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Molecular Cloning of ATPase α-Subunit Gene from Mitochondria of Korean Ginseng (*Panax ginseng* C.A. Meyer)

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Abstract□Molecular cloning and restriction mapping on ATPase α-subunit gene (*atpA*) were carried out to obtain genomic information concerned with the gene structure and organization in Korean ginseng mitochondria. Two different clones containing the homologous sequence of *atpA* gene were selected from *Sal*I and *Pst*I libraries of mitochondrial DNA (mtDNA) of Korean ginseng. The sizes of mtDNA fragments inserted in *Sal*I and *Pst*I clones were 3.4 kb and 13 kb, respectively. Southern blot analysis with [^{32}P] labelled *Oenothera atpA* gene probe showed that *atpA* gene sequence was located in 2.0 kb *Xba*I fragment in *Pst*I clone and in 1.7 kb *Xba*I fragment in *Sal*I clone. A partial sequening ascertained that the *Sal*I clone included about 1.2 kb fragment from *Sal*I restriction site to C-terminal sequence of this gene but about 0.3 kb N-terminal sequence of open reading frame was abscent. The *Pst*I fragment was enough large to cover the full sequence of *atpA* gene. The same restriction pattern of the overlapped region suggests that both clones include the same fragment of *atpA* locus. Data of Southern blot analysis and partial nucleotide sequencing suggested that mtDNA of Korean ginseng has a single copy of *atpA* gene.

Key words□ATPase α-subunit, mitochondrial DNA, *Panax ginseng*.

Introduction

Korean ginseng (*Panax ginseng* C.A. Meyer) is one of the most important medicinal herbs in the Orient. It is not able to grow under the full sun light as a semi-shade plant and grows so slowly even under the most favorable environmental condition. In order to improve its low productivity, physiological characteristics of ginseng plant as responses to the environmental factors were intensively investigated during the last decade.[1-3] However, characteristics of this plant such as a semi-shade plant, slow growth, long duration of one generation and no differentiation of cultivar make it very difficult to improve its low productivity by breeding a new variety. Only the newly developed technique in plant biotechnology, therefore, would be a tool to solve this problem by transformation with the genes which encode the enzymes to improve the efficiency of ginseng cell metabolism. In consideration of the low growth rate of ginseng cells, it would be of great worth to study energy supplying metabolism by mitochondria. However, few information on the genetical and molecular biological researches on energy metabolism in Korean ginseng is obtainable.

The mitochondrial (mt) genome size of Korean ginseng was estimated as about 160 kb by restriction endonuclease fragment analysis,[4] smaller than that of other plants, in which it varies from 208 to 2500 kb.[5,6] This variance in mt genome size is caused by recombination of repeated sequences. Sequence rearrangements in coding and intergenic regions can create another gene copy and pseudogenes in mt genome, which have been correlated with alterations in plant phenotypes, such as cytop-

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lasmic male-sterility (CMS) in many species,[7-10] and non-chromosomal stripe mutant in maize.[11]

In connection with the CMS, many investigators have concentrated on the regions of the genomes flanking the *atpA* (the alpha subunit of F_1-ATPase) locus, because these regions are very variable in normal and CMS lines of sunflower,[7] sugar beet,[8] radish,[9] pea,[12,13] and *Phaseolus vulgaris*,[14] and different expression of *atpA* gene and its flanking regions may affect the occurrence of CMS.[15]

In relation with the physiological phenomenon of low growth rate in Korean ginseng, a study on the structure of *atpA* gene in this plant is enough to arous our interest. In this paper, we have investigated whether the mt genome of Korean ginseng has a single copy or multiple repeated sequences of *atpA* gene, and cloned 3.4 kb *Sal*I and 13 kb *Pst*I fragments, which include a similar sequence to *Oenothera atpA* gene. We present here the restriction map of these fragments and in the following paper the result of nucleotide sequence analysis.

Materials and Methods

1. Isolation of total & mt DNA and construction of library

Shoots grown from 3 year-old ginseng roots in dark condition were harvested, when they were 10~12 cm long, stored at −70℃, and used for the isolation of total DNA by phenol-chloroform method and mt DNA, which was carried out as described by Lim and Kim.[16]

Mt DNA was digested with *Sal*I or *Pst*I and ligated with pBluescript II SK⁻ vector (Stratagene) digested with the same enzyme and dephosphorylated with the alkaline phosphatase (BM). After transformation to DH5α strain of *Escherichia coli* and amplification, the aliquots of the clones were stored at −70℃ and used for screening by colony lift hybridization.

2. Isolation and purification of plasmid DNA

Plasmid DNA was isolated by alkaline lysis method presented in Molecular Cloning : A Laboratory Manual.[17] For the amplification of DNA, competent cells were prepared and transformed by electroporation in use of Gene Pulser as described in the booklet (BIO-RAD Co.).

3. Labeling of DNA probe and screening of *atpA* clones

Plasmid DNA containing *atpA* gene of *Oenothera* mtDNA kindly provided by Dr. Axel Brennicke (Institut fuer Genbiologische Forschung GmbH Berlin, Germany) was digested with *Pst*I/*Hin*dIII. A 1.9 kb DNA fragment containing *atpA* gene was purified from agarose gel by Gene Clean II protocol (Bio 101 Inc.) and labeled with [^{32}P]dCTP by 'random primed DNA labeling' method (Molecular Biology Boehringer Mammheim Co.).

Screening for the presence of the *atpA* gene in recombinant plasmid libraries was performed as colony lift hybridization with *Oenothera atpA* gene probe.

4. Mapping of *atpA* clones and DNA sequencing

The plasmid DNA of the clones selected from genomic library were digested with many kinds of restriction endonuclease *Bam*HI, *Cla*I, *Eco*RI, *Pst*I, *Sal*I, and *Xba*I, separated by electrophoresis in a 0.8% agarose gel and transferred to b Biodyne™ nylon membrane (Pall) by capillary transfer method. Total DNA digested with *Xba*I and *Sal*I, and mt DNA with *Eco*RI, *Pst*I, and *Sal*I, were used for the Southern blot. DNA fragments were bound to nylon membranes by UV cross-linking (120,000 microjoules). Hybridizations were carried out overnight at 65℃ in hybridization solution (5× Denhardt's solution, 5× SSC, 0.1% SDS). Membranes were washed with wash buffer (2× SSC, 0.1% SDS) and exposed for autoradiography on Kodak XAR 5 films. From the autoradiography data, the homologous sequence position was ascertained and restriction map of these clones were constructed.

Sequencing was performed on double-strand plasmid templates by the dideoxy chain-termination procedure. Sequencing was accomplished through extension of T3 and T7 primer by Sequenase™ (United States Biochemical) in the presence of [^{35}S] dCTP or [^{35}S]dATP. Labelled extension products were analyzed on 7% polyacrylamide-urea gels. The similarities of nucleotide sequence Korean ginseng and other plants were analysed by searching with the GenBank and EMBL database.

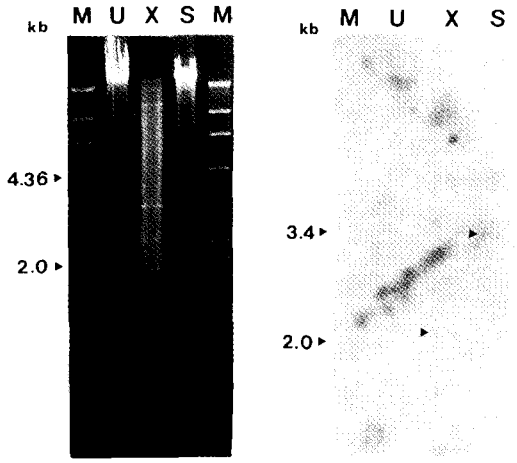

Fig. 1. Southern blot analysis of total DNA of Korean ginseng with [^{32}P] labelled *Oenothera atpA* gene probe : Total DNA was restricted with *Xba*I (X) and *Sal*I (S), and fractionated on 0.8% agarose gel (left). Only single band in each lane was observed in Southern blot hybridization (right).

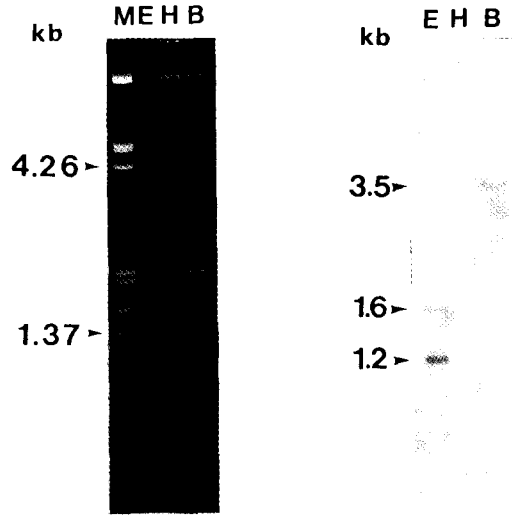

Fig. 2. Southern blot analysis of mitochondrial DNA of Korean ginseng with [^{32}P] labelled *Oenothera atpA* gene probe : Mt DNA was digested with *Eco*RI (E), *Hin*dIII (H), and *Bam*HI (B), and separated on 0.8% agarose gel.

Results and Discussion

1. Southern blot of total and mt DNA

Southern blot analysis with total DNA of Korean ginseng showed only a single band, 2.0 kb fragment in *Xba*I digestion and 3.4 kb in *Sal*I (Fig. 1), which are corresponding in size to the *Xba*I fragment coding mt *atpA* gene in *Pst*I clone (Fig. 7B) and inserted mt fragment in *Sal*I clones, respectively (Fig. 5 B, lane mt & S). This result suggests that the homologous sequence to *atpA* gene in Korean ginseng is located in mt genome. Form the digestion of mt DNA, two *Eco*RI fragment (1.6 kb, 1.2 kb) and one *Bam*HI fragment (3.5 kb) were detected, of which

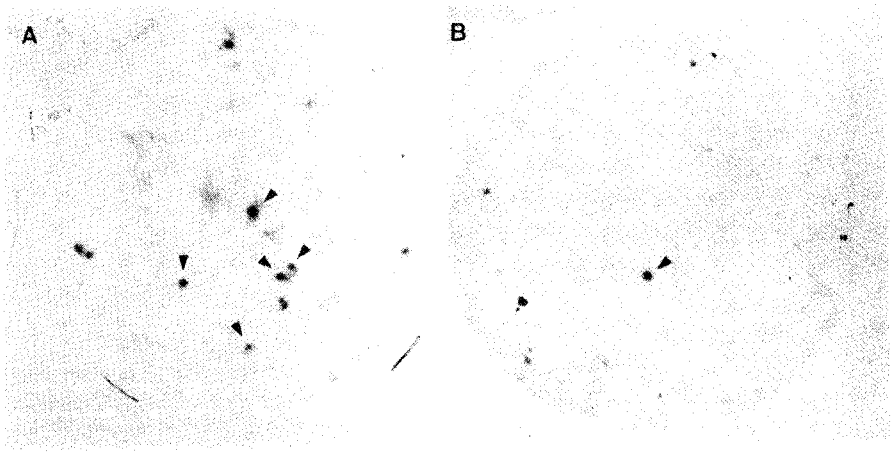

Fig. 3. Autoradiogram of membranes hybridized with the [^{32}P] labelled probe of *Oenothera atpA* gene after transfer of the colonies of *Sal*I (A) and *Pst*I (B) genomic libraries of Korean ginseng mitochondrial DNA.

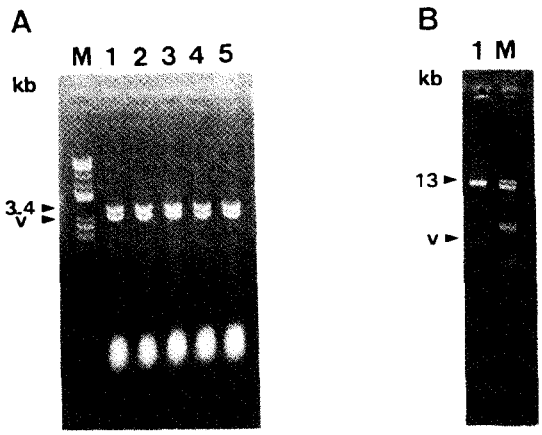

Fig. 4. Recombinant plasmid DNA of the *Sal*I (A : *Sal*I digestion) and *Pst*I (B : *Pst*I digestion) *atpA* clones selected by colony lift hybridization with *Oenothera atpA* gene probe from genomic libraries of Korean ginseng mitochondria (V : pBluescript II vector, 2.96 kb, M : marker DNA).

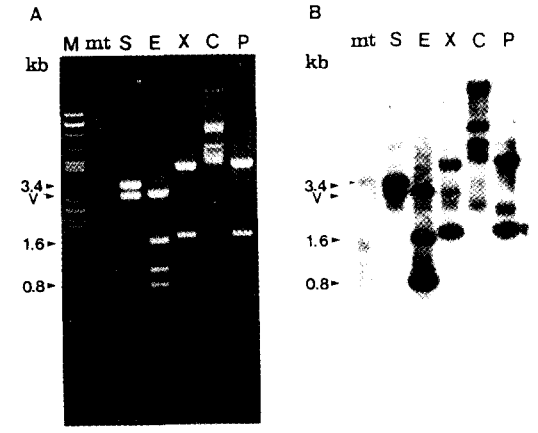

Fig. 5. Southern blot analysis of *Sal*I *atpA* clone DNA (3.4 kb DNA fragment) : DNA was separated by 0.8% agarose gel after digestion with *Sal*I (S), *Eco*RI (E), *Xba* (X), *Cla* (C), and *Pst*I (P) restriction enzymes (A), transferred to nylon membrane, and hybridized with [^{32}P] labelled *Oenothera atpA* gene probe (B). M : Marker DNA, mt : mtDNA digested with *Sal*I endonuclease.

the sizes could be identifiable in the restriction map of *Sal*I and *Pst*I clones (Fig. 7). Beyond our expection for the complex organization, it could be concluded that Korean ginseng contain only a single copy of *atpA* gene in the mt genome.

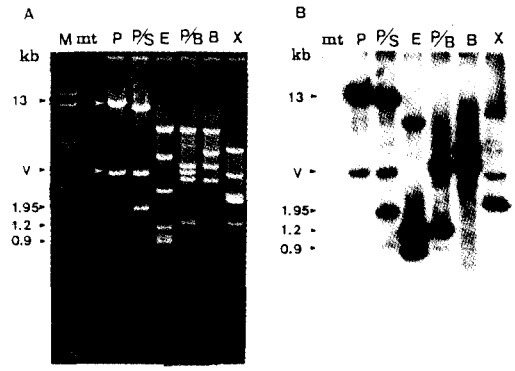

Fig. 6. Southern blot analysis of *Pst*I *atpA* clone DNA (13 kb DNA fragment). DNA was separated by 0.8% agarose gel after digestion with *Pst*I (P), *Pst*I/*Sal*I (P/S), *Eco*RI (E), *Pst*I/*Bam*HI (P/B), *Bam*HI (B), and *Xba*I (X) restriction enzymes (A), transferred to nylon membrane, and hybridized with [^{32}P] labelled *Oenothera atpA* gene probe (B). M : Marker DNA, mt : mtDNA digested with *Pst*I endonuclease.

2. Screening of Korean ginseng *atpA* gene

By the hybridization with the labelled *atpA* gene probe of *Oenothera* to the colony-lifted membranes of *Sal*I and *Pst*I libraries of Korean ginseng mtDNA, five positive clones from *Sal*I library and one positive clone from *Pst*I library were selected (Fig. 3).

Their plasmid DNAs were amplified and digested with corresponding restriction enzymes. The sizes of inserted DNA fragments were 3.4 kb long in *Sal*I clones and ca. 13 kb long in *Pst*I clone (Fig. 4).

3. Restriction map constrution of selected clone

For the construction of physical maps of selected two different clones, recombinant plasmid DNAs and mtDNA of Korean ginseng were digested with several kinds of restriction enzymes, and electrophoretically analysed (Fig. 5A and Fig. 6A). Southern blot analysis of mt DNA after digestion with *Sal*I showed a faint band, which was the same size as 3.4 kb inserted DNA fragment of *Sal*I clone (Fig. 5B, lane mt and S). However, homologous DNA fragment in mtDNA digested with *Pst*I was not detected, even though 13 kb fragment of *Pst*I clone showed a positve band (Fig. 6B, lane mt and P). This may result from loading too small amount of mt DNA compared with that of plasmid DNA.

In case of *Sal*I clone, 0.8 kb and 1.6 kb fragment

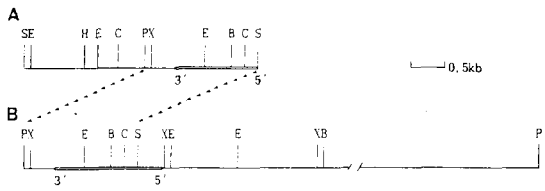

Fig. 7. Physical map of *Sal*I (3.4 kb, A) and *Pst*I (13 kb, B) *atpA* clone. Double line indicates the coding sequence of *atpA* gene. S, *Sal*I; E, *Eco*RI; H, *Hin*dIII; C, *Cla*I; X, *Xba*I; P, *Pst*I; B, *Bam*HI; S', *Sma*I.

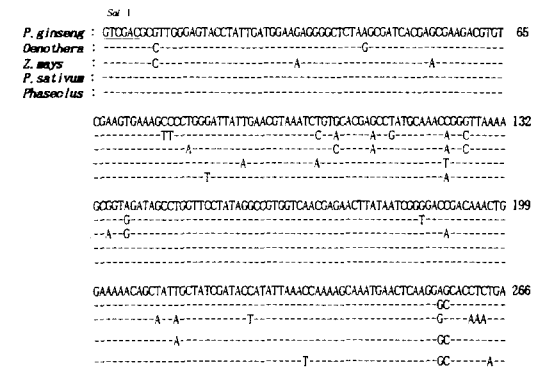

Fig. 8. The 5'-end nucleotide sequence of *Sal*I *atpA* clone. The partial sequence was compared with the coding sequences the *atpA* gene in other plants (*Oenothera*,[10] maize,[13] pea,[6] and *Phaseolus vulgaris*[9]). A *Sal*I site in the *atpA* gene was located at 328th nucleotide from initiation codon. Dash indicates identical nucleotide to that of Korean ginseng sequence.

in *Eco*RI digestion, 1.7 kb in *Xba*I digestion, and 1.75 kb in *Pst*I digestion were homologous to *Oenothera* gene probe (Fig. 5B) and a restriction map of this clone could be constructed as shown in Fig. 7A. A homologous sequence to *Oenothera atpA* gene was observed at one end of this fragment (Fig. 8), which suggests that this clone includes about 1.2 kb fragment from *Sal*I site in 3' direction to termination codon, not a complete coding sequence of *atpA* gene.

In Southern blot analysis of *Pst*I clone, the 0.9 kb and 1.2 kb fragments in *Eco*RI digestion and 2.0 kb fragment in *Xba*I digestion were homologous to the sequence of *Oenothera atpA* gene (Fig. 6B). The internal *Pst*I-*Sal*I fragment (ca. 1.6 kb) of *Pst*I clone was overlapped to that of *Sal*I clone (Fig. 7B), that was ascertained by the identical DNA band pattern of this fragment after digestion with six different restriction enzymes (Data not shown).

In the plant mitochondrial genome, the genes are widely scattered and the repeated gene structure are often found, which are generated by rearrangement of DNA fragments.[5,6] The homologous sequences of *atpA* gene in pea,[12] soybean,[18] and *Oenothera*,[19] mt DNA are located on four types of genomic segments : two of them include the uninterrupted α-subunit coding sequence, the others are pseudogenes, which contain only a partial sequence of *atpA* gene and do not express stable RNAs.[12]

The difference in organization of the *atpA* coding and the flanking sequences between fertile and CMS lines has been reported in many species,[7-14] that might be strongly correlated with the physiological abnomality. But no decisive evidence is not suggested, to date, that an abnormal expression of *atpA* gene in mitochondria causes the male sterility or other physiological disorder. Only in sunflower, a strong evidence is reported that DMS in the PET 1 cytoplasm is caused by alterations of the mt DNA in the region framed by the *atpA* gene and the *cob* gene.[15]

From the data of Southern blot analysis with total and mt DNA of Korean ginseng, it can be suggested that a homologous sequence of *atpA* gene is located in mt genome as a single copy and the low growth rate of this plant is not correlated with the complex organization of the *atpA* gene loci.

요 약

미토콘드리아내 유전자는 염기서열 재배치에 의해 단일 또는 여러 copy로 존재하는데 그 발현정도에 따라 생리적 특이 현상을 나타낼 것으로 생각되어 세포질 유전의 웅성불임 현상에 대한 연구가 활발히 이루어져 왔다. 반음지성이며 생육이 느린 고려인삼 (*Panax ginseng* C.A. Meyer)의 특성을 유전자 수준에서 접근하기 위해 F_1ATPase α-subunit 유전자 (*atpA*)에 대해 실험하였다.

고려인삼에는 *atpA* 유전자가 미토콘드리아 DNA에

단일 copy로 존재함을 확인하였으며, 미토콘드리아 DNA의 *Sal*I 및 *Pst*I 유전자은행으로부터 *Oenothera atpA* 유전자와 homology를 보이는 clone을 colony lift hybridization에 의해 선발하였는데, 각각 3.4 kb와 13 kb의 미토콘드리아 DNA조각을 함유하고 있었다. 이 *Sal*I clone과 *Pst*I clone에 대해 Southern blot hybridization과 부분적인 염기서열분석을 실시한 결과, 삽입된 3.4 kb와 13 kb의 미토콘드리아 DNA조각은 *Oenothera*, maize, pea, *Phaseolus vulgaris*의 *atpA*유전자와 homology를 보이는 sequence를 포함하고 있었다. 타 식물의 *atpA*유전자의 coding sequence와 비교해 볼때, *Sal*I clone은 initiation codon으로부터 약 300 bp를 제외한 *atpA*유전자의 나머지 약 1.2 kb sequence를 포함하고 있고, *Pst*I clone은 *atpA*유전자의 완전한 coding sequence를 포함하고 있는 것으로 여겨진다. 이들 두 clone의 제한효소 지도와 부분적인 염기서열이 제시되었다. 고려인삼은 단일 copy의 *atpA*유전자를 갖고 있기 때문에 이 타식물에서와 같이 복잡한 유전자 재배치에 따른 생리적 불균형의 가능성은 없는 것으로 사료된다.

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