlabelled uridine (40 µ moles; ca. 700 fCi. dilution of radioactivity) was added to the chase portion of the experiment. Protoplast samples were taken at set time intervals (see figure legends) and the trichloroacetic acid (TCA) insoluble fraction of the cells was isolated by the method of Francki et al. (18), collecting the TCA insoluble fractions on EHWP Millipore filters which were dried at 37°C in scintillation vials. Scintillant 1 ml; (4g PPO, 12g POPOP, 0.12g POPOP, 1,4-bis-(5-phenyloxazol-2-yl) benzene)/litre toluene) was added to each vial and the radioactivity due to (³H) incorporation was determined in a Phillips PW 4510/01 Automatic liquid scintillation Analyser. Where incorporation of radioactive precursor uridine into specific classes of cellular RNA was to be determined by polyacrylamide gel electrophoresis, protoplasts from both cotyledons and first leaves were suspended in the medium of Otsuki et al. (15) at a concentration of 1.5 x 10⁵ protoplasts/ml in 50ml Erlenmeyer flasks for pre-incubation. The medium also contained 10mM NaHCO₃, 0.1mM phosphoenolpyruvic acid (PEP), and 3mM sucrose to facilitate the uptake of (³H)- or (¹⁴C)-uridine and actinomycin D when tested in the dark (9). After pre-incubation, the protoplasts were sedimented.
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(100xg for 5 min) and the supernatant removed. The protoplasts were then resuspended in a similar medium at the same concentration, without NaHCO₃, and the radioactive label introduced aseptically. Incubation was then carried out for varying periods of time; details of incubation mixtures, protoplasts volume, label, lighting conditions and temperature are given in the figure legends.

RNA extraction for polyacrylamide gel electrophoresis

RNA was extracted from sedimented protoplasts (100xg for 5 min) by a modified method of Jackson et al. (9). The protoplasts, in a final volume of 1.0-1.5 ml, were suspended in 1.5 ml of TNE buffer (0.1M TRIS, 0.1M NaCl and 0.01M Na₂EDTA pH 8.5), 1.5 ml of water saturated phenol, (8.5 volumes) and m-cresol, (1.5 volumes) with 0.1% para-hydroxy-quinoline, 0.125 ml of a 20% sodium dodecyl sulphate solution (SDS) and 0.05 ml diethylpyrocarbonate. All buffers and glassware were autoclaved before use. The mixture was shaken at 4°C for 10 min, and the aqueous phase removed by centrifugation at 300 xg for 10 min. This phase was re-extracted with 1.5 ml of the phenol mixture and the aqueous phase recovered as before. Excess phenol was removed from the aqueous phase by shaking with four changes of water-saturated ether and the ether removed with air. The nucleic acids were precipitated at -20°C with two volumes of ethanol plus a few drops of 3M sodium acetate, pH 4.0 (9).

The nucleic acid precipitate was collected by centrifugation at 6000 xg for 15 min and an aliquot taken up in the electrophoresis buffer of Peacock and Dingman (19) containing 10% sucrose, prior to gel electrophoresis. The optical density of the solution was determined at 260nm and each gel loaded with 50 μl of solution having an optical density of 1.00-1.50 when diluted ten fold in TNE buffer.

Polyacrylamide-agarose gel electrophoresis

Protoplasts prepared from the two cultivars were incubated for various times in the presence of (3H)-uridine, RNA subjected to polyacrylamide gel electrophoresis in the presence of tobacco or E. coli ribosomal RNA as markers and radioactivity determined in 1 mm gel slices.

Gel electrophoresis was carried out in either 2.4% or 2.6% acrylamide gels stabilised with 0.5% agarose as previously described (20). Ten to fifty micrograms of E. coli RNA isolated by the method of Bolton (21), or tobacco
ribosomal RNA, were added to the RNA samples to provide optical reference markers when necessary. Six to seven cm. gels were run at 5 mA/gel for 2-3 hr at 4°C. The gels were removed and washed in electrophoresis buffer without SDS for 1 hr before staining with a solution of 0.1% toluidine blue in 40% 2-methoxyethanol for 3 min; destaining was achieved in 30% methoxyethanol by overnight rotation of the gels.

Gels were then scanned at 260 nm in a Unicam SP1800 spectrophotometer with a densitometer attachment, sliced into 1mm segments and treated overnight at 25°C with 0.5 ml soluene (Packard Instrument, Co., Inc., Illinois, U.S.A.) in scintillation vials. To each vial was added 8 ml. of scintillant and radioactivity counted as before.

**Autoradiography of polyacrylamide-agarose gels**

Samples were electrophoresed as described, the gels sliced longitudinally into four slices and the slices dried onto a sheet of chromatography paper (Whatman No. 1). The paper was then placed in contact with Kodirex X-ray film for 1-3 weeks and the film developed in Kodak D19 developer.

**RESULTS AND DISCUSSION**

The use of plant cells and protoplasts (12) has facilitated the study of metabolic processes in higher plants, particularly RNA metabolism (9-12), though attention has so far been focussed principally on tobacco cells; the preliminary results presented here indicate the feasibility of conducting such studies in protoplasts from cucumber tissue. The capacity of protoplasts from both cotyledon and first leaf mesophyll tissue of two cucumber cultivars to support RNA synthesis has been investigated, and some differences noted.

Firstly, protoplasts were prepared from first leaf mesophyll incubated in the presence of $^{3}H$-uridine and samples were taken at various times for determination of radioactivity incorporated into cold TCA-insoluble material. In a simple salts medium (15), protoplasts of both cultivars of cucumber incorporated radioactive uridine into total RNA at constant (but different) rates up to 25 hr (Fig. 1B). However, when the protoplasts were incubated in the growth medium of Harada (16), approximately constant incorporation could be achieved for periods up to 45 hr in both varieties. (Samples from both incorporations were routinely checked for micro-organism contamination, though none was detected). It appears that the metabolic stability of the protoplasts is maintained better in a medium containing the hormones 6-BAP, and 2,4-D.
Incorporation of \(^{3}H\)-uridine into leaf mesophyll protoplasts from Ashley (●) and China (○) cucumber plants. Protoplasts \(1.3 \times 10^{3}/\text{ml}\), were incubated in 5 ml of Takebe's simple salts incubation medium (B), or modified Harada's medium (A), in the light (2000 lux) at 25°C in the presence of 200 μCi of \(^{3}H\)-uridine. Samples (200 μl each) were taken at various time intervals and radioactivity incorporated into cold TCA insoluble material determined.

A finding previously reported for other protoplasts, (2).

One advantage of using isolated protoplasts for metabolic studies is the feasibility of conducting pulse-chase experiments (9,18) which cannot be satisfactorily conducted with intact leaf tissue. Such procedures are particularly valuable in studies of intermediates in viral RNA synthesis, for example (9). In all pulse-chase experiments performed here, protoplasts were cultured in the simple salts medium, samples of protoplasts taken at set time intervals and cold TCA-insoluble radioactivity determined. After a slight lag, \(^{3}H\)-uridine incorporation was essentially linear (Figs. 2A, B). However, upon the addition of unlabelled uridine to Ashley protoplasts (Fig. 2A), incorporation of radioactivity continued for a short period and then stopped, while addition of unlabelled uridine to China protoplasts permitted continued incorporation of labelled uridine, though at a reduced rate (Fig. 2B).

Continued incorporation after addition of excess unlabelled uridine probably reflected the time required for dilution of the intracellular \(^{3}H\)-
uridine pool(s), China protoplasts apparently allowing this to occur less efficiently than Ashley protoplasts. As demonstrated previously with tobacco cells (9), such pulse chase experiments can be performed satisfactorily with cucumber protoplasts, using high specific activity \(^{3}H\)-uridine as precursor.

**Fig. 2**

Effect of adding an excess of unlabelled uridine on the incorporation of \(^{3}H\)-uridine into leaf mesophyll protoplasts from Ashley (A), and China (B), cucumber plants. Protoplasts \((1.2 \times 10^{7}/\text{ml})\) in 5ml of Takebe's simple salts incubation medium, were incubated for 2hr in the absence of labelled uridine, as in Fig. 1. After this period, 200 µCi \(^{3}H\)-uridine was added to each assay and the incubation continued. After 1 hr pulse with \(^{3}H\)-uridine, 40 µ moles of unlabelled uridine (ca. 700 fold dilution of radioactivity) were added to the chase portion of the experiment. Samples (200 ul each) were taken, and the radioactivity determined at various time intervals, as in Fig 1: O--O \(^{3}H\)-uridine; O--O \(^{3}H\)-uridine for 1 hr followed by addition of unlabelled uridine

Ribosomal RNA synthesised de novo by both cotyledon and first leaf protoplasts was analysed by polyacrylamide gel electrophoresis. As expected, the ribosomal RNA species of both cytoplasmic and chloroplast ribosomes \((25s, 18s\) and \(23s, 16s\) respectively) were synthesised de novo in first leaf protoplasts (Fig. 3). There appeared also to be incorporation into a lower molecular weight component(s) (Fig. 5), possibly ribosomal RNA breakdown products \((9, 13, 23)\), and also into a higher molecular weight component(s), probably a precursor, analogous to that observed in several other plant species, \((24-27)\).
Incorporation of \(^{3}H\)-uridine into RNA of protoplasts from the leaf mesophyll of the (A), Ashley and (B), China cultivars of cucumber. Protoplasts (1.3 x 10^6/\text{ml}) were incubated in 5 ml of medium for 2 hr as in Fig. 2; 200 \text{mCi} of \(^{3}H\)-uridine was then added to each sample, and the samples incubated for a further 29 hr, in the light (2000 lux) at 25°C. RNA was extracted and subjected to electrophoresis in 2.6% acrylamide/agarose gels as outlined in the text. Prior to electrophoresis, tobacco leaf RNA (8 \text{ug}) was added as an optical marker to a duplicate Ashley sample, and E. coli RNA (0.1 \text{ug}) was added to a duplicate China sample. Electrophoresis was conducted for 2 hr in A, and 2.5 hr in B. After electrophoresis, the gels were scanned to determine the position of the optical markers, and radioactivity of gel slice fractions determined. Arrows indicate the position of the optical markers.

In Figs 3-5; \textcolor{red}{--------}, Optical densitometer trace at 260 nm; \textcolor{black}{

Incorporation of \(^{14}C\)-uridine into cotyledon protoplasts of both cultivars indicated \textit{de novo} synthesis of 25s and 18s ribosomal RNA, together with a possible precursor (25) (Fig.4). Little or no incorporation into the chloroplasts ribosomal RNA were evident, a finding previously reported in senescent or disease damaged cucumber cotyledons (13).

Similar results were obtained with 6 hr pulses of first leaf protoplasts with \(^{3}H\)-uridine, with less than 10% of the total label being incorporated into the chloroplast RNAs. (Fig. 5). However, these pulses were carried out in the dark for only 6 hr. Pulsing the same protoplast preparations in the
Incorporation of (\(^{14}\)C)-uridine into RNA of protoplasts isolated from cucumber cotyledon mesophyll of the (A) China, and (B) Ashley cultivars. Protoplasts were incubated in 8 ml of incubation medium (1.1 x 10\(^7\)/ml) in the light (2000 lux) for 2 hr prior to the addition of 2.5 \(\mu\)Ci of (\(^{14}\)C)-uridine. Incubation was then carried on for a further 5 hr, when RNA was extracted and analyzed as in Fig. 3 using 2.5% acrylamide gels, with optical density markers added in the same way. Arrows indicate the position of the optical markers. Figs 4C, D are representations of autoradiographs obtained by the methods described in the text. Fig. 4C indicates untreated cotyledon protoplast RNA extract from the Ashley cultivar pulsed in the dark for 6 hr with 2.5 \(\mu\)Ci of (\(^{14}\)C)-uridine; Fig. 4D indicates a similar protoplast sample, pulsed in the presence of 25 \(\mu\)g/ml actinomycin D.

Light for 29 hr indicated incorporation of the (\(^{2}\)H)-uridine into both classes of chloroplast ribosomal RNA along with increased incorporation into the cytoplasmic ribosomal RNA classes (Fig. 3). The relative rates of synthesis of mature chloroplast/cytoplasmic ribosomal RNA in the light and dark may also be a reflection of the absence of cell division in the protoplasts, in the medium in which they are being investigated in these experiments, similar to results found with cultured leaf discs (25). When assessing the effects of actinomycin D on the metabolism of RNA in the protoplasts all the experiments were conducted in the dark due to possible photo-degradation of the antibiotic (9).

From both the analysis of sliced polyacrylamide gels containing labelled RNA from pulsed first lead protoplasts (Figs. 5A, B) and autoradiographs of

![Image of polyacrylamide gel analysis]
Incorporation of \(^{(3)H}\)-uridine into cucumber first leaf mesophyll protoplasts of (A) Ashley and (B) China plants in the presence (\(-\)) or absence (\(\bigcirc\)) of actinomycin D. Two aliquots of protoplasts \((2.3 \times 10^5/ml)\) from both cultivars were pre-incubated in the light for 2 hr in 5 ml incubation medium, as in Fig. 3, the incubation medium containing 5mM NaHCO\(_3\), 3mM PEP, 3mM sucrose, pH 5.9. The protoplasts were then sedimented at 100 x g and each aliquot resuspended in similar medium (without NaHCO\(_3\)), one aliquot containing 25 \(\mu\)g/ml actinomycin D. Incubation was continued in the dark for 1 hr, when 150 \(\mu\)Ci of \(^{(3)H}\)-uridine was added to each sample. Incubation was then continued in the dark for a further 4 hr, RNA extracted from the protoplasts and subjected to electrophoresis; the gels were sliced and the radioactivity determined as in Fig. 3.

In gels containing labelled RNA from Ashley cotyledon protoplasts (Figs 4C, D), it is clear that actinomycin D (25 \(\mu\)g/ml) significantly reduced the uptake of labelled uridine into ribosomal RNA classes in first leaf and cotyledon protoplasts of both cultivars as reported in all plant cells and protoplasts so far investigated (28). Actinomycin D has been demonstrated to have a similar effect in intact cotyledons of these two cucumber cultivars, and its effect in other plant tissue, e.g. tobacco (29,30) is well documented. However, while actinomycin D almost completely inhibited ribosomal RNA synthesis in Ashley first leaf protoplasts, synthesis was reduced by only 66\% in China protoplasts.

Protoplasts prepared from cotyledons and first leaves of Ashley and China cucumbers have thus been demonstrated to synthesise ribosomal RNA...
species in culture, a de novo synthesis which is susceptible to actinomycin D. Further studies are in progress on the infection of such protoplasts with tobacco mosaic and cucumber mosaic viruses, and the effects of infection on metabolism of the host-cell.

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REFERENCES
