Isolation of Plant Mitochondrial RNA from Green Leaves

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1. Introduction

In plant cells, mitochondrial RNA (mtRNA) constitutes about only 1% of the total RNA. From this, most are ribosomal RNAs. Thus, isolation of high-purified mtRNA is necessary not only for construction of a mitochondrial cDNA library, but also for the analysis of plant mitochondrial transcription. Several methods have been frequently used for isolation of plant mtRNA (1–3). However, these mtRNA preparations may be heavily contaminated by chloroplast RNA (cpRNA), especially when mtRNA is isolated from green leaves (1,4). It is believed that the cpRNA sticks to the mitochondrial membrane and therefore persists after gradient purification of mitochondria. Although micrococcal nuclease would be the enzyme to remove the non-mtRNA from mitochondrial membranes prior to lysis of mitochondria, treatments with micrococcal nuclease for the mtRNA isolation from green leaves have not been effective (4).

We report here a modified procedure of mtRNA isolation based on the combination of RNase A/guanidine thiocyanate/CsCl centrifugation. In our procedure, mitochondria are first separated from other subcellular components, such as nuclei and plastids by differential centrifugation of leaf homogenates. The crude mitochondria are further purified by sucrose gradient centrifugation. To eliminate cpRNA, the purified mitochondria are treated with RNase A. Subsequently, RNase A is inactivated and mitochondria are lysed by adding guanidine thiocyanate in high concentration. As a strong protein denaturant, guanidine thiocyanate can inactivate nucleases very efficiently (5). mtRNA is pelleted through a CsCl gradient. Finally, coprecipitated single-stranded DNA in the CsCl gradient can be removed from mtRNA by LiCl precipitation (6).
2. Materials (see Notes 1 and 2)
1. 5.7M CsCl solution: 10 mM EDTA, pH 7.5, DEPC-treated.
2. Denaturation buffer: 50% formamide, 12% formaldehyde, 1X MOPS buffer (40 mM MOPS, 10 mM sodium acetate, 1 mM Na₂-EDTA, pH 7.0), freshly mixed before use.
3. 1 mg/mL Ethidium bromide: DEPC-treated, storage at −20°C.
4. Extraction buffer: 0.35M sorbitol, 50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 0.1% BSA, 0.25-mg/mL each spermine and spermidine, storage at 4°C, then add β-mercaptoethanol to 0.2% (final concentration) just before use.
5. 4M Guanidinium thiocyanate: in 100 mM Tris-HCl, pH 7.5 (storage at 4°C), add β-mercaptoethanol to 1% (final concentration) just before use. Storage at 4°C.
6. 7.5M Guanidinium-HCl: 10 mM DTT, pH 7.5 (adjusted with NaOH), filtrate, storage at 4°C.
7. 2M and 4M LiCl: DEPC-treated, storage at 4°C.
8. Loading buffer: 50% glycerol, 0.25% bromophenol blue, 1 mM EDTA, DEPC-treated, storage at −20°C.
9. 10X MOPS buffer: 0.4M MOPS, 0.1M sodium acetate, 10 mM Na₂-EDTA, pH 7.0, DEPC-treated.
10. 2M potassium acetate: pH 5.5, DEPC-treated.
11. 2M Sodium acetate: pH 7.0, DEPC-treated.
12. 5% Sodium lauryl sarcosinate
13. TE buffer: 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, DEPC-treated.
14. Wash buffer: 350 mM sorbitol, 50 mM Tris-HCl, pH 8.0, 20 mM EDTA.

3. Methods

3.1. Isolation of Mitochondria

All steps must be carried out at 4°C in a cold room. Solutions, bottles, and so on, should be kept in wet ice.

1. Harvest 20 g of 4-6-wk-old fresh green leaves from rapeseed or other plants, cut into small segments, and chill in 200 mL ice-cold extraction buffer.
2. Homogenize leaf tissue in a Waring blender at high speed three times (each time 5 s with 10 s breaks in between). Filter the homogenate through two layers of Miracloth into 250-mL cold centrifuge bottles.
3. Centrifuge the filtrate at 2000g for 10 min in a swing out rotor. Carefully transfer the supernatant to new bottles and centrifuge at 10,000g for 20 min in a swing out rotor.
4. Resuspend pellet in 100 mL extraction buffer and repeat step 3 once again.
5. Resuspend the mitochondrial pellet in 20 mL ice-cold wash buffer.
6. Carefully layer each 10 mL mitochondrial suspension on top of a sucrose step gradient (9 mL 0.9M/11 mL 1.5M/9 mL 1.75M in wash buffer) and centrifuge for 60 min at 80,000g in a swing out rotor (see Note 3). Collect the mitochondria from the 0.9M/1.5M sucrose interface (yellow band) with wide-bore pipets and then dilute with 5 vol of wash buffer over a period of 15–20 min (see Note 4).
7. Pellet the mitochondria by centrifugation at 10,000g for 20 min in a swing out rotor and resuspend in 1 mL ice-cold wash buffer.

3.2. Isolation of mtRNA

1. Coincubated mitochondria with 20 μg/mL RNase A for 60 min on the ice.
2. Add 5 vol of 4M guanidine thiocyanate solution to the mitochondria, then add 0.5 vol 5% sodium lauryl sarcosinate after 60 s at room temperature and mix by vortexing. Centrifuge the mixture at 5000g for 5 min in a swing out rotor to remove insoluble debris.
3. Layer each 3.2 mL mixture onto a 1.1 mL cushion of DEPC-treated 5.7M CsCl solution.
4. Carry out ultracentrifugation at 22,000g for 14 h in a swingout rotor.
5. Carefully aspirate the supernatant solution and cut off the top part of the centrifuge tube that was in contact with the homogenate (all steps should avoid contamination of finger RNase).
6. Dissolve the RNA pellet by extensive vortexing in 1 mL 7.5M guanidinium-HCl solution.
7. Add 0.05 vol of 2M potassium acetate pH (5.5) and 0.5 vol of ethanol to the mixture.
8. Incubate at −20°C for 4 h and precipitate the mtRNA at 5000g for 10 min in a swingout rotor.
9. Precipitate the recovered mtRNA by adding 0.1 vol of 2M sodium acetate (pH 7.0) and 2.5 vol of ethanol. Store at −20°C overnight. Centrifuge for 30 min at 10,000g.
10. Wash the mtRNA pellet with 70% ethanol, vacuum dry, and dissolve in 0.5 mL TE.
11. To obtain mtRNA free from single-strand DNA, add an equal volume of 4M LiCl to dissolved RNA, incubate at 4°C overnight, and collect the mtRNA by centrifugation at 10,000g for 20 min in a microcentrifuge. Wash once with 2M LiCl and two times with 70% ethanol.
12. Vacuum dry the mtRNA and dissolve in 50 μL DEPC-treated water. Estimate the yield of mtRNA by measuring the absorbance at 260 nm (see Chapter 12).
13. For long-term storage, then add 0.3 vol of sodium acetate and 2.5 vol of ethanol to the mtRNA, and store at −70°C. Precipitate the RNA just before use.

This mtRNA preparation procedure will yield 0.3–0.5 μg mtRNA per gram fresh leaves. As a control, MS2 phage RNA (Boehringer, Mannheim) may be treated using the same conditions as mtRNA isolation. We have tested that the isolated RNA is intact (Fig. 1) and the contaminating cpRNA is totally eliminated (Fig. 2).

3.3. Formaldehyde Gel Electrophoresis (see Notes 6 and 7)

1. Melt 3.75 g of agarose in 220 mL DEPC-H2O plus 30 mL 10X MOPS buffer. After the agarose is cooled to 60°C, add 50 mL of 37% (12.3 M) formaldehyde solution and pour the gel.
Fig. 1. Analysis of mtRNA preparation by electrophoresis in a 1.25% agarose-6% formaldehyde gel. Lane 1: mtRNA (5 µg) isolated with RNase treatment. Lane 2: mtRNA (5 µg) isolated without RNase treatment. Lane 3: MS2 phage RNA (10 µg) with RNase treatment. Lane 4: MS2 phage RNA (10 µg) without RNase treatment but using the conditions of mtRNA isolation. Lane 5: 10 µg MS2 phage RNA was directly loaded onto the gel. Lane m: RNA-ladder (BRL).

Fig. 2. Northern blot analysis of plant mtRNA preparations. The blots were probed with mitochondrial specific (rrn18), chloroplast-specific (psaA), and nucleus-specific (rbcS) gene probes. Lane 1: mtRNA (5 µg) isolated with RNase treatment. Lane 2: mtRNA (5 µg) isolated without RNase treatment. Lane m: RNA ladder (BRL).
2. Denature 9 μL RNA (5–10 μg) by adding an equal vol of RNA denaturation buffer, and incubate at 65°C for 5 min.
3. Add 1 μL of 1 mg/mL ethidium bromide, incubate at 65°C for an other 5 min, and place samples on ice for 5 min.
4. Add 2 μL of loading buffer to each sample and load on the prepared gel.
5. Carry out electrophoresis at 5 V/cm for 3–4 h. Soak the gel in DEPC-treated H₂O for 20 min to remove the formaldehyde, and photograph the gel.
6. Further Northern blot analysis can be carried out according standard methods (7) (see Chapters 15 and 16).

4. Notes
1. Wear disposable plastic or latex gloves during work. All reagents should be used for RNA work only and kept free of ribonuclease. All glassware should be baked at 160°C for 4 h. All plasticware to be used after the step of guanidinium thiocyanate treatment should be soaked in 0.2% DEPC for 12 h and autoclaved. All solutions to be used after the step of guanidinium thiocyanate treatment should be treated with 0.2% DEPC for 12 h and autoclaved.
2. DEPC is a carcinogen. DEPC-treatment of solutions and plasticware should be done in a chemical hood.
3. For sucrose gradient centrifugation, the prepared gradient should be allowed to equilibrate at 4°C overnight. After gradient centrifugation, wash buffer should be slowly added to collected mitochondria (over 15–20 min), this can minimize the osmotic shock.
4. For CsCl centrifugation, when different ultracentrifuge rotors are used, pay attention to maximum rotor speed and maximum run time.
5. The bulk of the DNA is removed using CsCl centrifugation. Since single-stranded DNA coprecipitates with the RNA in the CsCl gradient, 2M LiCl precipitations are necessary to obtain pure RNA preparations.
6. Formaldehyde is very toxic. Preparation and running of formaldehyde gels should be done in a chemical hood.
7. Reagent-grade formamide can be used directly. However, if any yellow color is present, formamide should be deionized by stirring it for 1 h with 5% (w/v) resin 501-X8 (D) (Bio-Rad). After filtration through Whatman No. 1 paper, deionized formamide should be stored in small aliquots at −70°C.

References

