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Genetic Similarity Between Jujube Witches' Broom and Mulberry Dwarf Phytoplasmas Transmitted by *Hishimonus sellatus* Uhler

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Using phytoplasma universal primer pair P1 and P7, a fragment of about 1.8 kb nucleotide sequences of 16S rRNA gene and 16S-23S rRNA intergenic spacer region, and a portion of 23S rRNA gene of jujube witches' broom (JWB) and mulberry dwarf (MD) phytoplasmas were determined. The nucleotide sequences of JWB and MD were 1,850 bp and 1,831 bp long, respectively. The JWB phytoplasma sequence was aligned with the homologous sequence of MD phytoplasma. Twenty-eight base insertions and nine base deletions were found in the JWB phytoplasma sequence compared with that of MD phytoplasma. The similarity of the aligned sequences of JWB and MD was 84.8%. The near-complete 16S rRNA gene DNA sequences of JWB and MD were 1,529 bp and 1,530 bp in length, respectively, and revealed 89.0% homology. The 16S-23S rRNA intergenic spacer region DNA sequences were 263 bp and 243 bp in length, respectively, while homology was only 70% and the conserved tRNA-Ile gene of JWB and MD was located into the intergenic space region between 16S-23S rRNA gene. The nucleotide sequences were 77 bp long in both JWB and MD, and showed 97.4% sequence homology. Based on the phylogenetic analysis of the two phytoplasmas, the JWB phytoplasma belongs to the Elm yellow phytoplasma group (16S rV), whereas, the MD phytoplasma belongs to the Aster yellow group (16S rI).

Keywords : mulberry, 16S rRNA gene, 16S-23S intergenic space region gene, *Zizyphus jujuba*.

Phytoplasmas, formerly called mycoplasma-like organisms, are wall-less prokaryotes that are associated with diseases in several hundreds of plant species in the world (McCoy et al., 1989).

The differentiation and classification of unculturable phytoplasmas relied primarily on biological properties such as symptom, host range, and insect vector specificity. The determination of biological properties has often been time-consuming, laborious and sometimes unreliable (Lee et al., 1992).

Recently, molecular-based analyses have been used increasingly to identify and differentiate unculturable phytoplasmas. The introduction of PCR for assay, in which universal primers and specific primers derived from 16S rRNA gene sequences are used, has improved the ability of researchers to identify and classify a broad range of phytoplasmas accurately (Ahrens et al., 1992; Davis et al., 1993; Lee et al., 1993; Lee et al., 1988; Namba et al., 1993).

In Korea, jujube witches' broom (JWB) and mulberry dwarf (MD) phytoplasmas cause sporadic to widespread damage on economically important trees. While these two phytoplasmas are transmitted by the same phloem-feeding insect vector, the leafhopper (*Hishimonus sellatus* Uhler.) (Chang et al., 1971; La et al., 1980), the genetic relatedness based on PCR-RFLP and sequences of 16S rRNA gene was distinct (Lee et al., 1993; Kim et al., 1994). However, this genetic relatedness between JWB and MD is not quite known. The objective of this study was to determine the phylogenetic position of JWB and MD phytoplasmas that are transmitted by the same vector, by sequencing of the complete 16S rRNA gene and 16S/23S rRNA spacer region gene.

Materials and Methods

Plant materials. Samples of naturally infected mulberry were collected from Chonbuk Sericulture Experiment Station, while jujube (*Zizyphus jujuba* M.) with jujube witches' broom disease was collected from major jujube growing regions of Songkyong in Chonbuk, Korea. Healthy mulberry and jujube samples grown from seedlings in the greenhouse were used as control.

Total DNA extraction. The DNA was extracted following the method of Kollar et al (1990) with minor modifications. One gram of the midrib 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 (polyvinyl-pyrrolidone-10) (Sigma, U.S.A), 1% (W/V) Cetavlon (hexadecyl-trimethylammonium 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 minutes at 65°C and centrifuged for 5 minutes 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

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5 minutes. The aqueous phase was mixed with 0.7 volume of isopropanol, and was left standing for 5 minutes at room temperature. The resulting pellet was washed with 70% ethanol and centrifuged at 1,200 g for 5 minutes, and then dried using a vacuum for 10 minutes and resuspended using 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 et al., 1991) and P7 (Schneider et al., 1995b) were used to amplify a region approximately 1,800 bp in length, consisting of the 16S rRNA gene, the 16S-23S rRNA intergenic spacer region and a portion of the 23S rRNA gene.

Primer pairs P3 (Schneider et al., 1995a) and P7 were used to amplify the 16S-23S rRNA intergenic spacer region. The DNA was amplified in a 50 μl reaction volume containing 20-30 ng/μl DNA, 150 μM of each dNTP, 2.5 mM $MgCl_2$, 0.5 μl M of each primer, 1 X PCR buffer and 1 U of AmpliTaq Gold DNA polymerase (Perkin-Elimer). PCRs were carried out for 38 cycles with the following conditions: 1 minute (9 minutes for the first cycle) at 94°C for denaturation; 2 minutes at 58°C for annealing; and 3 minutes at 72°C (7 minutes for the last cycle) for extension. PCR products were analyzed by electrophoresis in a 1% agarose gel, staining with ethidium bromide, and DNA bands were visualized using a UV transilluminator.

Cloning of PCR products and DNA sequencing. PCR products were gel purified with the gel clean kit (Qiagen) and ligated into the PCR 2.1 vector. The ligation product was then used to transform *Escherichia 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 picked from culture plates were added to 2 ml LB medium with ampicillin 0.1 mg/l, and grown overnight at 37°C. Plasmid DNAs were prepared by the Miniprep (QIAprep Spin, Qiagen). The first plasmid DNAs used the nested deletion approach (Yanisch et al., 1985). A series of exonuclease III digestion deletions were generated from plasmid DNAs (about 1.8 kb) by termination of reaction at 2-minute interval. Both strands of each DNA fragment 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 GenBank databases.

Results

Amplification of the 16S rRNA gene and the 16S-23S rRNA intergenic spacer region gene. The primer pair P1/P7 amplified a fragment of about 1.8 kb which contained the 16S rRNA gene, the 16S-23S intergenic spacer region and the 23S partial sequences from JWB and MD phytoplasmas (Fig. 1A). The primer pair P3/P7 for the 16S-23S spacer region of JWB phytoplasma yielded a product of approximately 350 bp, whereas, that of MD phytoplasma yielded a product of approximately 310 bp (Fig. 1B). No product was amplified from healthy plants (Fig. 1A lane 3).

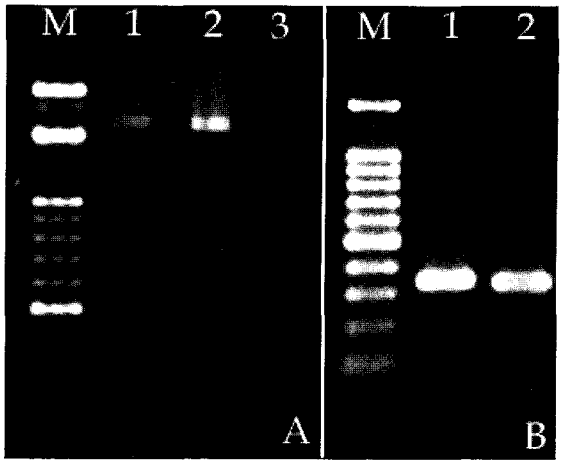

Fig. 1. Agarose gel electrophoresis of PCR products amplified from healthy and infected plant tissue with phytoplasmas using P1/P7 primer pairs (**A**) and P3/P7 primer pairs (**B**). Lane M, 100 bp DNA marker; Lane 1, *Zizyphus jujuba* witches' broom; Lane 2, mulberry dwarf; Lane 3, healthy *Z. jujuba*.

Sequence analysis. Using phytoplasma primer pair P1 and P7, the nucleotide sequences of 16S rRNA gene, 16S-23S rRNA intergenic spacer region and a portion of 23S rRNA gene of JWB and MD were determined. The nucleotide sequences were 1,850 bp (GenBank Accession No. AY072722) and 1,831 bp (AY075038) in length, respectively. Near-complete 16S rRNA gene sequences 1,529 bp long for JWB and 1,530 bp long for MD were obtained, except for 6 bp at the 5' end of the 16S RNA gene of both two amplimers. The 16S-23S spacer region DNA sequences were 263 bp long for JWB and 243 bp long for MD, while the tRNA-Ile gene sequences were both 77 bp long. The JWB phytoplasma sequence was aligned with the homologous sequence of MD phytoplasma using Genetyx-win (version 4.0) and GeneBank database. Twenty-eight base insertions and ten base deletions were found in the JWB phytoplasma sequence compared with that of MD phytoplasma sequence. The similarity of the aligned sequences of JWB and MD revealed 87% homology.

The near-complete 16S rRNA gene DNA sequences of JWB and MD revealed 90.0% homology. The 16S-23S intergenic spacer region DNA sequences revealed 70% homology. The tRNA-Ile gene of JWB and MD was located within 16S-23S rRNA spacer regions and the sequences were 77 bp in length for both JWB and MD, and showed 97.4% sequence homology. Fifty-eight base pairs of the 5' end of the 23S rRNA gene were determined for JWB and MD.

Phylogenetic analysis. In the phylogenetic analysis using GenBank database, JWB (AY 072722) was highly homologous to those of the 16Sr V (Elm yellow phytoplasma groups) such as *Zizyphus jujuba* witiches' broom (AF305240),

Elm yellow phytoplasma (AF189214), and Alder yellows phytoplasma (Y16387); and their similarities were 99.7%, 98% and 98%, respectively. The MD (AY 075038) was most similar to those of the Aster yellows phytoplasma group (16SrI), such as aster yellows (AF222063) and tomato big bud (AF222064) with 99% and 98% homology, respectively. In terms of sequence homology of 16S/23S space region in this study, JWB revealed high homology with JWB of China strain and FD (Flavescence doree) phytoplasma of grapevine (AF176319), with 100% and 98% homology, respectively. The conserved tRNA-Ile gene was different in only one position with FD (AF176319) phytoplasma, whereas, the JWB of China strain revealed 100% homology. The near-complete 16S rRNA gene of JWB showed high homology with JWB (AF305240), Alder yellow phytoplasma (Y16387) and EY phytoplasma (AF189214) with 99%, 98% and 98% homology, respectively. The MD phytoplasma showed high homology in sequence with AY (AF322645), clover phyllody (AF222066) and TBB (AF222064) at 99%, 98% and 98%, respectively. On the other hand, MD phytoplasma of 16S rRNA gene with MD strain of Japan and AY revealed 99.4% and 99.2% homology, respectively, whereas the 16S/23S space region showed 100% homology with AY phytoplasma. These results indicate that JWB belongs to group V (elm yellow group) phytoplasma while MD phytoplasma belongs to group I (aster yellow group).

Discussion

There has been little study of the genetic relationships between JWB and MD phytoplasmas, although these are transmitted by the same insect vector, *Hishimonus sellatus*, in Korea. In this paper, significant differences were found in the 16S rRNA gene, 16S-23S rRNA intergenic spacer region and a portion of 23S rRNA gene of JWB and MD. These results suggest that there are genetic diversity between JWB and MD phytoplasmas.

To date, many phytoplasmas have been classified by sequence analyses of the 16S rRNA gene and 16S-23S rRNA intergenic spacer region gene (Davis et al., 1997; Liefting et al., 1996; Macone et al., 1996; Schneider et al., 1995a, b; Seemüller et al., 1994; White et al., 1998). Namba et al. (1993) amplified the 16S rRNA genes of phytoplasma using a universal primer set (about 1.4 kb) by PCR from phytoplasma-enriched fraction of plants infected with each of six Asian phytoplasmas and two American phytoplasmas [O (*Oenothera hookeri*)-phytoplasma and SAY (severe strain of western aster yellows)-phytoplasma], and divided them into three groups: I = OY (onion yellows), TY (tomato yellows), MD (mulberry dwarf), PaWB (paulownia witches' broom) and SAY; II = TW (tsuwabuki witches' broom); and III = RY (rice yellow dwarf). Based on the comparison among these groups, Korean MD was closely related with Group I.

Based on near-complete 16S rDNA sequences of 21 phytoplasma strains, Seemüller et al. (1994) identified five groups: I = aster yellows group; II = apple proliferation group; III = Western X-disease group; and V = elm yellows group. In this study, using primer pairs P1/P7, approximately 1.8 kb of the nucleotide sequences, MD phytoplasma was closely related with group I, while JWB phytoplasma was closely related with group V. In the comparison of sequences, JWB phytopalsma was about 19 nucleotides longer than MD phytoplasma and showed 84.8% homology.

The analysis of JWB phytoplasma strains of Korea and China (AF305240) revealed five different positions: 428(G-A), 511(A-G), 724(C-G), 745(A-G) and 1216(A-T), and their homology was 99.7%. The present study showed that JWB phytoplasmas of Korea and China are very closely related with each other.

Previously, JWB and MD phytoplasmas have been considered to be strains of different organisms in Korea (Han, 1998), even though they are transmitted by the same leafhopper, and their 16S rRNA gene sequence homology was 90.7%. The near-complete 16S rDNA sequence analysis presented in this paper confirmed the close similarities of 89% homology. The complete 16S rRNA gene DNA sequence of JWB and MD revealed 90% homology. Comparison of the 16S-23S intergenic spacer region DNA sequences of JWB and MD revealed 70% homology, while the tRNA-Ile gene was located within 16S-23S rRNA spacer regions and showed 97.4% sequence homology. These results suggest that these two phytoplasmas highly differ in terms of the 16S rRNA gene sequence, even though the tRNA-Ile gene was conserved in intergenic space region.

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References

Ahrens, U. and Seemüller, E. 1992. Detection of DNA of plant pathogenic mycoplasma-like organisms by a polymerase chain reaction that amplifies a sequence of the 16S rRNA gene. *Phytoplathology* 82:828-832.

Chang, B. H. and Kim, C. J. 1971. Studies on the dwarf disease of mulberry tree. *Seri. J. Korea* 13:17-21.

Davis, R. E., Dally, E. L., Gundersen, D. E., Lee, I.-M. and Habili, N. 1997. "*Candidatus* Phytoplasma australiense," a new phytoplasma taxon associated with Austraian grapevine yellows. *Int. J. Syst. Bacteriol.* 47:262-269.

Davis, R. E. and Lee, I.-M. 1993. Cluster-specific polymerase chain reaction ampliofification of 16S rDNA sequences for detection and identification of mycoplasma-like organisms. *Phytopathology* 83:772-776.

Deng, S. and Hiruki, C. 1991. Amplification of 16S rRNA genes from culturable and non-culturable mollicutes. *J. Microbiol. Methods* 14:53-61.

Han, S. S. 1998. Studies on little leaf disease of chestnut tree (Castanea crenata Sieb. Et Zucc.) in Korea. -Detection of phytoplasma and its sequence-. *Ph.D thesis. Chonbuk National University.* pp. 1-91.

Kim, Y. H., So, I. Y. and Han, S. S. 1994. MLO-DNA nucleotide sequence and diversity of mycoplasmal tree disease in Korea. *Mycoplasmamology* 5:53-59.

Kollar, A., Seemüller, E., Bonet, F., Saillaird, S. and Bové, J. M. 1990. Isolation of the DNA of various plant pathogenic mycoplasma-like organisms from infected plants. *Phytopathology* 80:233-3237.

La, Y. J. and Woo, K. S. 1980. Transmission of jujube witches' broom mycoplasma by leafhopper *Hishimonus sellatus* Uhler. *Korean J. Forestry* 48:29-39.

Lee, I-M., Hammond, R. W., Davis, R. E. and Gundersen, D. E. 1993. Universal amplificatrion and analysis of pathogen 16S rDNA for classification and identification of mycoplasma-like organisms. *Phytopathology* 83:834-842.

Lee, I.-M., Davis, R. E., Chen, T.-A., Chiykowski, L. N., Fletcher, J., Hiruki, C. and Schaff, D. A. 1992. A genotype-based system for identification and classification of mycoplasma-like organisms (MLOs) in the aster yellows MLO strain cluster. *Phytopathology* 82:977-986.

Lee, I.-M. and Davis, R. E. 1988. Detection and investigation of genetic relatedness among aster yellows and other mycoplasma-like organisms using cloned DNA and RNA probes. *Mol. Plant-Microbe Interact.* 1:303-310.

Lee, J. T. and Yea, M. C. 1993. Detection of mycoplasma-like organisms (MLOs) using PCR from diseased plants in Korea. *Korean J. Mycoplasmamology* 4:36-44.

Leifting, L. W., Andersen, M. T., Beever, R. E., Gardner, R. C. and Forster, R. L. S. 1996. Sequence heterogeneity in the two 16S rRNA genes of phormium yellow leaf phytoplasma. *Appl. Environ. Microbiol.* 62:3133-3139.

Marcone, C., Ragozzino, A., Schneider, B., Lauer, U., Snart, C. D. and Seemüller, E. 1996. Genetic characterization and classification of two phytoplasma associated with spartium witches' broom disease. *Plant Dis.* 80:365-371.

McCoy, R. E., Caudwell, A. and Chang, C. J. 1989. Plant diseases associated with mycoplasmalike organisms. pp. 545-560. In: *The Mycoplasmas*, vol. 5. Academic Press, New York.

Namba, S., Kato, S., Iwanami, S., Oyaizu, H., Shiozawa, H. and Tsuchizaki, T. 1993. Detection and differentiation of plant-pathogenic mycoplasmalike organisms using polymerase chain reaction. *Phytopathology* 83:786-791.

Schneider, B., Cousin, M., Klinkong, S. and Seemüller, E. 1995a Taxanomic relatedness and phylogenetic positions of phytoplasmas associated with disease of faba bean, sunnhemp, sesame, soybean, and eggplant. *J. Plant Dis. Prot.* 102:225-232.

Schneider, B., Seemüller, E., Smart, C. D. and Kirkpatrick, B. C. 1995b. Phylogenetic classification of plant pathogenic mycoplasma-like organisms or phytoplasmas. In *Morecular and Diagnostic Procedures in Mycoplasmology*, vol. 1, pp. 369-380.

Seemüller, E., Schneider, B., Mäurer, R., Ahrens, U., Daire, X., Kison, H., Lorenz, K.-H., Firrao, G., Avinent, L., Sears, B. B. and Stackebrandt, E. 1994. Phylogenetic classification of phytopathogenic mollicutes by sequence analysis of 16S ribosomal DNA. *Int. J. Syst. Bacteriol.* 44:440-446.

White, D. T., Blackwall, L. L., Scott, P. T. and Walsh, K. B. 1998. Phylogenetic positions of phytoplasmas associated with dieback, yellow crinkle and mosaic diseases of papaya, and their proposed inclusion in "candidates Phytoplasma australiense" and a new taxon, "Candidatus Phytoplasma australasia". *Int. J. Syst. Bacteriol.* 48:941-951.

Yanisch-Perron, C., Vieira, J. and Messing, J. 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* 33:103-19.