

A Rapid and Efficient Method for the Isolation of Mitochondrial DNA From Wheat Crop

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Abstract

The mitochondrial DNA was isolated from wheat (*Triticum aestivum*) crop in combination of different centrifugation and density gradient ultracentrifugation, DNaseI enzyme treatment, sucrose sedimentation; lysis with SDS and potassium proteinase. Pure mitochondrial DNA was successfully obtained by phenol/chloroform/isoamyl alcohol extraction to remove protein, and digested with RNase phenol/chloroform extraction method. To detect mitochondrial DNA purity, specific primers were designed for nuclear (β -actin) and mitochondrial (COXII) gene. Isolated mitochondrial DNA is pure, suitable for PCR and genetic analysis.

Keywords: wheat, yellow etiolated shoots, mitochondrial DNA isolation

1. Introduction

Mitochondria play an important role in the development and reproduction of plant, encoding necessary proteins involved in the system of energy production. They occupy a specific evolutionary pattern relative to nuclear counterparts (Lynch et al., 2006). Numerous metabolic pathways at cellular site of mitochondria are essential for higher eukaryotic life (Logan, 2006; Reichert, 2004). Principal pathways are the tricarboxylic acid cycle, respiratory electron transfer and ATP synthesis (Logan, 2006; Barrientos, 2003). Isolation of mtDNA is particularly difficult and protocols developed for one species often do not work for other species (Hanson et al., 1986). This phenomenon is due to intact plant tissue may be the abundance of phenolic compounds which are known to exist in differentiated plant tissues. Moore and Proudlove (1983) reported that phenolic compounds bind strongly to mitochondrial membranes and are thought to be destructive to the integrity of the mitochondria. The protocol presented in this paper evolved from a protocol described by Li et al. (2007) for wheat crop and may serve as a guide for the development of other mtDNA isolation protocols.

2. Material and Method

2.1 Plant Material

In this investigation wheat (*Triticum aestivum*) line 1376 was used. The seed was soaked in water for 6 hours, Wheat yellow etiolated seedlings were grown in dark over a period of 7 to 10 days at 30°C in growth chamber and they meet their growth needs by on nutrients only supply sterile water. The plants were strictly protected from light. All the cultural practices were done in dark, prevent them to become green and get a high quality mtDNA which is free from chloroplast, genomic DNA and other impurities.

2.2 Mitochondria Isolation

We isolated mitochondria, ground 20 g of fresh etiolated yellow leaf, in high ionic-grinding buffer [50 mmol/L Tris-HCl pH=8, 0.3 mol/L mannitol, 0.2 mol/L sucrose, 25 mmol/L EDTA pH=8, 0.1% bovine serum albumin (BSA), 0.6% Polyvinylpyrrolidone (PVP) and 0.1% β -mercaptoethanol], BSA, PVP and β -mercaptoethanol added before use; 10 ml/g tissue; filtered it with 6 layer of Mira cloth. Centrifuged at 3500 rpm for 10 minutes on 4°C discarded the chloroplast and nuclear DNA pellets. The supernatant was centrifuged at 10 000 rpm for 20 minutes on 4°C. Re-suspended the pellets in buffer solution (50 mmol/L Tris-HCl pH=8, 0.3 mol/L mannitol, 0.2 mol/L sucrose, 0.1% BSA and 0.6% PVP) gently used soft paint brush, centrifuged at 3500 rpm on 4°C for 10 minutes discarded the nuclei pellets. Supernatant was centrifuged at 10 000 rpm for 20 minutes at 4°C. To eliminate nuclear DNA, the mitochondrial pellet was re-suspended in 10 ml of cold buffer (0.2 mol/L sucrose, 50 mmol/L Tris-HCl pH=8) added 100 μ l MgCl₂ (1 mol/L), 8 μ l DNaseI (50 ng/ml) (Sigma) enzyme and Incubated on ice for

90 minutes. The reaction was stopped by added EDTA- Na_2 to a final concentration was 0.2 mol/L. Washed the organelles from 25 ml buffer (50 mmol/L Tris-HCl pH=8, 0.3 mol/L mannitol and 0.6 mol/L sucrose) and Collected the organelle pellets of mitochondria by centrifuged at 10 000 rpm for 20 minute, at 4°C.

2.3 Mitochondrial DNA Isolation

The mitochondrial pellets was re-suspended in lysis buffer (25 mmol/L Tris-HCl pH=8, 25 mmol/L EDTA , 5% SDS, 0.1 mol/L NaCl and 0.1% β -mercaptoethanol) and added 100 $\mu\text{g/ml}$ potassium proteinase, incubated at 50°C and then 37°C on each temperature for 60 minutes. The mtDNA was cleaned with 2 mol/L NH_4 -acetate, phenol/chloroform/isoamyl alcohol (25:24:1), pH 6.7 and centrifuged at 18 000g for 5minutes on 4°C. (Repeat the step). mtDNA was washed with Chloroform/isoamyl alcohol (24:1) and Centrifuge at 18 000g for 5 minute at 4°C. RNA was removed from the samples with 10 $\mu\text{g}/\mu\text{l}$ DNase-free RNase enzyme (Sigma) and incubated at 37°C for 60 minutes. Purified the mtDNA by added ice cold anhydrous ethanol, 1/10th of 3mol/L CH_3COONa and incubated at -70°C for 60 minutes. Pelleted mtDNA by centrifuged at 18 000g for 15minutes on 4°C. mtDNA was washed with ethanol 70% (2 times) and 96% (1 time). Dry at room temperature, re-dissolved in TE-buffer (10mmol Tris and 1mmol EDTA (pH 8.0) and store at -20°C.

2.4 Quality Test

mtDNA Quality were measured at absorbance ratio A260nm, A280nm and calculated optical density (OD) value, used UV spectrophotometer for purity analysis.

2.5 Polymerase Chain Reaction and Electrophoresis

PCR reactions were performed in 20 μl volumes with 50 ng mtDNA, 6 μl ddH₂O, 1 μl (forward and reverse) primer of each specific-gene (β -actin and COXII gene) and 10 μl of master mix. Performed in an Eppendorf Mastercycler using the following program: 94°C for 5 min followed by 5 cycles at 94°C for 1 min, 60°C for 30 sec and 70°C for 1 min, 35 cycles of 50 sec at 94°C, 1min at 60°C, and 50 sec at 72°C and final extension at 72°C for 6 min. PCR products were separated on 1% (w/v) agarose gels electrophoresis and ethidium bromide staining.

3. Result and Discussion

The mtDNA was extracted from yellow etiolated shoots (20 g) of wheat crop (Scotti et al., 2001), used the method of Li et al. (2007) with some amendments. Removed the genomic, Plastid DNA by DNaseI enzyme treatment. Proteinase k was used to remove the protein. RNA was removed by RNase enzyme treatment. The mtDNA, OD values!ratio of A260/A280 was in between 1.81 to 1.99, at A260/A230 1.90 to 2.0 and the concentration of mtDNA/ μl was in between 559 to 590 (Table 1) which indicates the good quality mtDNA (Figure 1).

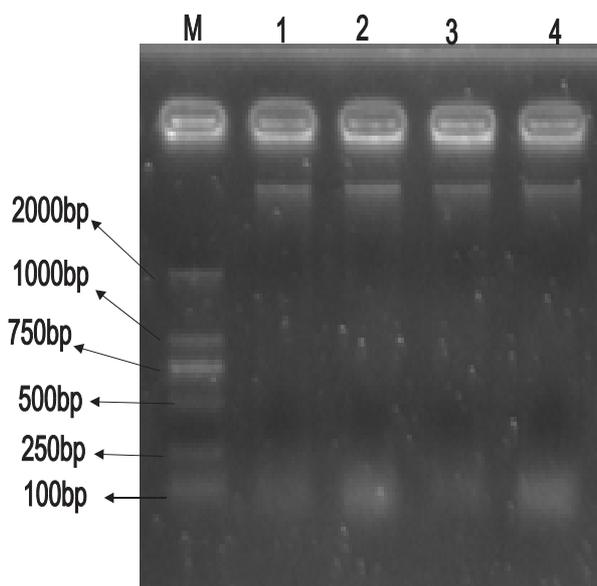


Figure 1. Results of mtDNA

M: Marker, Wheat line 1376 (1-4).

Purified mitochondrial pellets from genomic DNA organelles applied DNase I enzyme treatment. This DNase I enzyme concentration can be adjusted as needed for the different plant species until the nuclear DNA amplification is undetectable (Christine et al., 2004). For the mtDNA purity assessment, we analyzed samples through amplification of nuclear β subunit (β -actin) actin genes and the mitochondrial C oxidase subunit (COXII) gene. The products were separated on a 1% (w/v) agarose gel electrophoresis. Mitochondrial COXII gene 400bp was amplified and β -actin gene was not amplified, so the mtDNA was not contaminated by nuclear DNA, plastid DNA, RNA, protein, and was successfully used for PCR, cloning and southern blot analyses (Figure 2).

Table 1. Spectrophotometer values of mtDNA

Sr.No.	A260/A280	A260/A230	ng/ μ l
1.	1.99	2.00	559
2.	1.81	1.90	590
3.	1.92	1.99	585
4.	1.89	2.00	569

Values of Wheat line 1376 (1-4).

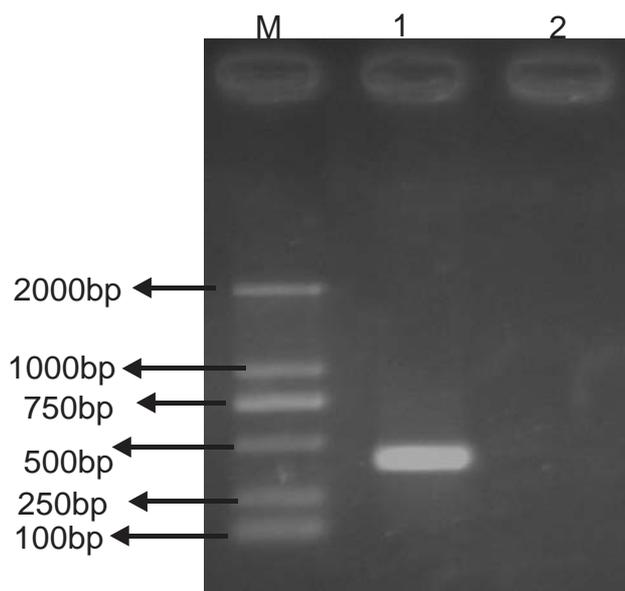


Figure 2. M. Marker 1. COXII gene 2. β -actin gene

M: Marker, Wheat line 1376 (1-2).

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