Synthesis and Stability of Chloroplast Ribosomal—RNAs

J. Ingle
Botany Department, King's Buildings, Edinburgh University, Edinburgh, Scotland

Received May 14, 1968.

Abstract. The chloroplast ribosomal-RNAs (1.1 × 10^6 and 0.56 × 10^6 mol wt) are synthesized in the normal ratio of 2:1. The non-ribosomal distribution observed after extraction and fractionation results from the liability of the 1.1 × 10^6 component, and a correction for this breakdown can be applied in certain cases. Newly synthesized 1.1 × 10^6 RNA is more stable than the older accumulated 1.1 × 10^6 RNA. Accumulation of the chloroplast RNA during growth of radish cotyledons occurs at a later time than the accumulation of cytoplasmic RNA, and its turnover is much less than that of the cytoplasmic ribosomal-RNA.

The presence of low and high molecular weight RNA components in the chloroplast, distinct in size from those of the cytoplasm, has been established in higher plants (3,8,11). The molecular weights of the large RNA component, 1.1 × 10^6 (23s) and 0.56 × 10^6 (16s), which are similar to those of bacterial ribosomes (8), and the fact that they can be prepared from isolated chloroplast ribosomes (11), suggest that these components are the chloroplast ribosomal-RNAs. However, the usual presence of a third component with a molecular weight of 0.40 × 10^6 (13s) (8), and the relative proportions of the chloroplast RNAs are not obviously correlated with ribosome structure. The ratio of 1.1 m to 0.56 m RNA is always less than 2, the value expected for ribosomal-RNAs. The values reported vary considerably, from a maximum value of around 1 (3,8,9,11) to the case where no 1.1 m RNA was present (10). This low, variable ratio of the RNA components, together with the fact that 1.1 m RNA is known to be unstable (8), suggests that the peculiar distributions which have been observed with chloroplast RNA preparations may be the result of a selective breakdown of the heavy chloroplast component. This paper describes experiments on the synthesis and stability of the chloroplast and cytoplasmic RNAs in the radish cotyledon, which, from a range of tissues previously studied was found to have relatively unstable chloroplast RNA (8). The timing of chloroplast and cytoplasmic RNA synthesis during development of the radish cotyledon was also determined.

Materials and Methods

Radish seedlings (Raphanus sativus L. var. Cherrybelle) were grown in a 16 hour photoperiod of 1600 ft-c at 22.5° as previously described (5). Although sterile techniques were employed, complete sterility was not always obtained. Samples of the cotyledons were routinely homogenized in water and plated on a peptone:yeast extract agar. The contamination varied from 0 to 1000 bacteria per pair of cotyledons in the different experiments.

Corn and barley were grown in soil under a 16 hour photoperiod of 600 ft-c at 22.5°, and leaf tissue was used after 10 to 14 days.

Total nucleic acid was extracted using either the sodium lauryl sulphate-phenol method or the naphthalene 1:5 disulphonate-tri-isopropynaphthalene sulphonate-p-amino-salicylate:phenol method and fractionated by polyacrylamide gel electrophoresis as previously described (8). The RNA components are referred to in terms of their molecular weight, since the relationship between electrophoretic mobility and log molecular weight has been shown to be linear with a range of viral RNAs (1) and with ribosomal RNAs (unpublished results). The relationship is largely independent of gel concentration and the structural properties of the RNA (8, and unpublished results). The fractionations were quantitated by measurement of the areas of the peaks, which were expressed as a percentage of the total area and related to the μg nucleic acid loaded onto the gel.

Synthesis of RNA in radish cotyledons was measured by the incorporation of 32P orthophosphate. Samples of cotyledons (10–30 pairs, dependent on size) were excised and incubated for 6 hours with continuous shaking under 400 ft-c at 25°, in 7 ml...
of medium containing 10 mM KCl and 250 μc 32P orthophosphate (neutralized to pH 7). After an incubation of this duration most (70-80%) of the high molecular weight radioactivity is ribosomal RNA (4). The other 20 to 30% of the RNA, the rapidly-labeled D-RNA (4), remains polydisperse on the gel, and is corrected for as the background level subtracted when calculating the radioactivity in the ribosomal RNA peaks. Experiments in which the 6 hour 32P orthophosphate incubation was followed by a 1 hour chase in 31P phosphate showed only a slightly lower background of polydisperse RNA, and gave identical results after correction. The cotyledons were then washed in 0.1 mM phosphate, pH 7.0, followed by distilled water, and total nucleic acid was prepared and fractionated. After scanning the gel at 265 μm, it was frozen in solid CO2, maintaining the scanned length of the gel, and then sliced into 0.5 mm sections using a modification of the McIlwain chopper (7). The sections were dried and counted using an automated Geiger counting system.

Chlorophyll content was determined on an 80% acetone extract and expressed as 655 μm OD per pair of cotyledons.

Results

Observed Changes in RNA Components During Development of the Radish Cotyledon. The change in total nucleic acid content of the radish cotyledon over a 6 day germination period is shown in figure 1. The major increase occurs over a relatively short period, during days 2 and 3, after which the amount of nucleic acid remains fairly constant for 2 days until decreasing on day 6. At least 80% of the total nucleic acid is accumulated before the cotyledons emerge on day 3 to day 4. The emergence and greening of the cotyledons is indicated in figure 1 by their chlorophyll content, which increases at a linear rate from the fourth day.

Gel electrophoretic fractionation of the RNA prepared from cotyledons of day 1 to 6 seedlings is shown in figure 2. The 265 μm OD scan represents the RNA accumulated prior to the time of sampling, and the radioactivity represents the RNA synthesized during a 6 hour period after excision of the cotyledons at various sampling times. The main components accumulated in the cotyledons of day 1 seedlings are the cytoplasmic ribosomal-RNAs, 1.3 M (25s) and 0.70 M (18s) and the low molecular weight transfer (4s) and 5s RNAs. There are small amounts of 0.56 M and 0.40 M RNAs, which are characteristic of the chloroplast, but there appears to be very little of the larger chloroplast component, 1.1 M RNA. The amount of this RNA is difficult to estimate due to the presence of a 0.98 M component, which appears to be characteristic of the dry seed, forming about 6% of the total RNA in both the cotyledon and embryo axis of the seed. The amount of the 0.98 M RNA decreases during growth, being 4% and 1% of the total RNA at days 1 and 2 respectively, and not being detected thereafter. The major change in the accumulation of RNA components during growth is the increase in the chloroplast RNAs, 1.1 M, 0.56 M, and 0.40 M, relative to the cytoplasmic ribosomal-RNA.

The cotyledons from day 1 seedlings synthesize only the cytoplasmic RNAs (1.3 M and 0.70 M) and low molecular weight RNA, as measured by the incorporation of 32P phosphate (fig 2). On days 2, 3 and 4 the cotyledons synthesize the chloroplast RNAs (1.1 M, 0.56 M, and 0.40 M) as well as those of the cytoplasm. Maximum synthesis of the chloroplast RNAs, relative to cytoplasmic, occurs on day 3, when about 30% of the incorporated radioactivity is in these components, and their specific activity is twice that of the cytoplasmic components. The cotyledons from day 5 and 6 seedlings, in which there is no further net increase in total nucleic acid (fig 1), still incorporate considerable radioactivity into the cytoplasmic RNAs but very little into the chloroplast components. The fractionations of RNA prepared from day 2, 3, and 4 seedlings show that although the ratio of the accumulated 1.1 M to 0.56 M RNAs (265 μm OD) is less than 1, the ratio of the newly synthesized RNAs (radioactivity) is much higher, approaching the expected value of 2 for ribosomal-RNA.

Stability of the RNA Components. The fractionations shown in figure 2 may be quantitated by the summation of the radioactive peaks, and the measurement of the areas of the 265 μm OD peaks. These values are expressed as the ratios of the heavy to light ribosomal-RNAs for the cytoplasmic and chloroplast components (table I). The observed ratio for the accumulated chloroplast RNAs (1.1:0.56) is less than 1, whereas for the newly synthesized chloroplast RNAs it is much higher, though still below the expected value, being fairly constant

![Fig. 1. Change in total nucleic acid and chlorophyll content during the development of the radish cotyledon. Total nucleic acid and chlorophyll were determined at daily intervals as described in Methods.](image-url)
at 1.4 to 1.5. In addition to the chloroplast ratio being very low, the ratio of the accumulated cytoplasmic RNAs (1.3:0.70) is also lower than expected, starting at 1.4 and dropping even lower during growth of the cotyledon, when chloroplast RNA is accumulated. (The maximum ratio of 1.3:0.70 obtainable was 1.8 rather than 2, due to non-linearity of the scanning under the conditions of loading and scanning used in these experiments.) The newly synthesized cytoplasmic RNAs are present with a ratio of 2 on day 1, but this value drops to 1.6 by day 3, when there is maximum chloroplast RNA synthesis, and then increases back to the theoretical value. The additional 0.40 M component, which is present in large amounts when there is little 1.1 M RNA (compare day 5 with days 4 and 6 in fig 2), and the decrease in the cytoplasmic ratio when chloroplast RNA is accumulated or synthesized,
Table 1. Chloroplast and Cytoplasmic Ribosomal-RNAs During the Development of the Radish Cotyledon

The amounts of the individual RNAs were determined from the fractionations in figure 2 and, expressed as the ratio of heavy to light components, are shown as the observed results. Corrected ratios were calculated assuming a breakdown of the 1.1 M RNA into 0.70 M plus 0.40 M pieces. The percentage breakdown of the 1.1 M RNA was determined from the observed and corrected values for this component. RNA accumulation was determined from the 265 mrm OD and newly synthesized RNA was determined from the incorporation of radioactivity.

<table>
<thead>
<tr>
<th>Age of seedling (days)</th>
<th>RNA accumulated</th>
<th>1.1 M breakdown</th>
<th>Newly synthesized RNA</th>
<th>Corrected</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Observed</td>
<td>Corrected</td>
<td>% 1.1 M</td>
<td>Observed</td>
</tr>
<tr>
<td></td>
<td>1.1 M: 0.56 M</td>
<td>1.3 M: 0.70 M</td>
<td>correction</td>
<td>0.56 M</td>
</tr>
<tr>
<td>1</td>
<td>1.44</td>
<td>1.67</td>
<td>1.64</td>
<td>0.56 M</td>
</tr>
<tr>
<td>2</td>
<td>1.44</td>
<td>2.08</td>
<td>1.65</td>
<td>0.40 M</td>
</tr>
<tr>
<td>3</td>
<td>1.35</td>
<td>1.97</td>
<td>1.60</td>
<td>0.40 M</td>
</tr>
<tr>
<td>4</td>
<td>1.19</td>
<td>1.77</td>
<td>1.71</td>
<td>0.40 M</td>
</tr>
<tr>
<td>5</td>
<td>1.00</td>
<td>1.97</td>
<td>2.11</td>
<td>0.40 M</td>
</tr>
<tr>
<td>6</td>
<td>1.17</td>
<td>1.85</td>
<td>1.92</td>
<td>0.40 M</td>
</tr>
</tbody>
</table>

Fig. 3. Degradation of radish chloroplast RNA. Total nucleic acid was prepared from day 6 radish cotyledons and fractionated for 3 hours (A). The sample remaining from A, dissolved in the electrophoresis buffer, was frozen and thawed 3 times, and fractionated (B). RNA prepared from a chloroplast preparation, which was the 1000g pellet from an homogenate of day 6 radish cotyledons in a medium containing 0.25 M sucrose, 25 mM tris pH 7.4, 2 mM Mg acetate and 1 mM Cleland Reagent, was fractionated for 2.5 hours (C).

suggests that the chloroplast 1.1 M RNA may be breaking down into 0.70 M plus 0.40 M pieces. If the observed results are corrected for such a breakdown, using the size of the 0.40 M RNA to correct both the chloroplast 1.1 M (observed 1.1 M + observed 0.40 M X 1.1:0.4) and the cytoplasmic 0.70 M RNA (observed 0.70 M - observed 0.40 M X 0.7:0.4), then the ratios, both accumulated and newly synthesized, of cytoplasmic and chloroplast RNAs are close to the values expected for ribosomal-RNA (table I). It should be noted that small errors in the measurement of the 0.40 M peak will be amplified in making this correction.

This particular pattern of breakdown of the 1.1 M RNA into 0.70 M plus 0.40 M pieces, which occurs in these experiments, is in fact a simplified case of chloroplast RNA degradation. If radish RNA, dissolved in the electrophoresis buffer, is frozen and thawed several times, there is a loss of the 1.1 M RNA and an increase in the 0.40 M component. There is also the appearance of several new components, such as 0.48 M, 0.22 M and a trace of 0.90 M (fig 3, a and b). If the correction is applied to this RNA, then although the chloroplast ratio is suitably increased (table II), indicating that the assumed split into 0.70 M and 0.40 M has occurred, the cytoplasmic ratio is over-corrected, suggesting secondary breakdown of the 0.70 M product, perhaps into 0.48 M plus 0.22 M components. Similar treatment of pea root tip RNA, which contains only the cytoplasmic components, shows that very little degradation of the cytoplasmic 1.3 M and 0.70 M RNAs occurs, indicating that practically all of the degradation observed with total leaf RNA is the result of chloroplast RNA breakdown. An extreme case of secondary breakdown occurs when RNA is extracted from a chloroplast preparation of radish cotyledons (fig 3c). The 0.40 M RNA is the major component. This shows that much of the 0.70 M and also some of the 0.56 M RNAs have broken down during the isolation.
Table II. Chloroplast and Cytoplasmic Ribosomal-RNA Ratios of Corn, Barley, and Radish Preparations

The amounts of the individual RNA components were determined from the fractionations in figures 3 and 4, and are shown as the observed results. Corrections were applied assuming a breakdown of the 1.1 M RNA into 0.70 M plus 0.40 M pieces.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Observed 1.1 M</th>
<th>0.56 M</th>
<th>RNA accumulated 1.3 M</th>
<th>0.70 M</th>
<th>Corrected 1.1 M</th>
<th>0.56 M</th>
<th>1.3 M</th>
<th>0.70 M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Radish, 6 day (fig 3)</td>
<td>0.56</td>
<td>1.44</td>
<td>1.34</td>
<td>1.94</td>
<td>0.29</td>
<td>1.29</td>
<td>1.70</td>
<td>2.46</td>
</tr>
<tr>
<td>Control</td>
<td>0.99</td>
<td>1.61</td>
<td>1.83</td>
<td>2.35</td>
<td>0.97</td>
<td>1.62</td>
<td>1.89</td>
<td>2.80</td>
</tr>
<tr>
<td>Degraded</td>
<td>0.77</td>
<td>1.56</td>
<td>1.50</td>
<td>1.85</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corn (fig 4)</td>
<td>2.04</td>
<td>1.92</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Barley (fig 4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Radish, 3 day (fig 4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Newly synthesized RNA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 The control RNA sample, in the electrophoresis buffer, was frozen and thawed 3 times, and then rerun.

down, since the 0.40 M component should be only two-thirds of the 0.56 M RNA, and only four-sevenths the size of the 0.70 M RNA, assuming essentially no cytoplasmic contamination as indicated by the small amount of 1.3 M RNA present. In addition there are 0.08 M, 0.31 M, 0.22 M and smaller RNAs.

This method of correction does not operate with some plant tissues, such as corn and barley leaves. RNA preparations from these tissues show low levels of 1.1 M RNA and the presence of the 0.40 M component (fig 4a and b), but although the chloroplast ratio is corrected to a reasonable value (table II), the cytoplasmic ratio is over-corrected, suggesting that the 0.70 M breakdown product is further degraded. Both tissues contain some 0.48 M RNA (fig 4).

Breakdown of the newly synthesized (radioactive) 1.1 M RNA is much less than that of the accumulated 1.1 M RNA (265 mg OD). This is shown in figure 2 (day 3) where the specific activity of the 0.40 M RNA is much lower than that of the 1.1 M RNA, from which it is assumedly derived. The data in table I show that whereas 60% or more of the accumulated 1.1 M RNA is degraded, only 25 to 30% of the newly synthesized 1.1 M RNA is so broken down.

This difference in stability can be seen more readily with better RNA preparations than those obtained in the experiments described. The observed ratios of the newly synthesized ribosomal-RNAs, both cytoplasmic and chloroplast are 2 (fig 4c, table II), but the accumulated chloroplast RNA ratio is still low, and there is accumulation of 0.40 M RNA which contains essentially no radioactivity. Under these rather better conditions of RNA preparation the degradation of the RNA seems to be rather different, since, after correction, the ratio of accumulated chloroplast RNAs is still low (table II).

Changes in RNA Components During Growth

![Diagram](image-url)
of the Radish Cotyledon After Correcting for Lability of 1.1 M RNA. The accumulation of the individual RNAs (after making corrections for breakdown of the 1.1 M RNA) during the growth of the cotyledon is shown in figure 5. Accumulation of the cytoplasmic RNAs is very rapid, and maximum content per pair of cotyledons is reached by day 3, after which the content slowly decreases. Chloroplast RNA accumulation lags behind that of the cytoplasm, reaching its maximum value by day 5, and then decreasing. The maximum chloroplast RNA content is normally 35 to 40% of the total ribosomal RNA. The value of 50% chloroplast RNA for day 5 cotyledons (fig 5) may be high due to error in the correction factor, which is particularly large with this sample (see fig 2).

Discussion

The contamination present in these experiments varied from 0 to 1000 bacteria per pair of cotyledons. Since a bacterium contains around 2 \times 10^{-14} g of RNA, this would account for a maximum content of 2 \times 10^{-11} g bacterial RNA per pair of cotyledons, which contain at least 20 \mu g chloroplast RNA, i.e., 2 \times 10^{-3} g. Consequently, although bacterial RNA contamination would not be detected in the fractionation in the presence of similar-sized, chloroplast RNA, the million-fold excess of chloroplast RNA present must render any contribution by bacterial-RNA insignificant, both in terms of accumulation of RNA, and with the incorporation of radioactive precursor into RNA. This is further substantiated by the fact that no differences in chloroplast RNA synthesis were observed in experiments which differed a thousand-fold in their bacterial contamination.

Although the normal observed distribution of high molecular weight chloroplast RNAs is far from that expected for ribosomal-RNA (3, 8, 9, 10, 11), i.e., a 2:1 ratio of the 1.1 M to 0.56 M RNAs, the incorporation of radioactive precursors shows that these 2 components are in fact synthesized in this 2:1 ratio (fig 4, table II). Similar results have been obtained in radish using \(^3\)H-uridine and \(^14\)C-carbon dioxide as the precursor, and also with whole plants of Phaseolus vulgaris (U. E. Loening, unpublished results). With RNA from the radish cotyledon there appears to be an initial breakdown of the 1.1 M RNA into 0.70 M plus 0.40 M pieces, and if a correction is made for such a breakdown then both the synthesis and accumulation of chloroplast RNAs is close to that expected for ribosomal-RNAs (table I). Such a correction seems to apply only to the initial stages of breakdown of the 1.1 M RNA, which in the case of radish RNA are themselves relatively stable. Under other conditions of breakdown, and in other tissues, such as corn and barley, further breakdown occurs (fig 3 and 4, table II) such that this correction is no longer valid. The chloroplast ribosomal-RNAs are therefore synthesized and accumulated in the normal manner, with a ratio of 1.1 M to 0.56 M of 2, but the lability of the 1.1 M RNA results in non-ribosomal distributions after extraction and fractionation. The cytoplasmic RNAs, 1.3 M and 0.70 M, and the smaller chloroplast component 0.56 M, are stable under conditions which degrade the 1.1 M RNA. Since 1.1 M RNA from bacteria is stable, chloroplast 1.1 M RNA lability cannot be simply related to the molecular size of the RNA. Another example of extreme lability of an RNA component is that of the large cytoplasmic ribosomal RNA of Euglena (2, 12, 13).

The differences in the specific activities of the 1.1 M RNA and its assumed breakdown product, 0.40 M, indicate a large difference in the stability of the newly-synthesized 1.1 M RNA, as measured by incorporation, and the older 1.1 M RNA, measured by accumulation of 265 m\(^4\) OD. The results in table I show that whereas there is 60% breakdown of the older 1.1 M RNA, newly synthesized 1.1 M RNA is broken down by less than 30%. Similarly, better preparations of RNA show no breakdown of the newly synthesized component, but there is still about 50% breakdown of the accumulated 1.1 M RNA. Such results suggest that during maturation or aging of the ribosome some change occurs which influences the future stability of the 1.1 M RNA. This may be due to (A) a change in the ribosome itself, such as an increase in ribosomal-ribonuclease activity during aging, which could render the RNA prepared from older ribosomes more subject to degradation than RNA from younger ones. (B) changes in the secondary or tertiary structure of the RNA which would effect its subsequent stability or (C) an in vivo occurrence of breaks in the RNA, which is held together within the ribosome structure but separates into pieces when it is extracted and fractionated. A somewhat similar difference between newly synthesized and older RNA has been
observed with the 0.70 m RNA of soybean hypocotyl (6). In this case there is some physical change in the RNA such that older 0.70 m RNA aggregates much more than the newly synthesized component under the high salt conditions of methylated-albumin kieselguhr chromatography.

During growth of the radish cotyledon the accumulation of chloroplast RNA occurs rather later than the accumulation of cytoplasmic ribosomal-RNA. Approximately 60% of the chloroplast RNA is accumulated after the maximum cytoplasmic RNA content has been reached (fig 5). The synthesis of the chloroplast RNAs, as measured by the incorporation of radioactive precursor, occurs during only a relatively short period, on days 2, 3, and 4. On days 5 and 6, when there is no net increase in either cytoplasmic or chloroplast RNA, 32P orthophosphate is incorporated into the cytoplasmic RNA, but not into that of the chloroplast (fig 2). This indicates that the turnover of chloroplast ribosomal-RNA is less than that of the cytoplasmic ribosomal-RNA, suggesting that, in this sense, the ribosomes of the chloroplast are more stable, or are more conserved, than cytoplasmic ribosomes. The differences in synthesis and stability, shown by these experiments, suggest that different control mechanisms exist for the regulation of chloroplast and cytoplasmic RNAs.

Acknowledgments

The author thanks Dr. U. E. Loening for helpful discussions throughout the course of this work, and Professor R. Brown for his interest and encouragement.

Literature Cited