

Rapid Analysis of Genetic Relationship of Phytoplasma Isolates by a DNA Heteroduplex Mobility Assay

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DNA Heteroduplex Mobility Assay법을 이용한 파이토플라스마 병원체의 유연관계 분석

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ABSTRACT: Molecular identification and genetic relationships between a phytoplasma associated with chestnut little leaf (CLL) and phytoplasma isolates of other trees in Korea were amplified by polymerase chain reaction (PCR). These 16S rDNA sequences amplified from the various phytoplasmas were used in DNA heteroduplex mobility assays (HMA). In DNA HMA combined with PCR, the mobility shift was observed for a heteroduplex formed in combined with CLL and jujube witches' broom, but not for those formed in combined with CLL and each of sumac witches' broom, paulownia witches' broom, and mulberry dwarf. HMA combined with PCR has been shown to be a very useful method for detection and differentiation of phytoplasmas.

Key words: Heteroduplex mobility assay, PCR, Phytoplasma.

Heteroduplex mobility assay (HMA) is a new method for the detection and estimation of genetic divergence between different genotypes and their strains without large-scale DNA sequencing which needs cost and laborious (3). This is a rapid, accurate and simple quantitative screening tool based on the observation that DNA heteroduplexes formed between related sequences have a reduced mobility in polyacrylamide gels proportional to their degree of divergence. Heteroduplexes, or DNA hybrids, are generated by base pairing between complementary single strands derived from the different parental duplex molecules, it occurs during genetic recombination. Unknown DNA sequences of genetically common and rare variants could, therefore, be determined on a selective rather than random base.

Recently, the use of HMA has allowed simple and accurate classification of various different phytoplasma isolates (1, 12).

The objective of this study is finding out simple, fast, and reliable method for detection and differentiation of different strains and groups of phytoplasmas.

MATERIALS AND METHODS

Plant material. Naturally infected samples of chestnut (*Castanea crenata* Sieb. et Zucc.) with typical little leaf symptoms were obtained from Songkwang, Chonbuk and Practice Farm of University of Chonnam in Korea. Healthy chestnut samples were collected from chestnut seedling grown in the greenhouse. Sumac (*Rhus japonica* L.) samples with witches' broom were collected from Songkwang, Chonbuk. The phytoplasma was transmitted to healthy sumac seedlings grown in the greenhouse by dodder (*Cuscuta japonica* C.).

Mulberry (*Morus* spp.) samples with dwarf symptom were collected from Chonbuk Sericulture Experiment Station. Paulownia (*Paulownia tomentosa* S.) with witches' broom in Anyang, Jujube (*Zizyphus jujuba* M.)

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with witches' broom in Songkwang, Chonbuk in Korea were collected, too. All samples were stored at -80°C until DNA extraction.

DNA extraction. The procedure described by Kollar *et al.* (8) was used with minor modifications. One gram each of midrib was pulverized in liquid nitrogen with a mortar and pestle. The powder was homogenized in 3 ml of cetyltri-methylammonium bromide (CTAB) extraction buffer (2.5 M NaCl, 0.5% (W/V) polyvinylpyrrolidone-10 (Sigma), 1% (W/V) Cetavlon (hexadecyltrimethylammonium bromide), 0.5 M Tris-HCl (pH 8.0), 0.25 M EDTA (pH 8.0), 0.2% 2-mercaptoethanol). The suspension was incubated for 40 min at 65°C and centrifuged for 5 min at 1,200 g. The supernatant was extracted with an equal volume of chloroform-isoamyl alcohol (24:1) by centrifugation at 1,200 g for 5 min. The aqueous phase was mixed with 0.7 vol of isopropanol, left standing for 5 min at room temperature and centrifugation at 1,200 g for 5 min. The resulting pellet was washed with 70% ethanol and centrifugation at 1,200 g for 5 min, dried under vacuum for 10-20 min. and resuspended by 150 μl of distilled water and digested with RNase. The concentration of DNA in a sample was calculated with a spectrophotometer at 260 nm.

Primers and PCR. Universal primers were selected for PCR and HMA. The sequence of upstream primer R16F2 is 5'-GAAACGACTGCTAAGACTGG-3' and that of downstream primer R16R2 is 5'-TGACGGGC-GGTGTGTACAAACCCCG-3' (10).

The amplification was carried out in 80 μl of reaction mixture, containing 200 μM of dNTPs, 1 μM of each primer, 10 X PCR buffer, 2.5 mM MgCl_2 , 2U Taq DNA polymerase (Promega Corp.). The mixture was covered with 25 μl of mineral oil. Thirty PCR cycles were conducted in an automated thermocycler (Perkin Elmer Cetus, Norwalk, CT). The following parameters were used: a 1-min (4-min for the first cycle) denaturation step at 94°C , annealing for 2 min at 54°C , and primer extension for 3 min (7 min in the final cycle) at 72°C . To analyze undigested PCR amplification products obtained from DNA of healthy and infected plants, 10 μl of the reaction mixture were analyzed by electrophoresis in 1.0% agarose gel followed by staining in ethidium bromide and visualization of DNA bands using a UV transilluminator.

Heteroduplex mobility analysis. A sample of 10 μl of PCR products from each of the phytoplasma isolates was combined with 8 μl of chestnut little leaf (CLL) PCR product, then 2 μl of 10 \times annealing buffer

was added (1 M NaCl, 100 mM Tris-HCl [pH 7.8], 20 mM EDTA). DNA was denatured at 94°C for 2 min and renatured by rapid cooling on ice. The DNA fragments were separated in 5% polyacrylamide gel (acrylamide:bis=29:1) in 1 \times TBE buffer at 200 V for 3 hrs, followed by staining in ethidium bromide and visualization of DNA bands using UV transilluminator.

RESULTS

PCR amplification. Using a universal primer sets, R16F2/R2, the 1.2 kb DNA fragment of the 16S rDNA was amplified with CLL and other diseased tree samples, but control reaction containing template DNA extracted from healthy chestnut and other tree phytoplasma isolates did not produce any PCR products (Fig. 1).

Heteroduplex mobility analysis. The corresponding fragments (1.2 kb) of 16S rDNA were amplified using universal primer pair from a number of phytoplasma isolates, which represent a variety of epidemiological origins. Heteroduplexes were formed by denaturing and reannealing mixtures of amplified DNA fragments from divergent phytoplasma isolates. Pairwise combinations of amplified products resulted in formation of heteroduplexes between CLL (used as a standard) and JuWB, but did not formation between CLL (used as a standard) and SuWB, PaWB or MD (Fig. 2).

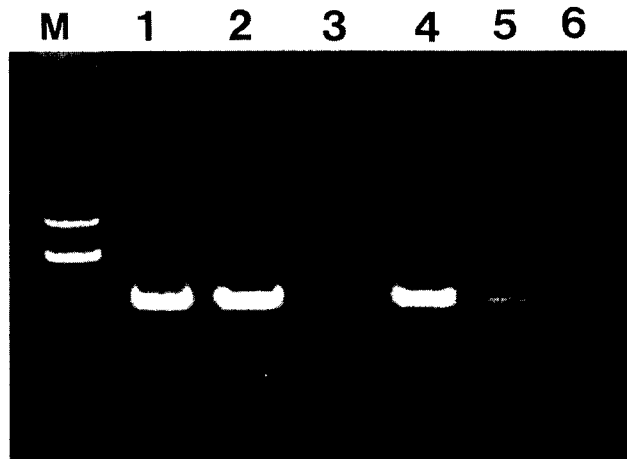


Fig. 1. Analysis of PCR products amplified from DNA samples extracted from healthy chestnut leaves and various phytoplasma infected leaves using R16F2/R2 primer pairs. M: 1 kb DNA marker, 1: chestnut little leaf, 2: paulownia witches' broom, 3: sumac witches' broom, 4: mulberry dwarf, 5: jujube witches' broom, 6: healthy chestnut.

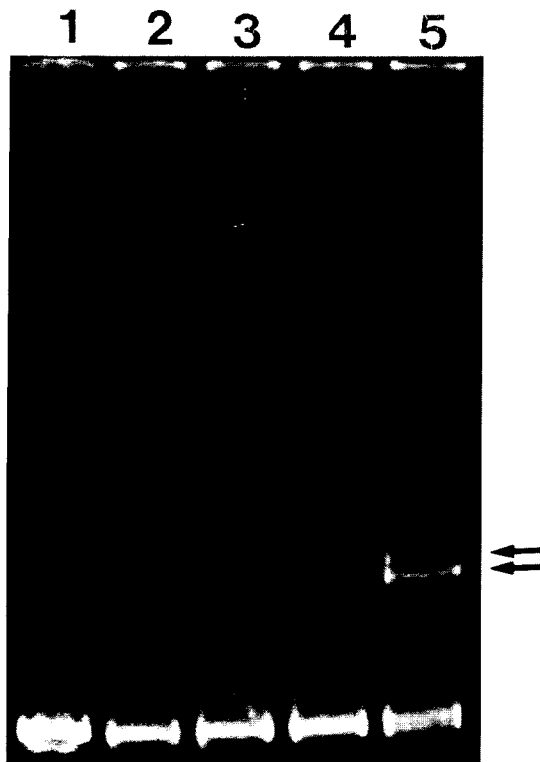


Fig. 2. Heteroduplex mobilities of PCR products of different phytoplasma isolates. Heteroduplexes were formed between CLL and JuWB. Lanes 1: CLL, 2: PaWB, 3: SuWB, 4: MD, and 5: JuWB.: ← indicate heteroduplex.

DISCUSSION

The recent development of PCR-based assays provides a convenient means of obtaining 16S rRNA genes from phytoplasmas and other mollicutes without removing eukaryotic and organelle genomic DNAs. A PCR-based procedure using a "universal" primer pair designed on the basis of 16S rDNA sequence from a strain of Michigan AY phytoplasma (MIAY-86-7) by Lee *et al.* (10) can be employed effectively to detect and identify a broad array of known and unknown phytoplasmas from various host plants. Enrichment of phytoplasma DNA also improved PCR amplification of 16S rRNA genes. In this study, by employing "universal" primer pair in PCR amplification, phytoplasmas of 16S rDNA sequence were amplified from nucleic acid samples extracted from all 5 phytoplasma-infected plants. No PCR products were obtained from samples extracted from healthy plants.

Earlier report on RFLP analysis of 16S rDNA from PaWB and JuWB phytoplasma had shown different band patterns (11) and Kim *et al.* (6) reported that SuWB and MD were the same group and JuWB was in different

group on the basis of sequencing analysis. In this study, the result of HMA analysis from five phytoplasma isolates clearly indicated that PaWB-, SuWB-, MD-phytoplasma are same groups with CLL phytoplasma, but JuWB is shown different group.

As seen in the present work, genetic classification of phytoplasmas using HMA method provides a simple screening tool for determining relationships among phytoplasma isolates. The band of a heteroduplex could be located easily in the position expected on the gel, because even a single 3- base pair gap can induce a noticeable mobility retardation in a heteroduplex formed (3). The degree of mobility retardations of heteroduplexes in polyacrylamide gels was in proportion with the degree of genetic divergence between analyzed phytoplasma isolates. A major advantage of HMA resides in the high accuracy of the procedure to differentiate among different phytoplasma isolates (1, 12). RFLP-based phytoplasma differentiation requires that certain enzyme-recognition sequences exist in the phytoplasma DNA. HMA is performed on the basis that DNA heteroduplexes formed between related sequences have reduced mobilities in polyacrylamide gels in proportion to their degrees of divergence. Our results suggest that HMA will prove important in phytoplasma differentiation when other methods, e.g. RFLP, are not readily applicable to differentiation of very close phytoplasma. Based on the results obtained, HMA, combined with PCR, has been shown to be a very simple, fast, and reliable method for detection and differentiation of different strains and groups of phytoplasmas.

요 약

밤나무 소엽병 파이토플라스마와 다른 수목성 파이토플라스마간의 분자학적 동정과 유전학적 유연관계를 PCR를 통하여 증폭한 후, 이 증폭된 16S rDNA를 Heteroduplex mobility assay방법에 의해 분석한 결과, 밤나무 소엽병 파이토플라스마 병원체와 대추나무 빗자루병 파이토플라스마 병원체에만 heteroduplex가 형성되었고, 밤나무 소엽병과 결합한 붉나무 빗자루병, 오동나무 빗자루병, 뽕나무 오갈병 파이토플라스마 병원체에서는 heteroduplex를 형성하지 않았다. PCR법과 병행한 HMA법은 파이토플라스마를 검출하고 유연관계를 알아볼 수 있는 매우 유용한 방법이었다.

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