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Clone Identification of *Cudraria Tricuspidata* and *Hibiscus Syriacus* by Using PCR and Southern Hybridization

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Abstract : Polymerase chain reaction (PCR) and Southern hybridization analyses were carried out to identify clones of silk worm thorn (*Cudraria tricuspidata*) and Rose of sharon (*Hibiscus syriacus*) which look like one tree with two or three branches or two or three different trees. For PCR five different PCR primers (17~24 nucleotides) are derived from CaMV 35S promoter, nopaline synthase terminator and coding region of thylakoid membrane protein gene. In the case of silk worm thorn, about 500 bp of PCR product was produced from DNAs of one tree or branch in the presence of 35S primer alone. Southern hybridization analysis of genomic DNAs hybridized with ^{32}P labeled PCR product showed that the same size of DNA fragments were hybridized with different intensities. In addition, PCR analyses using 20 different primers of OPERON 10-mer kits showed that only OPA01 primer produced PCR products of different size. These results indicate that two different trees of silk worm thorn combined to one tree. In the case of the Rose of Sharon, the same size of PCR products were produced from three different samples but Southern hybridization with the above PCR product as a probe did not show any hybridized bands. PCR analyses in the presence of OPERON 10-mers showed OPA04 and OPA13 produced different products including same sizes of products. These results indicate that three different trees of the Rose of Sharon seem to be derived from the tree.(Recevied November 24, 1997; accepted December 22, 1997)

Introduction

The introduction of recombinant DNA technology into genetics has given new opportunitiess in the basic genetics based on the direct analysis of DNA variations. The most convenient ways of monitoring DNA sequence variation is to use of the Restriction Fragment Length Polymorphism (RFLP). RFLPs could be used to map and manipulate polygenic systems, followed by the demonstration of numerous RFLPs in populations but need large number of RFLP markers in such populations.[1] Therefore DNA variation from randomly amplified polymorphic DNA (RAPD), which combines RFLP and polymerase chain reaction (PCR) techniques, has recently been shown advantages of solving the evolutionary problems including the determination of paternity and maternity.[2] The studies of parents and offspring using RAPD has been utilized for the parentage analysis. However RFLP and RAPD need two generations, parents and offsprings, for the determination of linkage and specific traits.

Therefore the oligonucleotide polymorphisms using short DNA oligomers has been introduced for a new tool for genomic genetics.[3] The defined unique 18-mers could be used in hybridization assays for their corresponding genes or DNA segments with a resonable degree of confidence. In principle, oligonucleotide probes could be as convenient and economical to use as isozyme determination, which has been used for the clonal identification of pine trees.[4,5] The clonal identification is very important especially in tree breeding and very old monumental trees whereas isozyme method is tedious.

In this report, PCR using 17~24-mers and Southern hybridization analysis were applied to identify clones of silkworm thorn and Rose of Sharon. In addition, PCRs using 20 different 10-mer primers were carried out for the verification of results. The silk worm thorn (*Cudraria tricuspidata*), which located at Bongdoo-ri of Keumsoo-myoen in Sungjoo-koon of Kyungsangbook-do,[6] looks like two people embraced each other (see Fig. 1A) indicating that two different trees combined to one tree or one tree with two different big branches. In addition to this, the Rose of Sharon (*Hibiscus syriacus*), which located in the Chungduck elementary school of Hapcheon-koon of Kyungsangnam-do,[7] looks like one tree in the view of distant place but three different trees in the view of near place (see Fig. 1B).

Key words : PCR, Southern hybridization, *Cudraria tricuspidata, Hibiscus syriacus*
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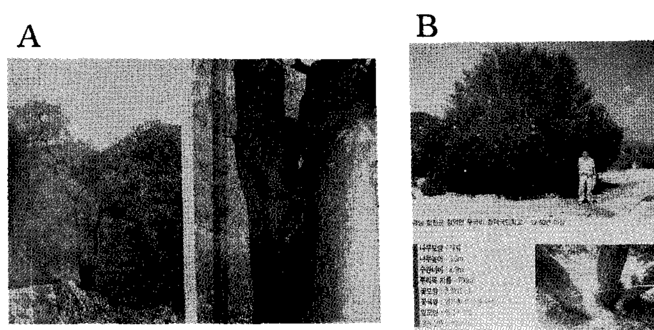

Fig. 1. Photographs of silk worm thorn(A) and Rose of Sharon(B). The silk worm thorn (*Cudraria tricuspidate*), which is located at Bongdoo-ri of Keumsoo-myoen in Sungjoo-koon of Kyungsangbook-do, looks like two people embraced each other in the view of near side. The Rose of Sharon (*Hibiscus syriacus*), which located in the Chungduck elementary school of Hapcheon-koon of Kyungsangnam-do, looks like one tree in the view of distant place but three different trees in the view of near place.

Materials and Methods

Plant leaf DNAs were isolated by the method of Doyle and Doyle.[8] Leaf tissue of 1.0 g was ground to a powder in liquid nitrogen in a chilled mortar and pestle. The powder was scraped into preheated extraction buffer [2% (w/v) CTAB (sigma), 1.4 M NaCl, 0.2% (v/v) 2-mercaptoethanol, 20 mM EDTA, 100 mM Tris-HCl, (pH 8.0)] and incubated at 60°C for 30 min with occasional swirling. After incubation, chloroform-isoamyl alcohol (24 : 1 ; v/v) were added and mixed thoroughly, followed by centrifugation at 1, 600x g for 5 min. The supernatant was transferred to new tube and 2/3 volume of cold isopropanol was added in order to precipitate nucleic acids. The nucleic acids were collected with centrifugation and dried at room temperature. The pellet was resuspended in 1 m*l* TE (10 mM Tris-HCl, pH7.4, 1 mM EDTA) and RNase was added to a final concentration of 10 ug/m*l* and incubated 30 min at 37°C. DNAs were precipitated by adding of ammonium acetate (7.5 M pH 7.7) to make 2.5 M and ethyl alcohol. The precipitated DNAs were dried on vacuum and resuspended in TE.

DNA oligonucleotides were synthesized by DNA synthesizer (Applied Biosystem Inc., model 381A) using phosphoramidite chemistry. After deprotection the oligonucleotides were purified by oligonucleotide purification cartridge (ABI). Five different oligonucleotides were utilized for the polymerase chain reaction (PCR) of the plant leaf DNAs. The 35S primer was derived from the cauliflower mosaic virus 35S promoter[9] and the nos primer was derived from the nopaline synthase terminator.[10] These two primers has been utilized for the determination of junction regions of the chimeric genes. The sequences of the psbA1, psbA2 and psbA3 primers were derived from the coding region of thylakoid membrane protein gene (psbA).[11] The psbA primers has been used for cloning of the psbA gene and determination of the cloned gene. In addition, the OPERON 10-mer kits were purchased and 20 different primers were utilized to confirm the clone identification. The sequence of these primers are in Table 1.

Table 1. Name and sequence of primers used in this research

Name of primer	Sequence	Size
35S	5'-AGCAAGTGGATTGATGTGAT-3'	20 mer
nos	5'-TCATCGCAAGACCGGCA-3'	17 mer
psbA1	5'-ATGACTGCAATTTTAGAGAGACGC-3'	24 mer
psbA2	5'-TTATCCATTTGTAGATGGAGCTTC-3'	24 mer
psbA3	5'-CACCAAACCATCCAATG-3'	17 mer
OPA01	5'-CAGGCCCTTC-3'	10 mer
OPA02	5'-TGCCGAGCTG-3'	10 mer
OPA03	5'-AGTCAGCCAC-3'	10 mer
OPA04	5'-AATCGGGCTG-3'	10 mer
OPA05	5'-AGGGGTCTTG-3'	10 mer
OPA06	5'-GGTCCCTAGC-3'	10 mer
OPA07	5'-GAAACGGGTG-3'	10 mer
OPA08	5'-GTGACGTAGG-3'	10 mer
OPA09	5'-GGGTAACGCC-3'	10 mer
OPA10	5'-GTGATCGCAG-3'	10 mer
OPA11	5'-CAATCGCCGT-3'	10 mer
OPA12	5'-TCGGCGATAG-3'	10 mer
OPA13	5'-CAGCACCCAC-3'	10 mer
OPA14	5'-TCTGTGCTGG-3'	10 mer
OPA15	5'-TTCCGAACCC-3'	10 mer
OPA16	5'-AGCCAGCGAA-3'	10 mer
OPA17	5'-GACCGCTTGT-3'	10 mer
OPA18	5'-AGGTGACCGT-3'	10 mer
OPA19	5'-CAAACGTCGG-3'	10 mer
OPA20	5'-GTTGCGATCC-3'	10 mer

The reaction conditions for PCR werefollowed published methods[12,13]; step 1: denaturation of DNA at 94°C for 5 min, annealing of primers at 37°C for 2 min, primer extension at 72°C for 3 min (1 cycle), step 2: denaturation of DNA at 94°C for 1 min, annealing of primers at 37°C for 2 min, primer extension at 72°C for 3 min (25 cycles), step 3: denaturation of DNA at 94°C for 1 min, annealing of primers at 37°C for 2 min, primer extension at 72°C for 10 min (1 cycle). PCR products were separated on a 1% agarose gel.

Southern blot hybridization was carried out according to the published method.[14] The probe was the PCR product derived from plant DNAs. Labelling of probe was carried out using a commercial kit from Amersham. Autoradiography was carried out with Kodak X-Omat films.

Results and Discussion

Synthetic primers which have been utilized for cloning of psbA gene and sequence analysis were first subjected to PCR using DNAs isolated from the oriental and west-

ern orchids to confirm quality of primers and usefulness in clonal identification. Different PCR products were made in the presence of 35S, nos and psbA1 primers as shown in Fig. 2A. In addition, DNAs from a ging ko tree and a silk worm thorn produced different PCR Products (data not shown). This result means primers used in PCR could make different products in DNAs of different species indicating that these primers can be utilized in distinction of clones. Therefore PCRs were carried out on DNAs isolated from silk worm thorn which looks like two people embraced each other. DNAs from one branch produced PCR product of 560 base pairs in size while DNAs from the other branch made nothing (Fig. 2B) suggesting of two different branches or trees. In addition, combind DNAs produced 560 dp PCR product (data not shown) suggesting that DNAs from one branch only produced PCR product. This PCR product will be cloned into a vector and sequence analysis will be carried out to know a putative function according to sequence search program.

The PCR product was labelled with ^{32}P and hybridized with BamHI, EcoRI and HindIII digested silk worm thorn DNAs in order to monitor a level of presence in genomic DNAs. Southern analysis showed that the same DNA fragments are in two different DNA samples with a different level (Fig. 3). The above PCR and Southern analyses indicate that silk worm thorn seems to be one tree with two big branches. To verify this hypothesis OPERON's 10-base oligonucleotide primers which are used in genetic mapping[15] and DNA fingerprinting[16] were subjected to PCR on silk worm thorn DNAs. Fig. 4 shows PCR products separated on 1% agarose gel in the presence of OPA01, OPA02, OPA04, OPA11 and OPA14 primers. OPA01 only gave three different PCR products with two identical products. Other 19 different primers gave same PCR products in the two DNAs samples. This results mean the two DNAs are almost identical. At this point we can conclude that two trees seem to be combined to one tree in the case of silk worm thorn.

In the case of the Rose of Sharon, which looks like one

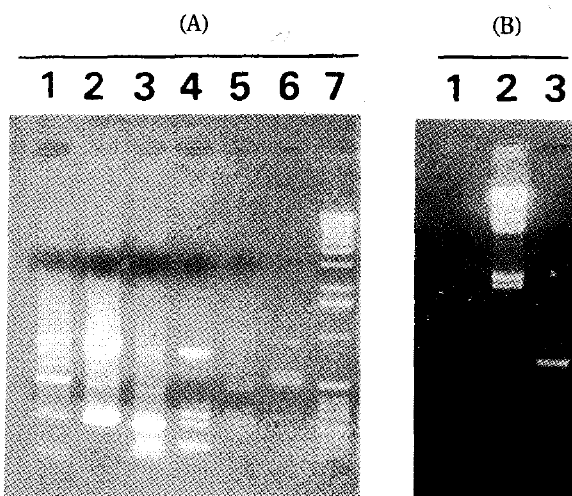

Fig. 2. PCR products from orchids(A) and silk worm thorn (B) DNAs. (A) Oriental orchid (lanes #1, #3, #5) and western orchid (lanes #2, #4, #6) DNAs were subjected to PCR for the verification of primers, 35S primer (lanes #1 & #2), nos primer (lanes #3 & #4) and psbA1 primer (lanes #5 & #6). Lane #7 contains DNA molecular weight standard (1 kb ladder). (B) Silk worm thorn DNAs were subjected to PCR using 35S primer. Lanes #1 and #3 contain PCR products from leaf DNAs of different branches. Lane #2 contains DNA molecular weight standard (HindIII digests of λ DNA).

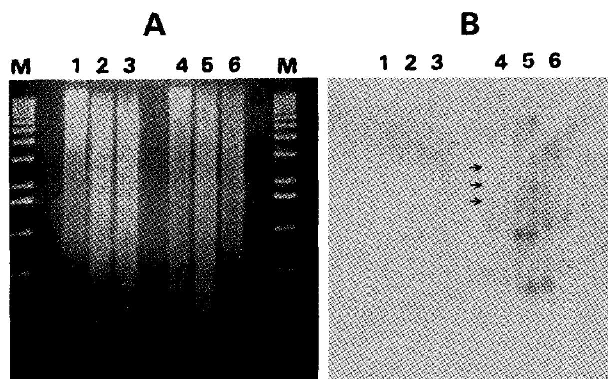

Fig. 3. Separation of restriction enzyme digests of silk worm thorn DNAs(A) and its autoradiogram(B) of Southern hybridization. (A) Ten μg of DNAs were digested with BamHI (lanes #1 & #4), EcoRI (lanes #2 & #5) and HindIII (lanes #3 & #6) and separated on 1% agarose. M stands for DNA molecular weight standard. (B) Southern hybridization was carried out with radiolabelled PCR product of 35S primer as a probe. Arrows mean 3 different fragments which are shown on X-ray film and they are in both samples.

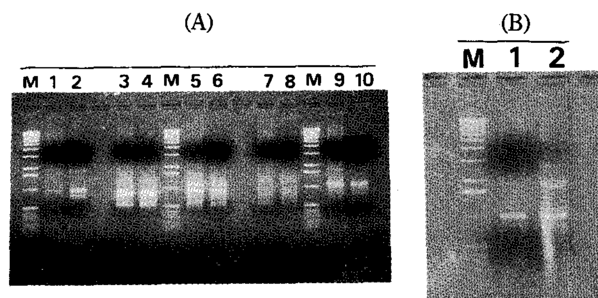

Fig. 4. Separation of PCR products of the silk worm thorn DNAs using OPERON 10-base oligonucleotide primers. (A) Five different primers, OPA01 (lanes #1 & #2), OPA02 (lanes #3 & #4), OPA04 (lanes #5 & #6), OPA11 (lanes #7 & #8) and OPA14 (lanes #9 & #10) were utilized for PCR and PCR products were separated on 1% agarose. M stands for DNA molecular weight standard. PCR products were obtained from DNAs of one branch (lanes #1, #3, #5, #7 and #9) and the other branch (lanes #2, #4, #6, #8 and #10). Twenty different primers were subjected to PCR and PCR products derived from five different primers are shown here. (B) The repetition of PCR on the same DNAs using OPA01 primer were carried out to show the only difference in PCR products of 20 different primers.

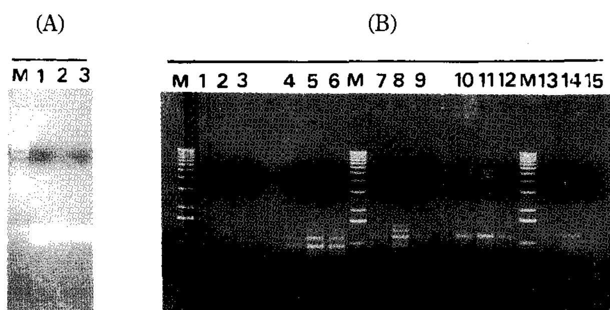

Fig. 5. Separation of PCR products of the Rose of Sharon DNAs using OPERON 10-base oligonucleotide primers. (A) PCR products derived from 35S primer are shown here. DNAs of three different branches (lanes #1, #2 & #3) were subjected to PCR and their products were separated on 1% agarose. M stands for DNA molecular weight standard(HindIII digests of λ DNA). (B) Five different primers, OPA01 (lanes #1, #2 & #3), OPA02 (lanes #4, #5 & #6), OPA04 (lanes #7, #8 & #9), OPA11 (lanes #10, #11 & #12) and OPA13 (lanes #13, #14 & #15) were utilized for PCR and PCR products were separated on 1% agarose. M stands for DNA molecular weight standard (1 kb ladder). PCR products were obtained from DNAs of the 1st branch (lanes #1, #4, #7, #10 & #13), the 2nd branch (lanes #2, #5, #8, #11 & #14) and the 3rd branch (lanes #3, #6, #9, #12 & #15). Twenty different primers were subjected to PCR and PCR products derived from 5 different primers are shown here.

tree in the view of distant place but three different trees in the view of near place, 35S primer produce three PCR products of the same size in three different DNA samples (Fig. 5A) and Southern analysis with the above PCR product from silk worm thorn as a probe did not show any hybridized bands (data not shown). In addition to these results, PCR analyses in the presence of OPERON 10-mers showed that OPA04 and OPA13 primers produced dif ferent products while other primers produce identical products (Fig. 5B). These results indicate that three different looking trees of the Rose of Sharon seem to be derived from one tree

At this point, the defined unique oligonucleotide primers can be used in general to identify clones of trees if oligonucleotide primer (s) can make different PCR products from DNAs of different clones. This idea actually comes from the fact that flowering plant genomes contain 10^8~10^{11} base pairs and the likelihood of encountering any specific arbitrary oligonucleotide sequence that is about 20-mers,[3] is for all intents, zero. Therefore PCR using unique ologonucleotide primers as well as Southern hybridization will be utilized to identify clones of other very old monumental trees, for example a zelcova tree (*Zelkova acuminata*) or a gingko tree (*Gingko biloba*) located at Jamo2-dong and Tae2-ri of Hyunpoong-myon, respectively. Both trees are of about 200 years old and are protected as monumental trees.

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PCR과 Southern hybridization을 이용한 구지뽕나무와 무궁화의 클론감별

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초 록 : 본 연구는 polymerase chain reaction (PCR)와 Southern hybridization을 이용하여 서로 붙어있는 나무가 한 나무인지 두 나무인지 규명하였다. 공시된 수종은 구지뽕나무와 무궁화이다. 구지뽕나무는 경상북도 성주군 금수면 봉두리 안새출 금수식당 앞에 있는 나무로 두 사람이 서로 안고 있는 형상인데, 한 나무의 두 가지인지 가까이 자란 두 나무가 붙었는지를 구별하기 어려운 나무였다. 다섯 종류의 PCR primer (17~24-mers) 중 35S primer의 경우 한가지의 잎 DNA에서만 PCR 산물이 검출되어 이것을 방사선표지한 후 genomic Southern hybridization을 행하였던 바 이 probe와 결합하는 DNA 단편이 동일한 위치에서 검출되었으나 정도는 다르게 나타났다. 아울러 OPERON 10-mer kits A에 있는 20종류의 primer를 이용하여 PCR을 행한 결과 OPA01 primer (CAGGCCCTTC)에 의한 PCR 생성물은 동일한 위치의 band 뿐만 아니라 추가로 4개가 한가지의 잎 DNA에서 더 나타났다. 따라서 구지뽕나무는 두 나무가 연결되어 한 나무를 이루는 것으로 짐작된다. 또한 무궁화는 경상남도 합천군 청덕면 두곡리 청덕초등학교 내에 있는데 수령 50년 이상으로 멀리서 보면 한 나무의 형상을 하고 있으나, 가까이 다가가서 줄기의 아래부분을 보면 세 줄기가 붙어있는 형상을 하고 있다. 따라서 이 무궁화 역시 한 나무의 세 가지인지 세나무가 가까이 자라 붙었는지 PCR과 Southern hybridization 방법을 이용하여 규명하려 하였다. Southern hybridization 결과에 의하면 구지뽕나무의 분석에 사용한 probe와 결합하는 DNA 단편은 검출되지 않았으며, 35S primer에 의한 PCR 생성물은 동일하였으나 OPA04와 OPA13 primer의 경우 약간의 상이성을 보였다. 이러한 결과에 따라 무궁화는 한 나무의 세 가지인 듯하나 추가적인 연구가 필요하다고 판단된다.

찾는 말 : PCR, Southern hybridization, *Cudraria tricuspidata, Hibiscus syriacus*

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