AMPLIFICATION AND SEQUENCE OF A 5S-rRNA GENE SPACER REGION FROM THE CRUDE DRUG "ANGELICA ROOT"

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A 5S-ribosomal gene spacer region was amplified by the polymerase chain reaction using a DNA preparation from the crude drug "Angelica Root" as a template. The nucleotide sequence of the amplified product was identical to that of *Angelica acutiloba*, the source plant of "Angelica Root," but different from that of *Bupleurum falcatum* or *Glehnia littoralis*. The possibility of discriminating "Angelica Root" from other umbelliferous crude drugs based on a *Hin* dIII restriction site inside the spacer region was suggested.

KEY WORDS DNA profiling; PCR; 5S-rRNA gene spacer; crude drug identification; Angelica Root; *Angelica acutiloba*

Correct identification of crude drugs is a prerequisite for chemical and pharmacological investigations of natural medicines, and also for their clinical applications. DNA-profiling (DNA-based polymorphism assay) has several advantages over morphological and chemical analysis for identification and/or discrimination of biological materials: (1) it analyzes the genotype rather than the phenotype and, therefore, it is not affected by environmental factors; (2) one or more sequences appropriate to solving the particular problem can be selected; and (3) DNA can be prepared from small amounts of samples and genetic information on rare or endangered specimens can be obtained without destructive sampling. In the present communication I describe amplification of a 5S-rRNA gene intergenic spacer region by PCR using DNA prepared from the powdered crude drug "Angelica Root" and its source plant, *Angelica acutiloba*, as templates and sequencing of the PCR products to examine the possibility of identifying or discriminating crude drugs by direct DNA analysis. To my knowledge this is the first report describing the cloning and sequencing of a gene region from crude drugs.

Total DNA was prepared from the powdered crude drug "Angelica Root" and from frozen leaf tissue of *A. acutiloba* (strains Yamato and Hokkai), *Bupleurum falcatum* and *Glehnia littoralis*, using the CTAB method of Rogers and Bendich¹). The PCR mixture was comprised of 0.2 mM each of deoxyribonucleotide triphosphate, 20 pmol of each oligonucleotide primer, 10-20 ng DNA and 1 unit of *Tth* polymerase (Toyobo) in 25 μ l of PCR buffer. The amplification was performed using 30 reaction cycles consisting of a 1-min denaturing step at 94°C, a 1-min annealing step at 55°C and a 2-min elongation step at 72°C. The amplification product was purified by agarose gel electrophoresis,

ligated to a pGEM-T vector (Promega) and sequenced by the dideoxyribonucleotide chain-termination method with a model 373A DNA sequencing system (Applied Biosystems).

DNA obtained from 0.1-0.3 g of the powdered crude drug was invariably shown to be degraded into smaller fragments with substantial amounts of DNA migrating in the range of 40-500 bp (data not shown). This is probably due to the oxidative process during drying and storage of crude drugs. Such fragmentation of DNA was also reported in the DNA extracted from dry remains of animal soft tissues.²⁾ Therefore, I examined enzymatic amplification of a specific and shorter length gene region using DNA preparations from the crude drug or fresh leaves as templates. The intergenic spacer region of 5S-rDNA was chosen for amplification. The 5S-rRNA genes occur in all of the higher eukaryotes in tandemly repeated units consisting of an approximately 120-bp coding region with a non-transcribed spacer of about 300 bp. Although the coding region is highly conservative, the non-transcribed spacer varies from species to species both in sequence and length.³⁾ Based on a comparison of compiled plant 5S-rRNA sequences,⁴⁾ two sequences (nt 58-77 and nt 4-23) in the coding region were selected as a forward primer (5SP1 = 5'-GTGCTTGGGCGAGAGTAGTA-3') and a reverse primer (5SP2 = 5'-TTAGTGCTGGTATGATCGCA-3'), respectively (Fig. 1).

Fig. 1. The Basic Structure of the Repeating Units in 5S-rRNA Gene Clusters in Higher Plants
The positions of primers used for PCR are indicated.

Fig. 2. Agarose Gel Electrophoresis of PCR Products Obtained from the Crude Drug, "Angelica Root," and Its Source Plant, *Angelica acutiloba*
Lanes A, *A. acutiloba* (Yamato); Lanes B, *A. acutiloba* (Hokkai); Lanes C, "Angelica Root"; Lanes D, size markers (pBR322/Msp I). Arrows indicate expected amplification products (ca. 300 bp).

Figure 2 shows an electrophoretic analysis of the amplification products after 30 cycles of PCR. The product of about 300 bp was only detected when DNA from the fresh leaves was used as a template. Then, the second round of PCR was attempted using 5- μ l aliquots of the first PCR mixture as templates for reamplification. After two rounds of PCR, a product of approximately 300 bp was obtained from the crude drug. The products were ligated to the pGEM-T vector and their nucleotide sequences were determined (Fig. 3). For comparison, PCR products obtained from *B. falcatum* and *G. littoralis* were also sequenced.⁵⁾ The nucleotide sequence of the product from "Angelica Root"

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1:  GTGCTTGGGCGAGAGTAGTACTAGGATGGGTGACCCCCTGGGAAGTCCTCGTGTGCACC 60
    └──────────────────────────────────────────────────────────────────┘
61:  CTTTTCGCTTTAATTTTTCGCCTGCAAATTATTTGCTTAATTTACGACACATTTTC 120
    └──────────────────────────────────────────────────────────────────┘
121: TTATATGAACAATTCATGTTTATCCACTTTAATATTGATTTTATAAATAATTTTATCATT 180
181: ATTTAATTATTTTATTATTATTTTCCAATTTATCGGAGCAAAATGCAAAAAAGCTTTTA 240
    ▲               ▲
241: ACGTTGGTATATAGAATTAGTTTGCACCTTCTTTGACGGGTGCGATCATACCAGCACTAA 299
    ▲               ▲

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Fig. 3. Nucleotide Sequence of a 5S-rRNA Gene Spacer Region Amplified from "Angelica Root"

The solid lines enclose regions encoding 5S rRNA. Primer sequences used for amplification are indicated by arrows. A *Hin* dIII restriction site is shown in bold print. Triangles show the nucleotides differing in the spacer amplified from *A. acutiloba* (Hokkai) (T for A, T for C, A for G). The sequence was identical to that from *A. acutiloba* (Yamato).

was identical to that from *A. acutiloba* (Yamato). The termination sequence of -CCCC- at the 3'-end of the coding region followed by a 21-bp T tract and the initiation sequence of -GGG- at the 5'-end in the coding region was detected at nt 59-72 and nt 277-279, respectively. Sequences of the tentatively assigned coding regions exhibited no difference among the fragments from different sources examined and showed high homology to the consensus sequence of 5S-rRNA of higher plants. These results indicate that the amplified fragments are the spacer regions of 5S-rRNA genes. The sequence homology between *A. acutiloba* (299 bp) and *G. littoralis* (303 bp) was 87% and that between *A. acutiloba* and *B. falcatum* (312 bp) was 48%, whereas the homology between the two strains of *A. acutiloba* (Yamato and Hokkai) was 99%. However, it is necessary to accumulate more molecular data to discuss the taxonomic relationship between these two geographical strains of *A. acutiloba*. A *Hin* dIII site was present inside the region of *A. acutiloba* but not in those of *G. littoralis* and *B. falcatum*, which provides us with a PCR-RFLP method to discriminate "Angelica Root" from other crude drugs of Umbelliferaceae origin.

In conclusion, the intergenic spacer region of 5S-rRNA gene can be amplified using DNA obtained from crude drugs. It may be possible to identify and/or discriminate crude drugs by comparing the sequences of the amplified products either directly (sequencing) or indirectly (PCR-RFLP).

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