A METHOD FOR EXTRACTING INTACT CHLOROPLAST AND CYTOPLASMIC RIBOSOMAL RNA FROM LEAVES

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SUMMARY: A method is described for extracting intact chloroplast and cytoplasmic ribosomal RNA from leaves of two higher plant species. Sodium dodecyl sulfate (1%) and 25 mM magnesium ions are required to inhibit ribonuclease action during RNA purification by phenol deproteinization. The ethanol-precipitated RNA product, including 23s chloroplast ribosomal RNA, is completely stable during electrophoresis in the absence of magnesium ions, even in the presence of EDTA. The in vivo mole fraction of chloroplast ribosomes relative to cytoplasmic ribosomes is estimated. Bentonite is shown to cause preferential losses of chloroplast RNA during extraction.

INTRODUCTION

Both cytoplasmic (25s and 18s) and chloroplast (23s and 16s) ribosomal RNA (rRNA) can be analytically resolved by electrophoresis on polyacrylamide gels (1, 2). Gel Analysis of purified RNA has shown that successful extraction of intact chloroplast rRNA from leaves is rarely achieved (1, 3-8), since chloroplast 23s rRNA is unusually susceptible to degradation. This paper reports a method for extracting intact chloroplast and cytoplasmic rRNA from higher plant leaves.

METHODS

Plant material: Tobacco (Nicotiana tabacum L. var Samsun) and jack bean (Canavalia ensiformis L. (DC.)) were grown under greenhouse conditions. Young tobacco leaves 3 to 5 cm long and primary leaves of 9 to 12 day-old jack beans were harvested, immediately

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wrapped in aluminum foil, and frozen between two blocks of dry ice.

RNA preparation: RNA was purified in the cold or at room temperature with an extraction buffer consisting of 1% SDS, 25 mM KCl, 25 mM Tris-HCl (pH 7.5), and 25 mM $MgCl_2$. Frozen leaf tissue was immersed in equal volumes of buffer (10 ml/g leaf fresh weight) and 90% phenol and immediately homogenized for 60 seconds. The preparation was shaken vigorously for 30 minutes and then centrifuged for 10 minutes at 12,000 x g. The phenol layer was aspirated and the aqueous phase, interphase, and pellet were shaken for 15 minutes with one-half volume of phenol followed by centrifugation. The aqueous phase was carefully collected and re-extracted with one-half volume of phenol followed by 15 minutes shaking and centrifugation. The aqueous phase from the third extraction was collected, and solid NaCl was added to 2.0 M final concentration. Two volumes of 95% ethanol were added and the nucleic acids precipitated overnight at -20°C. The precipitate was collected and washed successively with 95% ethanol and two changes of 100% ethanol. The final purified RNA pellet could then be used directly for gel analysis, stored for several months at -20 under 100% ethanol, or kept for at least a year after lyophilization without detectable degradation. RNA purified by this method routinely had an absorbance ratio $(A_{260}/A_{280}) \geq 2.1$.

Electrophoretic analysis of RNA: Chloroplast and cytoplasmic RNA species were separated on agarose-acrylamide gels by the method of Bourque and Naylor (9). Electrophoresis was normally for two hours in the cold at 5 ma/gel using the tris-phosphate-EDTA buffer of Loening and Ingle (1) without the addition of SDS, which was found to have no beneficial effect on the stability of RNA during electrophoresis.

Calculation of Molar ratios of RNA species and ribosomes: From the molecular weights (2,9) of cytoplasmic rRNA of 1.29×10^6 (25s) and 0.67×10^6 (18s) and that of chloroplast rRNA of 1.06×10^6 (23s) and 0.55×10^6 (16s), it follows that

$$X_{cyt} = A_{25s}/A_{18s} = 1.29/0.67 = 1.93 \quad (1)$$

and

$$X_{chl} = A_{23s}/A_{16s} = 1.06/0.55 = 1.93 \quad (2)$$

where X_{cyt} and X_{chl} are expected maximum values for the ratios of high to low molecular weight rRNA extracted from cytoplasmic and chloroplast ribosomes respectively, and A represents the area under each peak of undegraded RNA on a gel trace of 260 nm absorbance.

On an equivalent mass basis (i.e., number of nucleotide bases per molecule), the weight percentages of chloroplast rRNA (W_{chl}) and cytoplasmic rRNA (W_{cyt}) of the total high molecular weight rRNA (chloroplast plus cytoplasmic rRNA) observed in a gel absorbance profile is given by

$$W_{chl} = (A_{23s} + A_{16s})/(A_{25s} + A_{18s} + A_{23s} + A_{16s}) \times 100 \quad (3)$$

and

$$W_{cyt} = 100 - W_{chl}. \quad (4)$$

Since there is one large and one small RNA molecule in each 70s chloroplast and 80s cytoplasmic ribosome (5,10-13), one mole of chloroplast rRNA (Mol. wt.$_{23s}$ + Mol. wt.$_{16s}$ = 1.61×10^6 daltons) is equal to 0.827 moles of cytoplasmic rRNA (Mol. wt.$_{25s}$ + Mol. wt.$_{18s}$ = 1.96×10^6 daltons). Therefore, one mass unit of chloroplast rRNA represents 1/0.827 or 1.21 times the number of moles present in an equal mass of cytoplasmic rRNA. The mole percentages of chloroplast rRNA (M_{chl}) and cytoplasmic rRNA (M_{cyt}) expressed in terms of weight percentages are

$$M_{chl} = (1.21\ W_{chl}/(1.21\ W_{chl} + W_{cyt})) \times 100 \quad (5)$$

and

$$M_{cyt} = 100 - M_{chl} \quad (6)$$

and can be obtained from analysis of gel absorbance profiles.

RESULTS

Magnesium requirement for intact chloroplast RNA: When leaf RNA

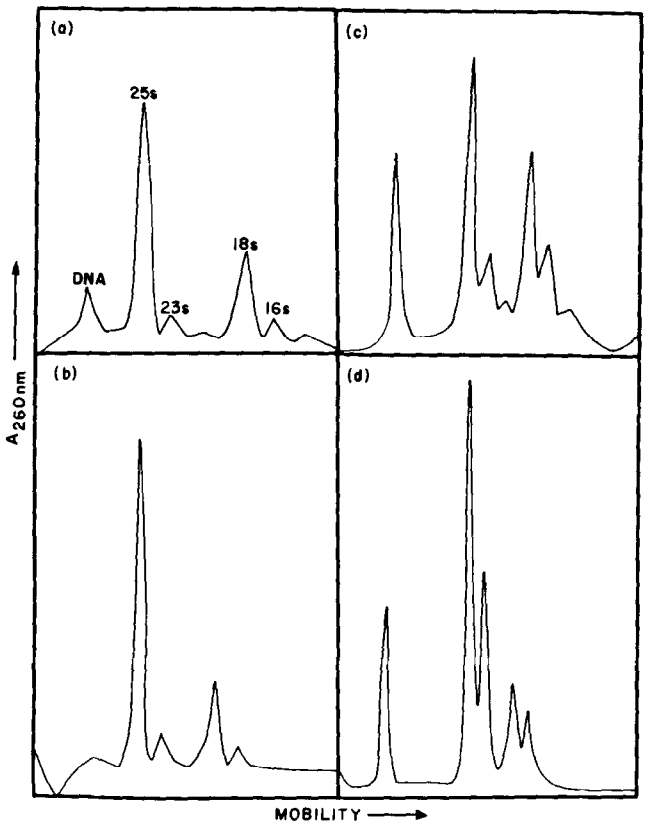

Figure 1. Electrophoretic profiles of jack bean and tobacco ribosomal RNA extracted with 10mM or 25mM Mg buffer as follows: (a) 10mM Mg extraction, jack bean RNA; (b) 25 mM Mg extraction, jcak bean RNA; (c) 10 mM Mg extraction, tobacco RNA; (d) 25 mM Mg extraction, tobacco RNA. Jack bean RNA(a,b) extracted in the presence of bentonite. Tobacco RNA (c,d) extracted without bentonite.

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from tobacco or jack bean was prepared by extraction with buffer containing 10 mM $MgCl_2$, chloroplast rRNA was highly degraded (Fig. 1a,c) and low ratios of 23s/16s RNA (X_{chl}, Table 1) were obtained. Similar results have been obtained by other investigators when only 10 mM magnesium was present in RNA extraction media and the same EDTA-containing electrophoretic buffer system (1-8) was used. Leaf RNA extracted with our standard buffer containing 25 mM $MgCl_2$ reproducibly yielded intact chloroplast 23s rRNA. Absorbance ratios (Fig. 1(b,d), X_{chl} in Table 1) of 23s/16s chloroplast RNA approached the predicted value of 1.93 within limits of experimental error

(±10%) in most cases. Although the average values of X_{chl} were usually lower than 1.93, when it exceeded 1.7 no evidence of degradation products was obtained from either A_{260}nm absorbance profiles or inspection of gels in which the RNA was quantitatively stained

Table 1. Effect of magnesium concentration and bentonite on (1) the intactness of cytoplasmic and chloroplast rRNA as measured by the absorbance ratios of high and low molecular weight RNA (X_{cyt} and X_{chl}), (2) the weight percentage of chloroplast rRNA (W_{chl}), and (3) the mole percentage of chloroplast rRNA (M_{chl}). Parameters calculated as described in Methods from area measurements of RNA peaks resolved by gel electrophoresis. Molecular weights of tobacco ribosomal RNA species were the same as that reported for jack bean (9).

A. Magnesium requirement for intact jack bean chloroplast RNA

$MgCl_2$	bentonite	X_{cyt}	X_{chl}	W_{chl} (%)	M_{chl} (%)	number of preparations
		(average values)				
10mM	+	1.9	1.0	17	20	9
25mM	+	2.5	1.9	13	15	11

B. Magnesium requirement and effect of bentonite on extraction of tobacco chloroplast RNA.

$MgCl_2$	bentonite	X_{cyt}	X_{chl}	W_{chl} (%)	M_{chl} (%)	number of preparations
		(average values)				
10mM	-	1.5	0.7	30	34	2
25mM	+	2.2	1.6	27	31	4
25mM	-	2.1	1.8	58	63	12

with methylene blue (9).

Effect of bentonite on mole fraction of chloroplast RNA: Jack bean RNA extracted with buffer containing bentonite (50 mg/ml) had low (13-20%) proportions of chloroplast rRNA and ribosomes as measured, respectively, by the weight (W_{chl}) and mole (M_{chl}) percentages (Table 1(A)). Similar values for the relative amounts of chloro-

plast rRNA have been reported for Phaseolus(14), but the mole percentage of 70S chloroplast ribosomes in Phaseolus estimated from the data of Boardman(15) was about twice (30-35%) that obtained for jack bean. Since it was suspected that the combination of high magnesium concentration and bentonite (16) caused selective losses of chloroplast rRNA, RNA was extracted from tobacco leaves with and without bentonite using 25 mM $MgCl_2$ buffer. In addition to finding a higher proportion (Table 1(B)) of chloroplast rRNA than observed in jack bean RNA, the percentage of chloroplast rRNA was doubled (Fig. 1(d), Table 1(b)) when bentonite was omitted from the procedure. Estimates of in vivo ribosomal proportions (Table 1(B)) indicate that chloroplast ribosomes represent as much as 63% of the ribosomes in a young, rapidly growing tobacco leaf.

DISCUSSION

Our results clearly demonstrate that the integrity of purified 23S chloroplast rRNA in solution is not dependent on magnesium concentration as others have concluded (6,7), since our samples were prepared for electrophoresis and electrophoretic separations were carried out in the absence of magnesium ions. In addition, the intactness of the chloroplast RNA in our preparations is confirmed by the lack of degradation products of 23s rRNA during electrophoresis in buffer containing EDTA. EDTA has been shown to reveal hidden strand breaks in RNA which are thought to be stabilized by magnesium during purification (6,7).

We have obtained intact chloroplast 23s RNA from pea leaves as well as from tobacco leaves infected with tobacco mosaic virus. This method may also prove to yield intact chloroplast rRNA from other plants. Furthermore, since all ribosomal RNA species remain intact, accurate measurements of molar ratios and labeling kinetics can be obtained from gel analysis in the absence of degradative artifacts.

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