Characterization of Cytoplasmic and Chloroplast 5S Ribosomal Ribonucleic Acid from Broad-Bean Leaves

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Green leaves of the broad bean (*Vicia faba*) contain two 5S RNA components that can be separated from each other by polyacrylamide-gel electrophoresis. The major component is located in the larger subunit of cytoplasmic ribosomes, whereas the minor component occurs in the larger subunit of chloroplast ribosomes. Their electrophoretic mobilities relative to those of *Escherichia coli* 5S RNA (120 nucleotides) and plant 4S RNA (78 nucleotides) suggest that they consist of 118 and 122 nucleotide residues respectively. Thermal 'melting' profiles of plant cytoplasmic and chloroplast 5S RNA species at 260nm indicate the similarity of their secondary structures, not only to each other, but also to those of *E. coli* and mammalian 5S RNA species. The base compositions of the two plant 5S RNA species have more in common with each other than with the corresponding molecules from either *E. coli* or mammalian cells.

The 70S ribosomes of prokaryotes contain 23S and 16S rRNA whereas 80S ribosomes, which occur in the cytoplasm of eukaryotes, contain 28–25S and 18S rRNA (Loening, 1968). The ribosomes of chloroplasts are of the 70S class (Stutz & Noll, 1967) and their ribosomal RNA molecules are of a similar molecular weight to those of prokaryotes (Loening & Ingle, 1967). These findings have contributed to the endosymbiont theory of the origin of chloroplasts (Sagan, 1967).

Few comparisons have been made between the 5S rRNA species of different organisms, although *Escherichia coli* and mammalian 5S RNA molecules have very similar molecular weights, as shown by the determination of their nucleotide sequences (Brownlee, Sanger & Barrell, 1967; Forget & Weissman, 1967; Williamson & Brownlee, 1969). There is some evidence, however, that the two 5S RNA species of green leaves differ in size, because the chloroplast 5S RNA elutes from methylated serum albumin–kieselguhr columns at a higher salt concentration (Dyer & Leech, 1968; Ruppel, 1969), and migrates more slowly in polyacrylamide gels (Dyer, Miller & Greenwood, 1971), than the corresponding cytoplasmic ribosomal component.

In this study the 5S RNA components of broad-bean leaves were further characterized and compared with those of *E. coli* and mammals.

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MATERIALS AND METHODS

Growth of plants. Seeds of the broad bean, *Vicia faba* var. White Fan (Carters Tested Seeds Ltd., London S.W.20, U.K.), were grown in John Innes no. 1 compost in a greenhouse maintained at 25°C and supplied with a 12h photoperiod. Leaves and roots were used when the seedlings were 3–4 weeks old.

Extraction of RNA. Nucleic acid was extracted by the method of Parish & Kirby (1966) as modified by Loening (1969). The method uses sodium tri-isopropyl-naphthalenesulphonate and sodium 4-aminosalicylate as detergents and a phenol–cresol solution for deproteinization. In some instances RNA but not DNA was extracted by the method of Hastings & Kirby (1966) as modified by Loening (1969). Tissues by this method were homogenized in 0.5% (w/v) sodium naphthalene-1,5-disulphonate and a phenol–cresol solution. Tissues were disrupted by using a VirTis homogenizer.

Highly purified 5S RNA from plants was prepared as described by Payne & Dyer (1971). The method involves extraction of RNA by the naphthalenedisulphonate–phenol method, the removal of carbohydrates and polyphenols by DEAE-cellulose chromatography, the separation of high-molecular-weight RNA from low-molecular-weight RNA by high-speed centrifugation, and the separation of 5S RNA from 4S RNA by filtration through Sephadex G-100 and then Sephadex G-75. The 5S RNA was similarly isolated from *E. coli* B ribosomes and from isolated chloroplasts, with the omission of the DEAE-cellulose stage.

Polyacrylamide-gel electrophoresis. High-molecular-weight RNA was fractionated in 2.4% gels at room temperature as described by Loening (1967, 1969). Gels

were scanned at 260 nm with a 0.2 mm-width light-beam, with a modified Hilger-Gilford spectrophotometer.

Low-molecular-weight RNA was fractionated in 7.5% gels, as described by Loening (1967, 1969), or in 12.5% gels when very high resolution was required, as described by Richards, Coll & Gratzer (1965), by using 0.03 M-diethylbarbituric acid–3.6 mM-tris, pH 8.9, as the electrophoresis buffer. The 12.5% gels were stained with Toluidine Blue (Payne & Dyer, 1971) and were subsequently scanned at 630 nm. Both types of gel contained 1 cm-long spacer gels of 2.4% acrylamide.

Preparation of cytoplasmic ribosomes and their subunits. Lateral roots were detached from broad-bean seedlings, suspended in water and flushed with O₂-free N₂ for 3 h. They were then homogenized in a VirTis blender at a concentration of 4 g of roots to 40 ml of homogenization medium (0.5 M-sucrose–5 mM-MgCl₂–16 mM-KCl–50 mM-tris-HCl, pH 7.6 at 0°C). The homogenate was filtered through one layer of Miracloth and the filtrate, adjusted to 4% (w/v) with respect to Triton X-100, was centrifuged at 25 000 g_{av}. for 10 min. The supernatant obtained was centrifuged at 105 000 g_{av}. for 90 min to sediment the ribosomes. All operations were carried out at 0–4°C.

The ribosome pellet was rinsed with several portions of homogenization medium and then resuspended in a small volume of 'high-KCl buffer', which contained 3 mM-MgCl₂, 0.5 M-KCl and 10 mM-tris-HCl, pH 7.4 at 0°C. Samples (0.5 ml) of ribosome solution, with $E_{260}^{1\text{cm}}$ about 40, were loaded on to three discontinuous sucrose gradients. The gradients, which consisted of six 4 ml layers of sucrose dissolved in high-KCl buffer at concentrations of 34, 29, 24, 19, 14 and 10% (w/v), were centrifuged at 20 000 rev./min in a Spinco SW 25.1 swing-out rotor for 17 h at 0°C. After centrifugation, the base of each tube was pierced and 50% (w/v) sucrose injected to displace the gradients upwards through a coned cap with an outlet tube connected to a u.v. analyser. The extinction of the gradients at 254 nm was measured and the ribosomal subunits were collected separately.

Preparation of chloroplasts and chloroplast ribosomal subunits. Chloroplasts were isolated by a modification of the method of Cockburn, Walker & Baldry (1968). Plants were de-starched in the dark for periods of 24–48 h before isolation of chloroplasts. Laminiae (25 g) were homogenized for 5 s in a Polytron PT 35 homogenizer (The Northern Media Supply Ltd., Hull, U.K.) with 100 ml of semi-frozen grinding medium (0.33 M-sorbitol, 5 mM-MgCl₂, 1 mM-NaCl, 2 mM-sodium ascorbate and 50 mM-Hepes [2-(N-2-hydroxyethyl)piperazin-N'-yl)ethanesulphonic acid], pH 6.5 at 0°C). The homogenate was squeezed through four layers of muslin, filtered through a further eight layers and centrifuged at 4000 g_{av}. for 30 s. The chloroplast pellet was rinsed with 5 ml of homogenization medium and resuspended in a medium containing 0.5 M-sucrose, 5 mM-MgCl₂, 16 mM-KCl, 50 mM-tris-HCl, pH 7.6 at 0°C, and 4% (w/v) Triton X-100, by using a camel-hair paint brush. The Triton procedure dissolved the chloroplast membranes. Intact nuclei and other contaminating particles were sedimented by centrifuging at 25 000 g_{av}. for 10 min. All operations were carried out between 0° and 4°C.

In large-scale experiments when milligram quantities of pure chloroplast 5S RNA were isolated, 80 g batches of leaves were homogenized in an MSE Ato-Mix blender at

full speed for 10 s. The blender contained 320 ml of the same homogenization medium as described above, with 50 mM-sodium phosphate buffer, pH 6.5, instead of the Hepes buffer.

Equally pure chloroplast preparations were obtained by both methods on the basis of cytoplasmic and chloroplast high-molecular-weight RNA content (Fig. 1), though larger quantities of the labile 1.03 × 10⁶-mol.wt. chloroplast RNA component are consistently recovered by the small-scale method.

Chloroplast ribosomes were pelleted from the Triton X-100-treated chloroplast preparations by centrifugation at 105 000 g_{av}. for 2 h. Chloroplast ribosomal subunits were separated in high-KCl sucrose gradients as described for cytoplasmic ribosomal subunits.

Thermal 'melting' profiles. Thermal 'melting' profiles of purified 5S RNA preparations were determined in 0.15 M-NaCl–15 mM-sodium citrate, pH 7.2, by using a Gilford 2000 spectrophotometer fitted with a thermostat-controlled cell housing. The instrument was operated manually and, for each determination, the temperature was maintained until the extinction became constant. Less than 0.05% of the sample evaporated during an experiment.

Analysis of nucleotide base composition. Base compositions of RNA samples were determined as described by Leech, Dyer, Poole & Smith (1968) with the following modifications. RNA at a concentration of 10 µg/µl of 1 M-HCl was hydrolysed in sealed capillary tubes at 100°C for 1 h, and 10 µl samples were spotted on thin layers of cellulose (Schleicher and Schuell 'F1440 TLC-ready plastic sheets') previously washed in 2 M-acetic acid and then water. The chromatograms were developed for 18 h at 20°C with propan-2-ol–HCl–water (65:17.2:17.8, by vol.)

Fig. 1. Fractionation of the high-molecular-weight RNA components from a large-scale chloroplast preparation. RNA was subjected to electrophoresis in a 2.4% gel for 4 h at 7 V/cm length of gel. The numbers beneath the peaks refer to molecular weights in millions and the abbreviations cyt. and chl. refer to cytoplasmic and chloroplast components respectively. The relative amounts of chloroplast and cytoplasmic ribosomal RNA were calculated from the areas of the 1.28 × 10⁶-mol.wt. cytoplasmic and 0.56 × 10⁶-mol.wt. chloroplast RNA peaks (Ingle, 1968).

and regions containing the nucleic acid derivatives (purine bases and pyrimidine nucleotides) were cut out and floated, cellulose layer downwards, on 1 ml of HCl for 1 h with agitation. The concentration of the HCl was altered according to which base or nucleotide was being eluted (Leech *et al.* 1968). Dislodged cellulose particles were removed from the solutions by centrifugation and the extinctions of the clear supernatants were determined by using a Gilford 200 spectrophotometer. No corrections were made either for deamination of cytidine or for dephosphorylation of pyrimidine nucleotides, which occur to a limited extent during acid hydrolysis (Loring, 1955).

RESULTS

Localization of cytoplasmic and chloroplast 5S RNA. When a preparation of low-molecular-weight RNA from broad-bean leaves was fractionated by polyacrylamide-gel electrophoresis, two 5S RNA components separated (Fig. 2a). The major, more mobile, component was shown to be derived from the cytoplasm and the minor component from chloroplasts by the electrophoresis of low-molecular-weight RNA prepared from a non-green tissue (roots) (Fig. 2b), isolated chloroplasts (Fig. 2c) and from a mixture of these two samples (Fig. 2d). Comparable 5S RNA components were also extracted from most higher plants that have been studied so far, but two components have not been clearly distinguished in preparations from the majority of lower plants examined (R. M. Carter, P. I. Payne & T. A. Dyer, unpublished work).

Plant 5S RNA has been shown to be a constituent of cytoplasmic ribosomes (Dyer & Leech, 1968; Vedel & Guitton, 1968; Li & Fox, 1969) and chloroplast ribosomes (Dyer & Leech, 1968; Ruppel, 1969). In this study, cytoplasmic and chloroplast ribosomes were dissociated into subunits by suspension in a medium containing 0.5M-potassium chloride and 3mM-Mg²⁺ to determine which contained 5S RNA. Mammalian subunits dissociated by this method regain their biological activity on reassociation, whereas those derived by Mg²⁺-chelation methods do not (Martin & Wool, 1968). A disadvantage of this method is that 80S ribosomes that are active in protein synthesis do not dissociate into subunits (Martin & Hartwell, 1970). Accordingly, broad-bean roots were flushed with oxygen-free nitrogen to minimize their polyribosome content before ribosome extraction (Lin & Key, 1967).

Despite the presence of RNA fragments derived from high-molecular-weight RNA in the 4–5S

Fig. 2. Fractionation of the low-molecular-weight RNA component from: (a) leaves; (b) roots; (c) chloroplasts; (d) roots and chloroplasts. RNA was subjected to electrophoresis in 12.5% polyacrylamide gels at 5mA/tube. Most of the high-molecular-weight RNA was removed from the sample by high-speed centrifugation before electrophoresis.

RNA region of the gels, it is clear that 5S RNA is located solely within the larger subunits of both 70S and 80S ribosomes (Figs. 3 and 4).

Electrophoretic properties of 5S RNA from roots, leaves, isolated chloroplasts, rat liver and E. coli. Low-molecular-weight RNA extracts from *E. coli* and rat liver were subjected to electrophoresis together in 12.5% polyacrylamide gels. These 5S RNA species migrated as one band throughout the length of the gel (Fig. 5). When separately combined with plant RNA samples, each formed a band at an intermediate position between the two leaf RNA species and each migrated more slowly than root 5S RNA. The greater mobility of plant cytoplasmic 5S RNA compared with *E. coli* 5S RNA is consistent with the finding that wheat-germ 5S RNA has a slightly lower sedimentation coefficient (4.48S; Soave, Galante & Torti, 1970) than that reported for *E. coli* (4.55S) by Boedtker & Kelling (1967).

Aubert, Scott, Reynier & Monier (1968) showed that denatured *E. coli* 5S RNA could be obtained by treatment with urea, or by heating and then cooling rapidly. The denatured form eluted more slowly from Sephadex G-100 columns and migrated more rapidly in polyacrylamide gels, and could be renatured by heating the sample in the presence of 10mM-Mg²⁺ and cooling slowly. To test that denaturation of 5S RNA had not taken place during extraction in this study, low-molecular-weight RNA samples were treated by the method of Aubert *et al.* (1968) for renaturation. None of the 5S RNA species showed a change in mobility on second electrophoresis (not shown).

Thermal 'melting' profiles. Thermal 'melting' profiles were determined of purified preparations of cytoplasmic and chloroplast 5S RNA. The sample of cytoplasmic 5S RNA was virtually pure as determined by electrophoresis (Fig. 6a), whereas 6% of the chloroplast 5S RNA sample consisted of the analogous cytoplasmic component (Fig. 6b) (see the legend of Fig. 1 for the method of determination of purity). Both species had similar 'melting' spectra (Fig. 7). The loss of secondary structure resulting in the increase in absorption characteristically occurs in a more restricted temperature range than for 4S RNA. The melting temperature (*T_m*) and the increase in hypochromicity at 260nm are 56°C and 27% respectively for plant cytoplasmic 5S RNA and 57°C and 28% for chloroplast 5S RNA. These values are similar to those deter-

Fig. 3. Location of 5S RNA in cytoplasmic ribosomes. Ribosomal subunits were separated in sucrose gradients (a). The RNA of the larger (b) and smaller (c) subunit was fractionated in 7.5% polyacrylamide gels at 7V/cm length of gel for 3h.

Fig. 4. Location of 5S RNA in chloroplast ribosomes. Ribosomal subunits were separated in sucrose gradients (a). The RNA of the larger (b) and smaller (c) subunit was fractionated in 7.5% polyacrylamide gels at 7V/cm length of gel for 3 h.

mined for 5S RNA isolated from rat liver (Bachvaroff & Tongur, 1966), *E. coli* (Boedtner & Kelling, 1967) and *Blastocladiella emersonii* (Comb & Zehavi-Willner, 1967).

Analysis of nucleotide base composition. The accuracy of the base-composition method was assessed by determining the base composition of electrophoretically pure *E. coli* 5S RNA (Fig. 6c;

Fig. 5. Electrophoretic separations of mixtures of 5S RNA species prepared from various sources: (1) *E. coli* and rat; (2) plant chloroplasts; (3) rat; (4) plant cytoplasm; (5) *E. coli*; (6) plant cytoplasm. High-molecular-weight RNA was removed from the samples before electrophoresis by high-speed centrifugation.

Fig. 6. Purity of 5S RNA samples prepared from: (a) roots; (b) chloroplasts; (c) *E. coli* ribosomes. RNA was fractionated in 12.5% polyacrylamide gels.

Table 1). The results compare favourably with values calculated from the base sequence of this RNA (Brownlee *et al.* 1967).

The base compositions of the plant cytoplasmic and chloroplast 5S RNA species differ from those of either *E. coli* or human KB cells, but they are similar to each other. Particularly noticeable is the much lower G+C content of the plant RNA samples.

The base composition of chloroplast 5S RNA has not been recorded previously. Chakravorty (1969) reported the base composition of pea cytoplasmic 5S RNA to be: A, 21.2%; U, 22.0%; C, 25.0%; G, 31.8%; G+C, 56.8%. This composition is unlike that of broad-bean 5S RNA reported here, which is surprising in view of their close taxonomic relationship. However, the purity of the pea RNA was not established by polyacrylamide-gel electrophoresis and the base-composition method was not checked with an RNA sample of known nucleotide content. As ribosomes were prepared before RNA extraction, it is probable that the 5S RNA fraction, separated by Sephadex G-100 column chromatography, was contaminated with a breakdown fragment of

the labile 18S rRNA, which has mol.wt. 100000 (Payne & Loening, 1970). Our results are supported by the report by Soave *et al.* (1970). They give the base composition of purified wheat 5S RNA as: A, 23.4%; U, 22.1%; C, 25.5%; G, 28.7%; G+C, 54.2% (values that are similar to ours for the broad bean).

DISCUSSION

The results show that plant cells, in common with those of bacteria and animals, contain 5S RNA as a constituent of the larger ribosomal subunit.

The thermal 'melting' profiles of plant cytoplasmic and chloroplast 5S RNA are similar not only to each other but also to those published for 5S RNA from *E. coli* and mammals. This indicates that the number and length of the helical regions in these molecules, together with the distribution and degree of G·C and A·U base-pairing, are alike. As, in addition, the G+C contents of these RNA species do not vary greatly (Table 1), it is likely that, in common with high-molecular-weight RNA species, their mobilities in polyacrylamide gels are inversely related to the log of their molecular weights (Loening, 1969). This is supported by the finding reported here that rat liver and *E. coli* 5S RNA, both of which have nucleotide chain lengths of 120 (Williamson & Brownlee, 1969; Brownlee *et al.* 1967) and hence very similar molecular weights, migrate together in 12.5% polyacrylamide gels.

If it is assumed that plant 4S RNA has mol.wt. 25000 and *E. coli* 5S RNA 38750, then, the molecular weight of plant cytoplasmic 5S RNA is chloroplast is 39400 (122 nucleotide residues). The 37900 (118 nucleotide residues) and that of the base compositions of these RNA species were taken into account in the calculations involved.

Unlike the corresponding high-molecular-weight RNA species (Loening, 1968), 5S RNA has not increased in molecular weight during evolution. The most advanced species (mammals) have RNA with the same molecular weight as the most primitive

Fig. 7. Thermal 'melting' profiles at 260nm of root 5S RNA (●), chloroplast 5S RNA (○) and root 4S RNA (▲). The spectrophotometer cell had a 1cm light-path and contained approx. 40 µg of RNA/ml.

Table 1. Base composition of 5S RNA from various sources

The experimental errors are expressed as standard deviations. The base compositions of *E. coli* and KB-cell 5S RNA were calculated from their nucleotide sequences deduced by Brownlee *et al.* (1967) and Forget & Weissman (1967) respectively.

Source	Composition (mol%)					No. of preparations	No. of determinations
	A	U	C	G	G+C		
<i>E. coli</i> M.R.E. 600 (calculated)	19.2	16.7	30.0	34.1	64.1	—	—
<i>E. coli</i> B (experimental)	20.8±0.8	17.0±0.7	28.8±0.4	33.4±0.3	62.3	1	6
KB cell (calculated)	18.2	22.3	27.3	32.2	59.5	—	—
Plant cytoplasm	24.1±0.7	23.5±0.8	23.8±0.6	28.6±1.1	52.4	2	11
Plant chloroplast	26.5±0.8	22.2±1.0	22.6±0.8	28.7±0.8	51.2	2	6

organism studied (*E. coli*), whereas plant cytoplasmic 5S RNA is smaller than these two and chloroplast 5S RNA is larger. Separations of mixed 5S RNA preparations from different species have been reported. Comb & Zehavi-Willner (1967) separated *E. coli* and *Blastocladiella* 5S RNA species by DEAE-cellulose chromatography at 90°C; the latter was eluted at a higher salt concentration and presumably had a greater molecular weight. Tartof & Perry (1970) showed that *Drosophila* 5S RNA migrated fractionally faster in polyacrylamide gels than that obtained from mouse L cells.

The primary structures of the two plant 5S RNA species were compared with those from bacteria and mammals by determination of nucleotide base compositions (Table 1). Those from *E. coli* and human KB cells, calculated from their nucleotide sequences (Brownlee *et al.* 1967; Forget & Weissman, 1967), are similar, although differences in the proportions of C and particularly U are evident. There is less resemblance between the 5S RNA species from plant cytoplasm and *E. coli*, as all bases are present in different proportions. Plant cytoplasmic 5S RNA is also different from human 5S RNA, though it has in common a relatively high proportion of U. In contrast, it has a similar base composition to chloroplast 5S RNA, the main difference being a slightly higher A content, but there are no obvious similarities between chloroplast and *E. coli* 5S RNA.

The significance of these differences in electrophoretic mobility and base composition in relation to the endosymbiont hypothesis for the origin of chloroplasts (see Sagan, 1967) cannot be established until the variability of 5S RNA structure within the prokaryotes and of chloroplasts from different species is known. Nevertheless, broad-bean chloroplast 5S RNA, although generally similar to analogous RNA from other organisms, has properties which are unique.

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