Korean J. Ginseng Sci.
Vol. 14, No. 2, 310–316 (1990)

The Characterization of Mitochondrial DNA of Korean Ginseng (*Panax ginseng* C.A. Meyer)

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Abstract □ This study was focused on the characterization of mitochondrial DNA (mtDNA) for molecular genetical approach of energy production related mechanism in *Panax ginseng*. The simple and efficient method of mtDNA isolation from ginseng has been developed by modification of recently advanced methods. This procedure can successfully apply to mtDNA isolation of several plants. MtDNA of etiolated shoot and one-year root were digested with restriction endonucleases, but that of 6-year root not. Any difference was not observed in the restriction endonuclease digestion patterns among the ginseng variants. Molecular size of ginseng mtDNA was estimated at least 159 kb by the restriction endonuclease fragment analysis. The 4.5 kb extra band at the lane of EcoRII treatment could be observed in restriction patterns digested with the methylation sensitive endonucleases, BstN1 and EcoRII. For construction of mitochondrial genomic library of ginseng, mtDNA was partially digested with EcoRI, and packaged with EMBL4 phage vector. Genomic library was screened and purified for further research including restriction mapping of ginseng mtDNA, and cloning of the genes. The gene of ATP synthase A subunit was cloned from the purified EMBL4 library clone No. 16. Now, clone No. 16 is subcloned for structure gene sequence analysis.

Introduction

Since mitochondrial DNA (mtDNA) was isolated from vertebrate mitochondria[2,15,20], Suyama and Bonner[23] isolated DNA from mitochondrial fractions derived from a number of *angiosperm* plants. Since this finding, a great deal of interest has been stimulated concerning the isolation of mtDNA from several species such as maize[11], wheat[7,18], *Nicotiana*[26], *Petunia*[1], etc.

This interests in higher plant mtDNA has focused research on the range of complexity in mitochondrial genomes, cytoplasmic male sterility, molecular mapping of mitochondrial genomes, isolation of mitochondrial structural and non-structural genes, and molecular polymorphisms in mtDNA restriction fragment profiles.

The mitochondrial genome of higher plants is significantly larger and more complex than the mitochondrial genomes of other groups of organisms. They have a heterogeneous array of molecules, mostly linear, with a low percentage of circles[9,24], and exhibit very complex restriction endonuclease digestion patterns. Therefore, in spite of extensive research on mitochondrial genomes, plant mitochondrial genomes are the most poorly understood not only with respect to their structure but also in their coding capacity.

Korean ginseng (*Panax ginseng* C.A. Meyer) is one of the rarest of medicinal herbs in prescriptions of traditional Oriental medicine, with the mysterious efficacies which works on "all-healing". Korean ginseng grows so slowly and has the low productivity. In order to improve its low productivity, physiological characteristics of ginseng plant as responces to environmental factors were intensively investigated during last decade[10,17]. However, the informations on the genetical and molecular biological researches were very rare, and the molecular biological approach on mtDNA of ginseng has not been reported.

This study was focused on the characterization and cloning of mitochondrial genome for molecular genetical information of ginseng mtDNA.

Materials and Methods

Materials

The experiment was conducted with 1 year old root, seed, and callus of *Panax ginseng* obtained from Korea Ginseng and Tobacco Research Institute. The maize (*Zea mays*) was used as a control. Pancreatic DNAse I, pronase K, restriction endonuclease, and electrophoresis grade agarose were perchased from commercial source including Bethesda Research Laboratories and International Biotechnological Inc. All other chemicals were analytical grade or higher grade.

Mitochondrial DNA (MtDNA) isolation from ginseng and maize

The isolation procedure of mitochondria was used with some modifications of Hsu and Mullin method[6]. *Panax ginseng* and maize seeds were germinated in five and three rolls of germination paper (Anchor Paper Co.), respectively, in the dark at room temperature. Two-week and 5-day old etiolated shoots of ginseng and maize, respectively, were used for homogenization. The purification methods of ginseng mitochondria was described by Lim[12].

Mitochondrial DNA was then isolated by a series of precipitation steps described in Lim and Kim[13]. After lysis with SDS, the 1.6 m*l* 5M potassium acetate was added. After incubation at 0 °C for 20 min, this mixture was centrifuged at 10,000 × g for 5 min. Supernatant was poured into a solution (3.2 m*l* chroloform/octanol and 0.32 m*l* 5M ammonium acetate) and vortexed. After centrifugation at 10,000 × g for 5 min, the upper layer was transferred to a siliconized Corex centrifuge tube, mixed with 2 volume of ethanol, and stored at −70 °C for 30 min. After centrifugation at 10,000 × g for 5 min, the mitochondrial DNA was pelleted, washed 70% ethanol, and dried.

Construction of genomic library of ginseng mtDNA

For construction of genomic library, mtDNA of ginseng was partially digested with EcoRI. After agarose gel electrophoresis of partial digested mtDNA, DNA ranged from 20 to 9kb was sliced and electroeluted by IBI electroelutor. The size selected mtDNA was cloned into the phage EMBL4 vector. Recombinant phage DNA was packaged using Lambda Gigapack II Gold packaging extract. For amplification of library, P2392 was used as host bacteria. Recombinant phage library was spreaded with proper pfu on the plate, and each single plaques were selected. Phage DNA was isolated, and digested with EcoRI. Each digested DNA of purified clones was fractionated on the agarose gel, denatured and transferred to nitrocellulose according to the method of Southern[15]. Plasmid DNA of ATP A a subunit gene was isolated by the alkaline method[8], and electroeluted and oligolabelled as discribed by commercial manual of Pharmacia Biotechnology Company. Filters were prehybridized at 65 °C in 10% dextran sulfate, 6X SCP, 2% sarkosine and 500 μg/m*l* heparin, and hybridized in the same buffer containing 100 μg/m*l* denatured salmon testis DNA and denatured oligolabelled probe. The filters were washed in 0.2X SCP, 0.2% SDS at 65 °C[3].

Results and Discussion

The methods of mtDNA isolation

Plant mtDNA isolation procedure is generally difficult, time consuming, and expensive. The isolation of Korean ginseng mtDNA is difficult comparing to other plants, because it contains the abundance of polysaccharide and phenolic compounds. By modification of recently advanced methods[6,12,13,25], the simple and inexpensive methods of mtDNA isolation from ginseng root, etiolated shoot, and/or callus have been developed.

DNase treatment was not applied for isolation of ginseng mtDNA. A degraded and/or restriction digested nuclear smear background was only slightly detectable in isolated mtDNA of ginseng. DNase treatment as control experiment reduced the degraded nuclear background only slightly (data not shown). Therefore, DNase treatment was eliminated during the mtDNA isolation procedure of ginseng.

The incorporation of pronase K step was applied prior to the potasium acetate-SDS precipitation step in the isolation procedure. Pronase K treatment can remove the DNA binding proteins which bind to the terminal portion of mtDNA, so that the mtDNA is not precipitated in the protein-potasium acetate-SDS complex[25].

This method for isolation of mtDNA is more simple, and time saving than that of other methods. It spends only 5-6 hours, and does not require DNAse and phenol treatment. Additionally, this procedure can be successfully applied to mtDNA isolation of maize, chinese cabbage, rape seed, radish, soybean, or pearl millet (data not shown).

Restriction digestion and determination of molecular weight

Restriction endonuclease analysis of mtDNA was conducted with etiolated shoot, one-year old root, and six-year old root. Fig. 1 shows the digestion pattern of mtDNA with restriction endonuclease HindIII. MtDNAs of both etiolated shoot (lane 2) and one-year root (lane 3) revealed sharp restriction fragment pattern, but mtDNA of the 6-year root (lane 4) was not digested. It is suspected that the 6-year root contains some enzyme inhibitors such as polysaccharide, or phenolic compounds. To check the possibility of incomplete digestion of the mtDNA by restriction enzyme, control experiment was performed by increasing enzyme units combined with extensive incubation for period up to 24 hr. No difference in pattern was observed (data not shown).

In the agarose gel electrophoregram presented in Fig. 2, over 30 restriction bands are present in each restriction digestion. Restriction patterns with EcoRI (lane 1), HindIII (lane 2), and SalI (lane 3) were more or less evenly distributed across the gel ranged from 15kb to below 1kb, while the patterns with Sau3AI (lane 4) distributed below 3 to 4 kb.

5 MtDNA size of ginseng might be smaller than mtDNA size of maize. Agarose gel electrophoresis of HindIII restriction digests of mtDNA from ginseng exhibits a simple pattern of bands as compare with maize. Inspection of pattern from UV photography reveals at least 30 fragments in ginseng mtDNA, but 50 in maize. Generally, mtDNAs are circular double strand DNA. However, the plant mitochondrial genome is diverse in size and, in some species, has in addition low-molecular-weight, circular and linear DNAs and single and double-stranded RNAs. Heterogeneity of mtDNA size was

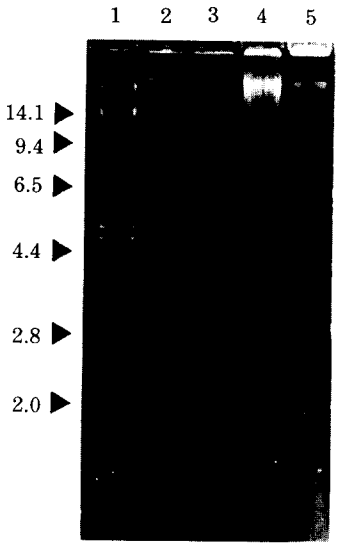

Fig. 1. Agarose gel electrophoretic patterns of Korean ginseng mitochondrial DNA digested by HindII. Lane 1. Lambda DNA digested with HinndIII and PstI. Lane 2. Etiolated shoot. 3. One-year old root. Lane 4. Six-year old root. Lane 5. Etiolated shoot of maize.

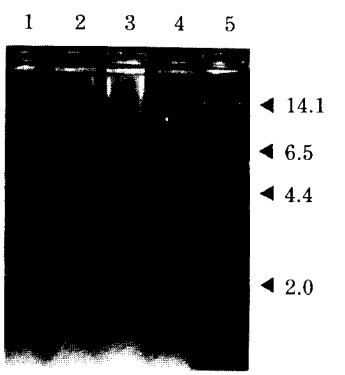

Fig. 2. Agarose gel electrophoretic patterns of Panax ginseng mitochondrial DNA digested by different restriction endonuclease Lane 1. EcoRI. Lane 2. HindIII. Lane 3. SalI. Lane 4. Sau3A1. Lane 5. Lambda DNA digested with HindIII and PstI.

distributed from 208kb (*Brassica hirata*)[16] to 2400kb (muskmelon)[24] in plant. Estimations of mitochondrial genome size has been attempted by summation methods of molecular weights of restriction fragments using HindIII and EcoRI. Estimated molecular size of ginseng mtDNA was at least 159.75 kb (Table 1). For satisfactory data on genome size, more detailed experiment should be done such as restriction mapping, electron microscopy, or renaturation kinetics. Inspite of the possibility of repetitive DNA sequences of mtDNA, mitochondrial genome of ginseng can be included in the smallest group of plants.

The characterization of mtDNA of ginseng

The application of the conventional breeding method to ginseng plant is very difficult, because there are no other ginseng varieties and it takes about 3 years for one generation. Four variants of Korea ginseng are only released by ginseng breeding program, i.e. green-stem, violet-stem, yellow-berry, and redish yellow-berry variants. For taxonomical characterization and identification of plant, many studies[4,19,22] were conducted using morphological, cytological, anatomical, physiological, ecological methods, etc. Recently, chemotaxonomical, and molecular biological techniques were developed for plant taxonomy[5,14]. For comparison among the variants of ginseng, restriction endonuclease analysis was applied. However, no difference was observed in restriction endonuclease digestion patterns among the ginseng variants (Fig. 3).

When DNA methylation sensitive restriction en-

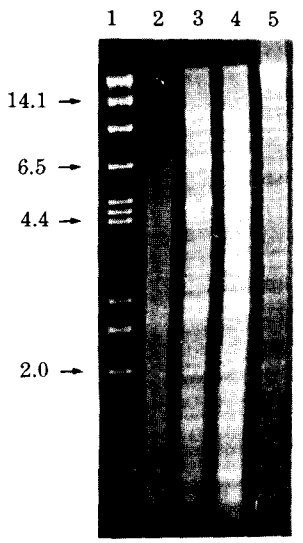

Fig. 3. Agarose gel electrophoresis patterns of HindIII digested mt DNA of ginseng violet-stem variant (lane 2), green-stem variant (lane 3), yellow-berry variant (lane 4), and maize (lane 5). Lane 1 is marker.

Table 1. Estimation of me DNA molecular weight of the etiolated shoot of violet-stem ginseng.

Band No.	M.W.		Band No.	M.W		Band No.	M.W	
	EcoRI	HindIII		EcoRI	HindIII		EcoRI	HindIII
1	18.5	10.0	13	4.5	5.25	25	2.75	3.1
2	9.5	9.1	14	4.35	5.1	26	2.65	3.05
3	9.3	8.7	15	4.3	4.9	27	2.6	3.0
4	8.05	7.9	16	4.0	4.75	28	2.4	2.9
5	7.8	7.3	17	3.9	4.6	29	2.3	2.9
6	6.2	6.6	18	3.65	4.45	30	2.25	2.75
7	6.1	6.5	19	3.5	4.25	31	2.2	2.6
8	5.7	6.4	20	3.4	3.75	32	2.05	2.3
9	5.5	6.2	21	3.3	3.65	33	1.85	2.05
10	5.0	5.7	22	3.2	3.45	34	1.8	1.95
11	4.85	5.55	23	3.05	3.35	35	1.6	
12	4.7	5.3	24	2.95	3.32	Total	159.75	162.55

donucleases were treated in ginseng mtDNA (Fig. 4), 4.5kb extra band at the lane of EcoRII treatment could be observed in restriction pattern digested with the methylation sensitive endonucleases, BstN1 and EcoRII. The data suggests that several portion of mtDNA of ginseng are hypomethylated, and that these methylated DNA may be related to the gene expression during ginseng development process.

The construction of mitochondrial genomic library

For construction of genomic library, mtDNA of ginseng was partially digested with EcoRI. The experiments were carried out to test which concentration and reaction time of restriction enzyme were proper to the partial digestion of ginseng mtDNA. Treatment of 0.1 unit EcoRI during 10 min is most proper to partial digestion of mtDNA (data not shown). After agarose gel electrophoresis of partial digested mtDNA, digested DNA, ranged from 20 to 9kb was sliced and electroeluted. The eluted DNA was ligated with EMBL4 arms by T4 DNA ligase, and ligated recombinat DNA was packaged. Single plaque of 61 clones was selected from amplified mt library.

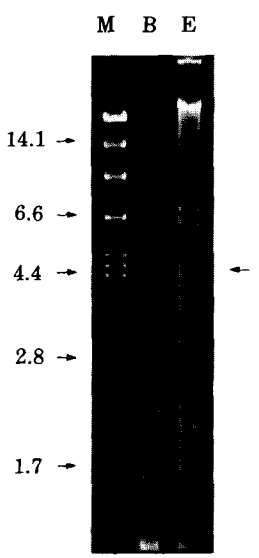

Fig. 4. Agrose gel electrophoresis band pattern of ginseng mitochondrial DNA digested with DNA methylation sensitive restriction endonuclease BstNI (B) and EcoRII (E). M indicates marker DNA.

The cloning of ATP A subunit gene from mtDNA of ginseng

For checking whether mitochondrial genome contains the homology of ATP A subunit gene of *Oenothera*, mtDNA was digested with several enzymes, and transfered to nitrocellulose paper (Fig.

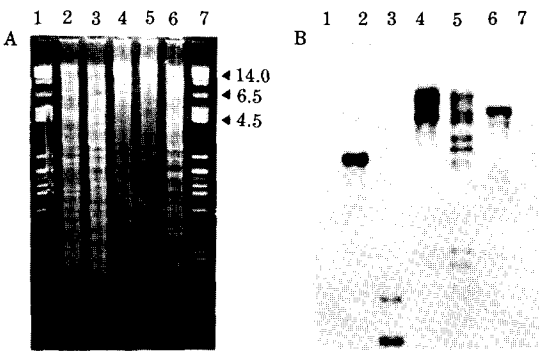

Fig. 5. (A) Agarose gel electrophoresis band pattern of ginseng me DNA digested with (lane 2) HindIII, (lane 3) EcoRI, (lane 4) XbaI, (lane 5) NheI, and (lane 6) Bg1II. Lane 1 and 7 indicate the marker DNA. (B) Southern analysis pattern of ginseng mt DNA digested with (lane 2) HindIII, (lane 3) EcoRI, (lane 4) XbaI, (lane 5) NheI, and (lane 6) Bg1II probed with ATP A subunit gene of *Oenothera*.

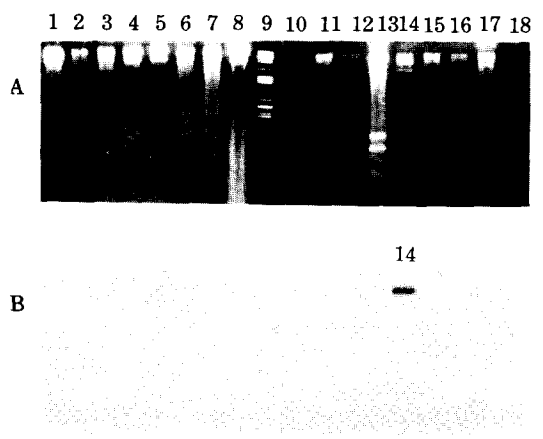

Fig. 6. (A) Agarose gel electrophoresis band pattern of recombinant phage DNA clones of ginseng mt DNA digested with EcoRI, (B) Southern analysis pattern of recombinant phage DNA clones of ginseng mt DNA digested with EcoRI probed with ATP A subunit gene of *Oenothera*.

5-A). Southern blots of mt DNA digested with enzyme were probed with the internal 1.9kb PstI/HindIII double digested fragment of pH 1/23 plasmid supported from Dr. A. Brennicke. Fig. 5-B shows that mtDNA of ginseng has the homology sequences of ATP A gene of *Oenothera*.

The DNAs of 17 clones from 61 clones were digested with EcoRI, and were fractionated on the agarose gel (Fig. 6-A), denatured and transferred to nitrocellulose. Fig. 6-B shows the Southern analysis pattern of each clones probed with the pH 1/23 plasmid. One homology band was observed in clone 16 of ginseng mt genomic library (lane 14). Now, we are trying to subclone the ATP A gene from clone 16.

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