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Association of Aster Yellow Phytoplasma with Witches' Broom Disease of Ash (*Fraxinus rhynchophylla* Hence) in Korea

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Abstract : Typical phytoplasma whiches' broom symptoms were observed in Ash (*Fraxinus rhynchophylla* Hence) in Korea. The symptoms of the disease were showing abnormally small leaves, shorted internodes and proliferation of shoots. Examination of fluorescent and electron microscopy of leaf midribs revealed numerous phytoplasma bodies localized in the phloem tube cells. The phytoplasmas were detected in all the symptomatic samples by the amplification with phytoplasma specific primer pair P1/P7 consistently, and the expected size was1.8 kb. However, the phytoplasma DNA was not detected in healthy seedlings. Based on sequence analysis of amplified region, this phytoplasma has close homologies with eqilodium phyllody, mulberry dwarf, and aster yellow phytoplasmas, 99.95%, 99.79% and 99.78%, respectively, This phylogetic analysis indicates that ash witches' broom phytoplasma should be classified in the aster yellow group 16SrVI and clearly distinct from the ash yellow group 16SrVII.

Key words **:** *AshWB,* Fraxinus rhynchophylla, *fluorescent microscopy, electron microscopy, PCR*

Introduction

Ash (*Fraxinus rhynchophylla* Hence) is widely cultivated throughout Asia, Europe and North America as well as in korea as an important component of the landscape and have been planted at urban region. The typicall witches' broom symptoms on ash trees were first identified in 2002 at the Jeonbuk province in Korea. In U.S.A, ash yellow caused by phytoplasma was shown symptoms such as growth reduction, decline, chlorotic, shoot growth from axillary and shortened internodes of white ash (Hibben and Wolanski., 1971; Schall and Agrios., 1973). Whereas showing abnormally small leaves, shorted internodes, and proliferation of shoots on ash tree in korea. Recently, PCR-RFLP and PCR-sequence analyses have been used extentively to identify and differentiate the unculturable phytoplasmas (Ahrens and Seemüller, 1992; Davis and Lee, 1993; Gundersen *et al.*, 1994; Schneider *et al.*, 1995). Based on 16S rRNA gene sequences, the ash yellow phytoplasma was identified as 16S rVII group (Ash yellow phytoplasma groups) (Davis *et al.*, 1992; Lee *et al.*, 1993: seemüller *et al.*, 1994). The objective of this study was 1) to detect of the phytoplasma from ash tree by fluorescent microscopy, Electron microscopy and PCR, and 2) to determine its phylogenetic relation other phytoplasmas by sequencing analyses of 16S rDNA and ITS gene.

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Materials and Methods

1. Plant materials

Samples of naturally infected ash (*Fraxinus rhynchophylla* Hence) were collected from Wanju, at Jeonbuk in Korea. Healthy ash samples grown from seedlings in the greenhouse were used as control.

2. Fluorescence microscopy

The procedure by Seemüller, E, (1976) was used in this study. Petioles and stem tissues from healthy and diseased samples were cut, about 0.5 cm thick. For DNA staining, sections were fixed in 5% glutaraldehyde in 0.1 M phosphate buffer pH 7.0 for 2 hrs. After rinsing, the samples were cut in hand section about 0.5 mm thick. After rinsing, they were stained in a solution (1 μg/ml) of 4', 6-diamidino-2-phenylinodole. 2HCl (DAPI, Sigma Chemical Co., St. Louis, U.S.A). The sections were mounted in a DAPI solution for 30 min. and examined for DNA-specific fluorescence under a fluorescent microscope (Nikon HFX-IIA).

2. Electron microscopy

Petiole and main vein obtained from healthy and dis-

eased trees were cut into small pieces, about 2 mm×2 mm thick, and fixed for 2 hrs at 4°C in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). The tissues were then washed in buffer and postfixed in 1% osmium tetroxide for 2 hrs. After another wash, the tissues were dehydrated in a graded ethanol series and placed in propylene oxide. Reinfitration was done by use of graduated concentrations of Epon 812 in propylene oxide. The tissues were immersed in 100% Epon, placed in flat molds, and cured for 24 hrs at 60°C. Thin sections of embedded tissue were cut on an LKB Ultrotome III equipped with a glass knife, and stained with uranyl acetate. Examinations were made with a Hitachi H-180 electron microscope.

3. Total DNA extraction

The DNA was extracted following Kollar *et al.* (1990) with minor modifications. One gram of 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 for 40 min. at 65°C and centrifuged for 5 min. at 1,200 g. The supernatant was extracted with an equal volume of chlorform/isoamyl alcohol (24:1) by centrifugation at 1,200 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 1,200 g for 5 min, and 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.

4. Primers and PCR

Phytoplasma universal primer pairs, P1 (Deng *et al.*, 1991) and P7 (Schneider *et al.*, 1995), were used to amplify a region approximately 1,800 bp in length, consist of the 16S rRNA gene, the 16S-23S rRNA intergenic spacer region and a portion of the 23S rRNA gene. 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 following conditions: 1 min. (9 min. for first cycle) at 94°C for denaturation, 2 min. at 50°C for annealing and 3 min. at 72°C (7 min. for last cycle) for extension. PCR products were analyzed by electrophoresis in a 1% agarose gel and staining with ethidium bromide. The DNA bands were visualized by using a UV transilluminator.

5. 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 and ligation product was used to transform *Escherchia coli* Top 10 competent cells according to the manufacturer's instructions (Origianl TA cloning kit, invitrogen). The transformed colonies were selected as a white colonies by blue-white screening method. Single white colonies were picked from culture plates, added in to 2 ml LB medium with ampicillin 0.1 mg/*l*, and grown overnight at 37°C. The plasmid DNAs were prepared by the Miniprep. (QIAprep Spin, Qiagen). The first plasmid DNAs are used the nested deletion approach (Yanisch et al, 1985). A series of exonuclease III digestion deletions were generated from pasmid DNAs (about 1.8 kb) by termination of reaction at 2 min. intervals. 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 Gene Bank databases.

Results

1. Symptomatology

The general symptoms were observed typical phytoplasma witches' broom such as abnormally small leaves, shortened internodes and proliferation of shoots of trees (Figure 1-a, b).

In affected plants, leaves were smaller in size and numerous in number than normal trees. Furthermore, the lateral buds on these shoots were abnormally activated in growth. In advanced stages of the disease, the new shoots were short and slender and failed to develop. In many cases, typical witches' broom symptoms developed in matured trees, 10-20 years old and most of the infected young branches were died during winter. This

Figure 1. Naturally infected with phytoplasma showing typical witches' broom on the top (A) and new branch of stem on ash trees (B).

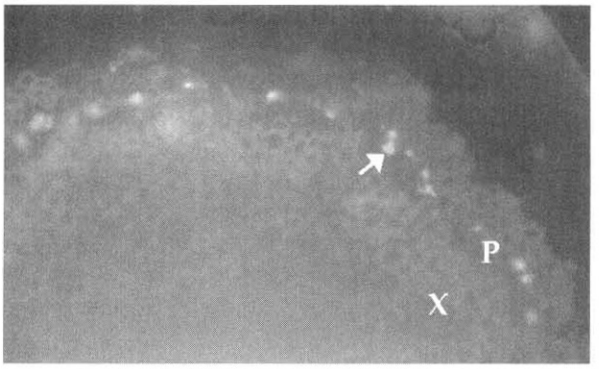

Figure 2. Fluorescence micrograph of cross section of ash twig stained with DAPI. Arrow indicates phytoplasma-specific fluorescence spots. X : xylem, P : phloem.

observation suggests that the infected plants are weakened by constant proliferation of new shoots so that they readily succumb to low temperatures, whereas healthy trees produced vigorous green leaves and branches.

2. Fluorescence microscopy

After staining with DAPI, fluorescent particles were showed characteristic fluorescence in the phloem elements (Figure 2).

Fluorescent particles were limited to sieve tube elements and readily distinguished from other sources of fluorescence such as host nuclei, mitochondria, and secondary xylem thickenings. Similar fluorescence was not observed in comparable sections from a healthy ash.

3. Electron microscopy

Examination of ultra-thin sections of leaf veins from affected leaves revealed numerous phytoplasmas ranging in diameter from 100-800 nm in phloem tissue. No phytoplasma were present in asymptomatic controls raised from healthy ash (Figure 3)

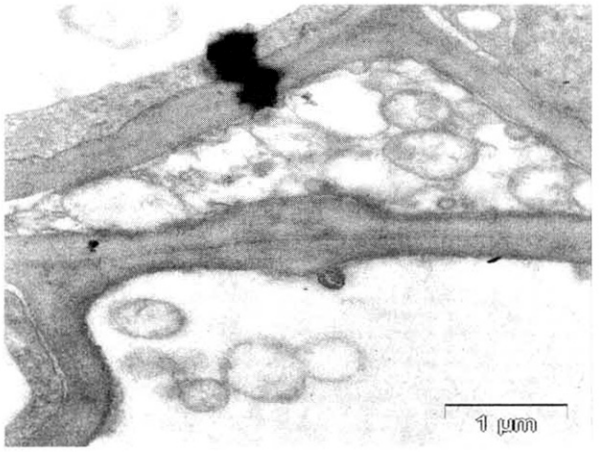

Figure 3. Transmission electron micrograph of sieve element of the leaf midrib of ash tree. Bar=1 μm.

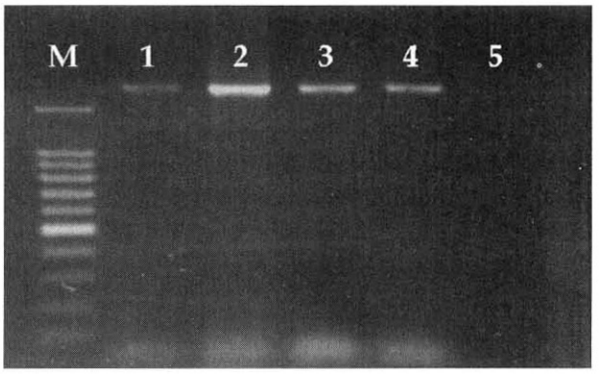

Figure 4. Amplifiction of PCR products from DNA samples extracted from ash leaves infected with phytoplasma using P1/P7 phytoplasma universal primer pair. M : 100 bp DNA marker, 1-4 : AshWB (ash witches' broom), 5: healthy ash.

The morphology of phytoplasmas was typically pleomorphic. Pleomorphism observed in this study was similar to that described previously by other workers (Chen, et al, 1971; Doi *et al*, 1967; Hibino *et al*, 1970) and was evidenced by a wide variety of sizes, shapes, and electron densities. Phytoplasmas were surrounded by a poorly defined membrane but lacked cell wall. The virus like particles or other plant pathogens were not observed.

4. PCR amplification

The universal primer pair P1/P7 amplified the expected 1. 8 kb DNA fragment of the 16S rRNA gene and ITS gene from infected phytoplasma. No amplified product was observed in healthy ash tree (Figure 4).

5. Sequences 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 AshWB was determined. The nucleotide sequences consist of 1,830 bp (GeneBank Accession No. AY 072722) in length. Near-complete sequences of 16S rRNA gene were obtained 1,529 bp. Sequences of the 16S-23S spacer region DNA and the tRNA-Ile gene were 263 bp and 77 bp, respectively.

In the phylogenetic analysis using GeneBank database, AshWB (AY 072722) was most closely related to the Epilobium phyllody (GeneBank Accession No. AY 101386), Mulberry dwarf (GeneBank Accession No. AY 075038), Aster yellow (GeneBank Accession No. AF 222063), and Maize bussy stunt phytoplasma (GeneBank Accession No. AF 487779), and their similarity were 99.7%, 99%, 99% and 98%, respectively which belong to the aster yellow phytoplasma groups, whereas the ash

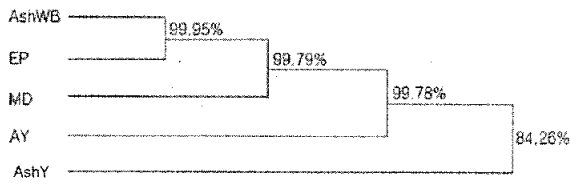

Figure 5. Phlogenic tree of nucleotide sequences from the 16S rDNA, ITS, and a part of 23S rRNA gene of AshWB and other various phytoplasma isolates. AshWB : Ash witches' broom, EP : Epilobium phyllody, MD : Mulberry dwarf, AY : Aster yellow and AshY : Ash yellow.

yellow phytoplasma (GeneBank Accession No. AY666302) revealed 84 % homologous (Figure 5).

Discussion

The association of phytoplasma with typical witches' broom disease of ash tree was confirmed by fluorescent, electron microscopy and PCR. Previously, phytoplasmas have been detected by serology, insect transmission, symptomatology, fluorescence and electron microscopy (Doi *et al.*, 1967; Seemüller *et al.*, 1976). In U. S. A, the ash tree infected by phytoplasma showed the characteristic symptoms of yellowing and dieback (Hibben and Wolanski., 1971; Schall and Agrios., 1973) but in the present study, the infected ash tree showed a typical witches' broom symptoms. The results suggest that the phytoplasma detected from the ash tree is same in Korea and U.S.A, but the symptoms are different. In this hypothesis, we investigated the phylogenic relationships of ash yellow and witches' broom phytoplasma isolates between U.S.A and Korea. The sequences analysis of 16S rDNA, ITS and a part of 23S rRNA gene of ash witches' broom phytoplasma isolates in Korea was most closely related to epidium phyllody, mulberry dwarf, and aster yellow phytoplasmas which belong to aster yellow phytoplasma groups (16S rI) however, the ash yellow phytoplasma isolates (16S rVII) in U.S.A was evidently distinct. These results indicate that AshWB phytoplasma belongs under group I (aster yellow group) phytopalsma. This is first report on observation of ash (*Fraxinus rhynchophylla* Hence) witches' broom phytoplasma in Korea.

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