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Mixed Infection of 16S rDNA I and V Groups of Phytoplasma in a Single Jujube Tree

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Jujube trees infected with phytoplasma exhibit symptoms of typical witches' broom, such as yellowing, abnormally small leaves, short internodes and proliferation of shoots. A 1.2 kb fragment of the 16S rDNA from jujube phytoplasma was generated by R16F2n/R16R2 primer pair from earlier amplified P1/P7 PCR products of cloned jujube witches' broom phytoplasmas. Enzymatic restriction fragment length polymorphism (RFLP) and sequence analysis of 16S rDNA revealed that the jujube tree was infected with 16S rDNA I and V groups of phytoplasmas. Extensive comparative analyses of restriction enzyme profiles from *Alu* I, *Hha* I, *Msp* I, and *Rsa* I clearly classified the two into different phytoplasma groups. The phylogenic analyses based on 16S rDNA showed that the similarity of the two different clones was 87.5%. This is the first report of a mixed phytoplasmal infection in a single jujube tree.

Keywords : 16S rDNA, jujube witches broom, mulberry dwarf phytoplasma, phytoplasma, RFLP

Phytoplasmas are associated with several hundreds of plant species worldwide (McCoy et al., 1989), and cause serious diseases to several economically important medicinal and fruit plants in Korea but thus far none have been pure cultured. In nature, phytoplasmas are transmitted and spread by insect vectors and disease or pathogen identification and classification have been primarily relied on disease symptoms, host range and insect vectors (Chiykowski et al., 1991, 1988; Shiomi et al., 1984). The multiple distinct phytoplasmas are associated with a particular disease in different hosts (Davis et al., 1993; Alma et al., 1996). Likewise, a particular species of phytoplasma can also cause different diseases (Liefting et al., 1998). Sometimes a single host plant is infected double or multiple with different strains of phytoplasmas (Bianco et al., 1993; Lee et al., 1994). For example, the clover dwarf phytoplamsa was associated with two different phytoplasma groups including both 16S rI-C and 16S rIII-B phytoplasmas (Staniulis et al., 2000)

Among the numerous tree phytoplasmas known in Korea, jujube witches' broom (JWB) phytoplasma is known as the most serious disease causing heavy losses to the trees throughout all parts of Korea and the yield was reduced approximately from 30 to 80% (Lee, 1988).

In nature, JWB, sumac witches' broom (SuWB), and mulberry witches' broom (MD) phytoplasmas were transmitted and spread by the same insect vector *Hishimonas sellatus* Uhler (Chang and Kim, 1971; Kim, 1980; La and Woo, 1980). However, the analysis of RFLP, JWB and MD phytoplasma was clearly distinct phytopalsma (Lee and Yea, 1993). Moreover, the relationships among JWB, SuWB and MD phytoplasmas in Korea based on 16S rRNA gene sequencing have indicated that SuWB and MD isolates were clearly related, whereas JWB phytopalsma was genetically distinct from SuWB and MD phytoplasmas, although, those phytoplasmas were transmitted by the same vector (Kim et al., 1994). The objectives of this study were to identify mixed or multiple phytoplasmas from a naturally infected jujube tree and to determine the relationships of mixed JWB phytoplamsas by RFLP and sequence analysis.

Materials and Methods

Plant materials. Jujube trees, which were naturally infected with JWB phytoplasma, were collected at Boeun-gun, Sericulture Experiment Station, and Wanju at Chonbuk in Korea. Healthy jujube trees were grown from seedlings in a netted greenhouse.

Total DNA extraction. DNA was extracted following Kollar et al. (1990) with minor modifications. One gram of jujube leaf midribs was pulverized in liquid nitrogen with a mortar and pestle. The powder was homogenized in 3 ml of CTAB extraction buffer (2.5 M NaCl, 0.5% (W/V) PVP-10 (polyvinylpyrrolidone-10) (Sigma, U.S.A), 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 at 65°C

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for 40 min and centrifuged at 1,200 *g* for 5 min. The supernatant was extracted with an equal volume of chloroform/isoamyl alcohol (24:1) by centrifugation at 1200 *g* for 5 min. The aqueous phase was mixed with 0.7 vol. of isopropanol, and left standing for 5 min at room temperature. The resulting pellet was washed with 70% ethanol and centrifuged at 1200 *g* for 5 min, then dried under vacuum for 10 min and resuspended by 150 μl of distilled water. The concentration of DNA in a sample was calculated with a spectrophotometer at 260 nm.

Primers and PCR. Phytoplasma universal primer pairs, P1 (Deng and Hiruki, 1991) and P7 (Schneider et al., 1995), were used to amplify a region approximately 1800 bp in length, consisting of the 16S rRNA gene, the 16S-23S rRNA intergenic spacer region and a portion of the 23S rRNA gene. The R16F2n/R16R2 (Lee et al., 1994) was used as a nested PCR. The direct PCR assays were performed using the P1/P7 primer pair. The amplified products were diluted 1:30 with sterile deionized water and reamplified in nested PCR with primer R16F2n/R16R2. Each reaction was performed in a total volume of 50 μl containing 20-30 ng/μl DNA, 150 μM of each dNTP, 2.5 mM $MgCl_2$, 0.5 μM of each primer, 1 × PCR buffer, and 1 U of AmpliTaq Gold DNA polymerase (Perkin-Elimer). The PCR was carry out thirty-six cycles following parameters step; 1 min (5 min for the first cycle) denaturation at 94°C, annealing for 2 min at 55°C, and extension for 3 min (10 min in final cycle) at 72°C. The nest PCR was carry out thirty cycles following parameters step; 1 min (5 min for the first cycle) denaturation at 94°C, annealing for 2 min at 54°C, and extension for 3 min (10 min in final cycle) at 72°C. PCR products were analyzed by 1% agarose gel electrophoresis stained with ethidium bromide and DNA bands were visualized using a UV transilluminator.

Restriction digestion of PCR products. Each PCR products which amplified using R16F2n/R16R2 primer pair were cloned into the PCR 2.1 vector. The final plasmid DNAs were digested for 2 h at 37°C in a 20 μl of reaction mixture with 4 restriction endonuclease, i.e., *Alu* I, *Hha* I, *Rsa* I and *Msp* I. The restriction products were then separated by electrophoresis through a 5% polyacrylamide gel stained with ethidium bromide. DNA bands were visualized using a UV transilluminator, and RFLP patterns were then compared. Molecular weights were determined using digestion fragments of 100 bp.

Cloning of PCR products and DNA sequencing. PCR products which amplified using R16F2n/R16R2 primer pair were gel-purified with the gel clean kit (Qiagen) and ligated into the PCR 2.1 vector and the ligation product was used to transform *Escherchia coli* Top 10 competent cells, according to the manufacturer's instructions (Original TA cloning kit, Invitrogen). Transformed colonies containing inserts were selected as white colonies by blue-white screening method, and single white colonies were picked from culture plates, and then inoculated in 2 ml LB broth, supplemented with 0.1 mg/*l* ampicillin, and grown overnight at 37°C. Plasmid DNAs were prepared by the Miniprep. (QIAprep. Spin, Qiagen). Both strands of each DNA fragments were sequenced with an ABI 373 automated sequencer using the Taq dideoxy terminator cycle sequencing method. All sequence data were analyzed using Genetyx-win (version 4.0) and Gene Bank databases.

Results

Symptoms. The general symptoms on diseased jujube trees were observed typical witches' broom such as abnormally small leaves, shortened internodes, stunting, yellowing and proliferation of shoots. These symptoms were very similar to previously reported JWB phytoplasma.

PCR amplification. PCR products were obtained from direct PCR with phytoplasma universal primer pair P1/P7 (1.8 kb) from all of diseased JWB and MD samples tested. But no amplification product was obtained from asymptomatic healthy jujube plants (Fig. 1). Nested-PCR assays with the primer pair P1/P7 followed by the phytoplasma universal primer pair R16F2n/R16R2 obtained an approximately 1.2 kb DNA fragment from all JWB and MD samples (Fig. 2).

RFLP analysis of PCR products. RFLP analysis of the R16F2n/R16R2 products (about 1.2 kb), with 4 restriction

Fig. 1. Agarose gel electrophoresis of PCR products amplified from healthy and phytoplasma infected plant tissues using P1/P7. M: Molecular weight marker (100 bp DNA ladder), Lane 1, JWB (Wanju); Lane 2, JWB (Boeun); Lane 3, Sericulture Experiment in Chonbuk (Group V); Lane 4, Sericulture Experiment in Chonbuk (Group I); Lane 5, MD; Lane 6, Healthy jujube tree.

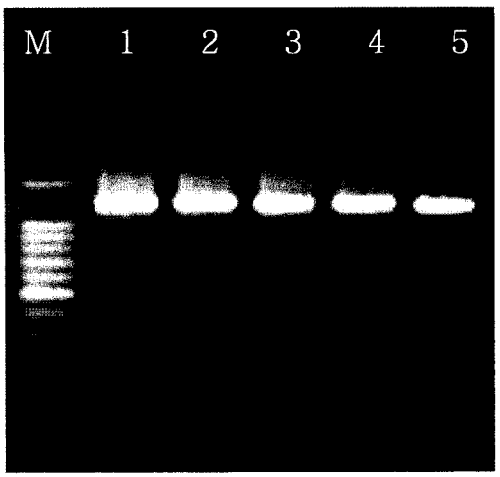

Fig. 2. Agarose gel electrophoresis of PCR products amplified from healthy and phytoplasma-infected plant tissues using R16F2n/R16R2. M: Molecular weight marker (100 bp DNA ladder), Lane 1, JWB (Wanju); Lane 2, JWB (Boeun); Lane 3, Sericulture Experiment in Chonbuk (Group V); Lane 4, Sericulture Experiment in Chonbuk (Group I); Lane 5, MD.

endonuclease, i.e., *Alu* I, *Hha* I, *Msp* I and *Rsa* I resolved two distinct RFLP patterns. The JWB (Wanju), JWB (Boeun) and JWB (Sericulture Experiment Station in Chonbuk, Group V) phytoplasma were produced the same restriction profiles belonging to 16SrV group (Elm yellows group), and JWB (Sericulture Experiment Station in Chonbuk, Group I) and MD phytoplasma were produced the same restriction profiles belonging to 16SrI (Aster yellows group) (Fig. 3). The RFLP patterns of 16S rDNA from JWB (Sericulture Experiment in Chonbuk, Group V) and JWB (Sericulture Experiment in Chonbuk, Group I) both of which were separated from one tree were slightly shown different RFLP profiles. These results indicated that one jujube tree showing JWB phytopalsma was infected with two different phytoplasmas.

Sequence analysis. Using the phytoplasma primer pair R16F2n/R16R2, the near complete nucleotide sequences of 16S rRNA gene of JWB (Sericulture Experiment in Chonbuk, Group V) and JWB (Sericulture Experiment in Chonbuk, Group I), both of which were separated from one tree, were determined. In the phylogenetic analysis with JWB (AY 072722) and MD (AY 075038), the clone of JWB (Sericulture Experiment in Chonbuk, Group V) was highly homologous with JWB and the similarity was 99.8%, but the other clone of JWB (Sericulture Experiment in Chonbuk, Group I) revealed 100% homology with MD phytoplasma (Fig. 4). These results indicated that the jujube tree infected with phytoplasma was mixed with two different phytoplasmas belonging to 16SrV and 16SrI group.

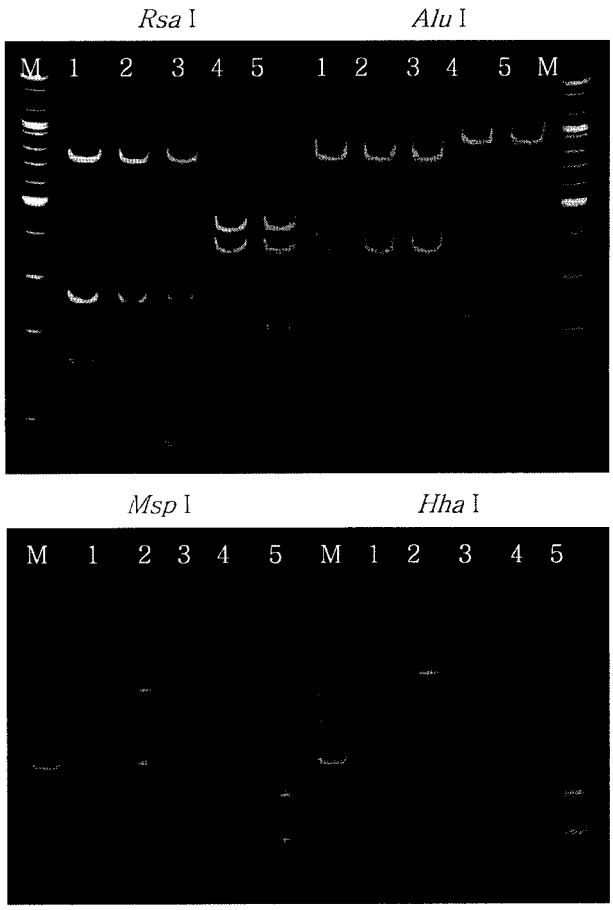

Fig. 3. Polyacrylamide gel electrophoresis of *Rsa* I, *Alu* I, *Msp* I and *Hha* I digest of phytoplasma 16S rDNA amplified by PCR using a primer pair R16F2n/R16R2. M, 100 bp DNA marker; Lane 1, JWB (Wanju); Lane 2, JWB (Boeun); Lane 3, Sericulture Experiment in Chonbuk (Group V); Lane 4, Sericulture Experiment in Chonbuk (Group I); Lane 5, MD.

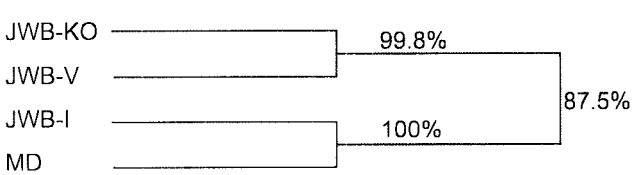

Fig. 4. Phylogenic tree constructed by parsimony analyses of 16S rDNA sequences of JWB phytoplasma and reference phytoplasmas. JWB-Ko (Gene Bank No. AY 072722), JWB-V (Sericulture Experiment in Chonbuk (Group V), JWB-I (Sericulture Exper. in Chonbuk (Group I), MD (GeneBank No. AY 075038).

Discussion

The present study separated the different phytoplasma groups belonging to 16SrV and 16SrI groups from a single jujube tree infected with JWB phytopalsma. Phytoplasmas are associated with both plants and insect vectors. Because of

overlapping vectors and host plant ranges shared by phytopalsmas, it has been suspected that a single host plant and insect vector can be infected by more than one type of phytoplasmas. In naturally cultivated areas, both of JWB and MD phytopalsmas are usually transmitted by the same insect vector, *Hishimonus sellatus* in Korea (La and Woo, 1980; Chang and Kim, 1971). The JWB phytoplasma belongs to 16SrV (Elm yellow phytopalsma) and MD phytoplasma belongs to 16SrI group (Aster yellow group) (Han and Cha, 2002). Even though, these two phytoplasmas are transmitted by the same phloem-feeding insect vector. The genetic relation- ships based on PCR-RFLP and sequences of 16S rRNA gene were clearly distinct (Han and Cha, 2002). But the results of PCR-RFLP and sequence analysis in our study clearly indicated that JWB phytoplasma had two types of phytoplasmas on a single trees. Previously, the mixed phytoplasmas from a single host plant separated using group-specific primers (Lee et al., 1994), and clover dwarf phytoplasma in Lithuania was infected with a mixture CPh and CYE phytopalsmas (Staniulis et al., 2000). In general, the phytoplasma was detected by direct PCR performing the genetic analysis. In this study, the JWB phytoplasma was amplified using phytoplasma universal primer and genetic analysis was carried out by nest-PCR. The final PCR products were cloned and the correct insert colonies were performed by RFLP analysis. Though these processes, mixed phytoplasmas were clearly separated and easily classified by RFLP or sequence analysis. This method was more sensitive and correct to divide more than one type of mixed phytopalsmas in a single host plant. It was the first report of two types of phytoplasmas in a naturally cultivated jujube tree infected with JWB phytoplasmas.

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