Purification of mitochondria and mitochondrial nucleic acids from embryogenic suspension cultures of a gymnosperm, Larix × leptoeuropaea

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Received 18 July 1994/Revised version received 20 October 1994 – Communicated by G. Pelletier

Abstract

After a number of attempts to isolate mitochondria from different conifer tissues, embryogenic suspension cultures of hybrid larch (Larix × leptoeuropaea) were developed which enabled the purification of mitochondria using slight modifications to standard techniques. The mitochondrial purity was verified by analysis of the mitochondrial RNA, DNA and proteins. The larch mitochondrial genome size is surprisingly large (> 1000 kbp) and the polypeptide pattern differs greatly from those of wheat or potato mitochondria, suggesting that valuable evolutionary insights will be gained from comparisons between gymnosperm and angiosperm mitochondria. The ease with which embryogenic conifer suspensions can be initiated and used for mitochondrial purification implies that they will be the material of choice for future studies of this type.

Introduction

Mitochondria are essential for the correct functioning of plant cells and their molecular biology constitutes an increasingly important area of plant biology (Brennicke and Kück 1993). Biochemical, physiological and genetic studies on angiosperm mitochondria have shown that this organelle is semi-autonomous with a wide variation in genome size and complexity between various plant species (Bonen and Brown 1993), ranging from the 200 kbp genomes of some Brassica (Palmer and Herbon 1987) to a reported 2400 kbp for muskmelon (Ward et al. 1981), with an average size of 400-500 kbp. Growing interest in the evolution of mitochondria necessitates detailed information on mitochondrial genomes from plant species representing other evolutionary groups, but in this regard hardly any data is available. This gap remains despite the complete sequencing of the mitochondrial genome from the bryophyte Marchantia polymorpha (Oda et al. 1992). Detailed analyses on the mitochondrial genomes of gymnosperms have been hampered by the unavaliability of a suitable mitochondrial purification method. The few published reports, concerning mainly inheritance patterns (Neale and Sederoff 1989; Sutton et al. 1991; Wagner et al. 1991; DeVerno et al. 1993) or the confirmation of the occurrence of RNA editing (Glaubitz and Carlson 1992) have relied on PCR methods or hybridization to detect mitochondrial DNA in crude preparations of total nucleic acids. Further advances require pure mitochondrial nucleic acid, but except for isolation of mitochondrial DNA (mtDNA) from an embryogenic culture of white spruce (Sutton et al. 1991) a successful separation of experimentally pure mitochondrial nucleic acids from a gymnosperm plant species has not been reported to our knowledge. This white spruce mtDNA was reported to contain contaminating plastid DNA and no restriction digest or hybridization patterns were shown, although the preparation did enable the cloning of two mitochondrial genes.

In an ongoing effort to catalogue the differences in the tRNA coding potential of mitochondrial genomes of gymnosperms, monocotyledenous and dicotyledenous plants, we have developed a method for isolation of mitochondrial nucleic acids from suspension cell cultures of a gymnosperm (Larix). Here the details of this method are provided along with experimental evidence supporting the fact that the mitochondrial components obtained (nucleic acids and proteins) are sufficiently pure for detailed molecular analyses.

Materials and Methods

Plant Materials

The experiments were conducted with a highly embryogenic line (ixd 69-3) of hybrid larch (Larix kaempferi × Larix decidua = L. × leptoeuropaea). Embryonal masses were obtained from mature somatic embryos (Lelu et al. 1994b) and transferred to proliferation medium which consisted of MSG (Becwar et al. 1990) containing 10 mM glutamine and supplemented with 9 μM 2,4-D, 2.25 μM BA and 60 mM sucrose (solidified with 4 g/l Gelrite). Approximately 10 to 12 pieces of embryogenic masses (1 cm in diameter) were cultured per Petri dish. Embryogenic masses were incubated in darkness at 25 ± 1 °C and subcultured every 2 weeks.

Establishment and subculture of embryogenic suspensions

Embryogenic suspensions were established from embryogenic masses 7 days after subculture on the proliferation medium. Small pieces of embryogenic masses were transferred to 250 ml Erlenmeyer flasks containing 20 ml of liquid culture medium of the same composition as that used for the proliferation but lacking gelling agent. The initial
inoculum density was about 2 grams fresh weight per flask. Cultures were placed on a rotary shaker (100 rpm) and maintained in darkness at 25 ± 1 °C. After the first week of culture, 20 ml of fresh medium was added to the flask. Subsequently, the suspension was subcultured weekly by adding 20 ml of cell suspension to 20 ml of fresh medium. Sustained growth was established in 3 weeks. For mitochondrial isolation, saturated 10 day old cultures of established cell lines were diluted 8-fold into fresh medium, and grown for 7-8 days.

**Mitochondrial purification**

Cells from one litre of suspension cultures were pelleted at 6000 x g (6000 rpm, Kontron A 6.14 rotor). The pellet (about 100 g) was mixed with acid-washed sand and 10 ml of extraction buffer (0.4 M sucrose, 50 mM Tris-HCl pH 7.5, 10 mM EDTA, 5 mM B-mercaptoethanol, 0.5% (w/v) BSA) and ground in a pre-chilled pestle and mortar. After thoroughly mixing the mixture, the volume of the homogenate was raised to 150 ml with extraction buffer, mixed and filtered through four layers of Miracloth (Calbiochem). The unbroken cell mass was carried through this procedure again. The filtrate was centrifuged at 15000 x g (3000 rpm, Kontron A 6.14 rotor) for 10 minutes and the pellet discarded. The supernatant was recentrifuged at 33000 x g (4300 rpm, Kontron A 6.14 rotor) and then 3500 x g (4600 rpm, Kontron A 6.14 rotor) for 10 minutes each and the pellets discarded. The liquid phase was spun at 24000 x g (12000 rpm, Kontron A 6.14 rotor) for 20 minutes to collect the crude mitochondrial pellet. Most of the buffer was removed and the pellet completely resuspended in 1 ml of resuspension buffer (0.4 M mannitol, 50 mM Tris-HCl pH 7.5, 0.5% (w/v) BSA) with a fine brush. Whereas a small part of this resuspended mix was saved as a crude mitochondrial fraction (P), the rest was layered on to three-step Percoll (Pharmacia) gradients prepared in 13 ml capped polypropylene tubes (4.0 ml 50%, 5.0 ml 25%, 3.5 ml 14% (w/v) Percoll in 0.25 M sucrose and 50 mM Tris-HCl pHe 7.5) and centrifuged at 17000 x g (12000 rpm, Kontron A 6.14 rotor) for 20 minutes without brake. Particulate fractions were collected: the top of the 14% step as interface 1 (I1), 14/25% as interface 2 (I2), 25/50% as interface 3 (I3) and the pellet (B). The volume of each fraction was raised with 5-6 volumes of resuspension medium and spun at 17000 x g (12000 rpm, Kontron A 8.24 rotor) for 20 minutes to collect the pellets. The analyses showed that I1 was most enriched in mitochondria. Maize (Zea mays) and muskmelon (Cucumis melo) mitochondria were isolated from 4-5 day old etiolated seedlings using the same procedure.

**Mitochondrial DNA isolation**

The mitochondrial pellet from I1 was resuspended in 1 ml of resuspension medium and treated with DNAse on ice for one hour by adding the enzyme at a final concentration of 100 µg/ml and 20 mM MgCl2. The volume was then raised to 30 ml with resuspension medium and the mitochondria pelleted as above. The pellet was lysed and treated with proteinase K (100 µg/ml) at 37 °C for 30 minutes in 0.5% (w/v) N-lauroylsarcosine, 100 mM EDTA, 100 mM Tris-HCl pH 8.0, and mtDNA was further purified by centrifugation in a CsCl/ethidium bromide gradient.

**Nuclear DNA isolation**

For nuclear DNA, larch nuclei were pelleted by spinning the initial homogenate at 350 x g (1500 rpm, Kontron A 6.14 rotor) for 10 minutes and the pellet processed according to Rivin et al. (1982). This DNA was used to assess the extent of nuclear DNA contamination in the mitochondrial DNA preparations.

**Mitochondrial RNA isolation**

Pellets of fractions P, I1, I2, I3 and B of larch and I3 of wheat were lysed directly in 100 mM NaCl, 10 mM Tris-HCl pH 9.0, 12.5 mM EGTA pH 7.5 and 1% (w/v) SDS. RNA was purified by chloroform-phenol extraction followed by ethanol precipitation. The RNA was pelleted, washed with 70% (v/v) ethanol and resuspended in DEPC-treated water.

**Total RNA extraction**

Total RNA was isolated from young tree needles of larch and etiolated wheat seedlings by the guanidine hydrochloride method (Logemann et al. 1987).

**Mitochondrial proteins**

A few microliters of water were added to the pellet from fraction I3 of larch and wheat, and the quantity of protein assayed using Bradford's reagent (Biord). For Southern blot analysis, 2 µg of digested DNAs were electrophoresed in 1 x TBE (0.045 M Tris-borate, 0.001 M EDTA) and blotted on to Hybond-N nylon membrane (Amersham) by capillary blotting in 10 x SSC with the procedure described by Sambrook et al. (1989). Prehybridization and hybridization was carried out in 6 x SSC, 50% (v/v) formamide, 0.1% (w/v) N-lauroylsarcosine, 0.02% (w/v) SDS and 2% (w/v) blocking agent (Boehringer Mannheim) at 42°C. Two post-hybridization washes were conducted in 2 x SSC with 0.1% (w/v) SDS at 55°C. Final washing was done in 0.1 x SSC with 0.1% (w/v) SDS at 55°C for 1 hour. Hybridized blots were exposed to X-ray film in the presence of an intensifying screen at -70°C. For northern blot analysis, RNA samples were electrophoresed on a 1.2% (w/v) agarose gel containing 6% (v/v) formaldehyde and transferred to Hybond-N+ (Amersham) by capillary blotting in 20 x SSC. Prehybridization, hybridization and washings were similar to those for Southern blot analysis.

**Hybridization probes**

A 1.276 kb HaeIII restriction fragment including nucleotides -41 to 1235 of the wheat mitochondrial coxl gene (Bonet et al. 1987) was purified by the Glassmill method (Bio101) and labelled with [α-32P]dCTP (Amersham) according to the manufacturer's instructions using the Random Primer Labelling kit (Pharmacia). Labelled antisense transcripts of Arabidopsis thaliana cytosolic 3.85S rRNA (a cDNA clone kindly provided by T. Desprez) were prepared by incorporation of [α-32P]UTP using a transcription kit (Promega).

**Protein analysis**

Five μg of mitochondrial protein from larch and wheat were analysed by SDS-PAGE (Laemmli 1970) on 10% gels and stained with Coomassie blue. For two-dimensional (2-D) electrophoresis, 40 μg of larch mitochondrial proteins were precipitated in 80% (v/v) acetone, pelleted, dried and resuspended according to Rémy and Ambard-Brettet (1987). The first dimensional IEF and second dimensional SDS-PAGE were resolved essentially according to the procedure described by Colas des Francis-Small et al. (1992) and the gels stained with Coomassie blue.