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Differentiation and Detection of Phytoplasma using PCR from Diseased Plant in Korea

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ABSTRACT

This test checked jujube witches' -broom disease, sumac witches' -broom disease, paulonia witches' - broom disease, and mulberry dwarf disease whether or not they were infected by phytoplasma, using universal and specific primers. Upon treatment of DNA amplified by PCR of phytoplasma with *Alu* Ⅰ, *Hpa* Ⅱ and *Sal* Ⅰ restricted enzymes, distinction of phytoplasmas was possible. Particularly, phytoplasma of each host was distinguishable by treatment of *Hpa* Ⅱ restricted enzyme. Meanwhile, analysis of restricted enzymes of jujube witches' -broom disease showed a higher infectivity of phytoplasmas of two origins. There were a lot of relations between jujube witches' -broom disease and sumac witches' -broom disease, and between paulonia witches' -broom disease and mulberry dwarf disease.

Key Words : Phytoplsama, PCR, RFLP, witches' -broom disease.

INTRODUCTION

Phytoplasma is generally known that it causes a witches' -broom disease, flower virescence and phyllody, and thus it neither produces the seeds, nor gets infected by seeds (Zhu, 1981). It is reported that transmission of phytoplasma is carried by the leafhoppers or dodders (Hibben *et al.*, 1970; Shiomi and Sugiura, 1984). Meanwhile, it is known that there are more than 300 kinds of plant diseases attacked by phytoplasma throughout the world and more than 20 kinds in our country (Hiruki, 1992). Analysis of 16S rRNA shows that phytoplasma has higher relations to *Acholeplasma palmae* and *A. modicum* than to other kinds in the mollicutes (Gundersen *et al.*, 1994). Meanwhile, 16S rRNAs of more than 20 kinds of phytoplasma were analyzed, and thereby classified into several groups (Seeüm ller *et al.*. 1998). As reliable and specific methods for classification and arrangement of phytoplasma, there are ELISA method, immune electron microscope method, etc. (Clark *et al.*, 1989; Lin *et al.*, 1985). Recently, as 16S rRNA genes of phytoplasma can be amplified by the PCR method, they are classified according to the interpretation of restriction fragment length polymorphism (RFLP) (Gibbs, 1996; Gundersen *et al.*, 1994; Hiruki, 1992; Lee *et al*, 1998). According to Namba et al. (1993),

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phytoplasmas are classified into three groups according to basic order of 16S rRNAs.

Like this, molecular-biological methods are widely used in recent times. This study was made of jujube witches' -broom disease (JW), mulberry dwarf disease (MD), paulonia witches' -broom disease (PW) and sumac witches' -broom disease (SW) mainly occurred in our country, to compare with the inpectivity of phytoplasma by treating the products amplified by PCR with restricted enzymes, using a primer for 16S rRNA amplification of phytoplasma.

MATERIALS AND METHODS

Phytoplasma source

For diseased plants used in this test, infected trees suffering serious economic losses from phytoplasma and indicating positive symptoms of phytoplasma were used as materials. For the subjects, JW was collected from Sintaein-up, Jeongup-city, Chonbuk, PW from Wansan-gu, Chonju, SW from Sinpyeong-myon, Imsil-gun, and MD from Bongdong-up, Wanju-gun, Chonbuk. The diseased plants were collected by four units per infected field from different places. For the test of collected materials, the DNAs of these were extracted immediately and then stored in a refrigerator (-70℃).

DNA extraction and PCR

The extracted DNAs were made for the total DNA, according to the methods performed by Namba *et al.*, by putting 1 g of veins of the infected plant with phytoplasma in 0.9*ml* of CTAB (hexaadenyl trimethyl ammonium bromide) extract. The extracted DNA was stored in the refrigerator for use in a test. Using the extracted DNA, phytoplasma infection was checked by a specific primer for phytoplasma amplification and a universal primer for mollicutes amplification designed by Namba et al. (1993). The primers used are shown in Table 1.

The conditions of PCR were adjusted to 30 cycles at 94℃/1 min, 50℃/2 min and 72℃/3 min for the mollicutes and at 94℃/1 min, 65℃/2 min and 72℃/3 min for the phytoplasma specific primer, and the both conditions were extended for 10 min at 72℃. For PCR-products, the amplified band was checked by electrophoresis in 1.5% agarose gel (1x TBE), 70mA for 1 hour.

RFLP analysis of PCR products

Treated DNA (80-100μg) of the amplified PCR-products with restricted enzymes of *Alu* Ⅰ, *Hpa* Ⅱ and *Sal* Ⅰ, and it was reacted at 37℃ for 1 hour, then checked DNA, whether it was digested or not, by electrophoresis in 3% agarose.

RESULTS AND DISCUSSIONS

Detection of phytoplasma by PCR

Upon investigation of infection of phytoplasma on test materials which indicated clear symptoms of JW, PW, SW and MD, using a specific primer designed for amplification of only phytoplasma and a universal primer for amplification of the mollicutes, the results

Table 1. Primers for polymerase chain reaction amplification of 16s rRNA gene of mollicutes and phytoplasma

Primers	Length	Sequence(5' -3')
SN910601-1	21 bases	GTTTGATCCTGGCTCAGGATT
SN910502-2	21 bases	AACCCCGAGAACGTATTCACC
SN920204-3	17 bases	CCTCAGCGTCAGTAA

Primer -2 and -3 are reverse primer.

showed as in Fig. 1 and Fig. 2. All of the test materials showing symptoms of phytoplasma indicated a positive PCR reaction. Upon electrophoresis of amplified PCR-products, all collected materials showed one and the same band at 1,300 bp (Fig. 1). Meanwhile, upon amplification using a phytoplasma specific primer, they showed the same band at about 750 bp (Fig. 2). Consequently, upon amplification of DNA by universal and specific primers, the collected test materials showed the same band at the same position and was as certained as infected with phytoplasma accordingly. These results were in according to the report by Namba et al. that the mollicutes-genus and the amplified phytoplasma PCR products were 1,370 bp and 750 bp respectively (Namba *et al.*, 1983).

Recently, the PCR method, which is more convenient and can diagnose within a shorter period than the existing methods, is widely used as a method of diagnosing phytoplasma-infected plants. The PCR method is the one detectable even in the properties of phytoplasma being parasistic only on phloem of a plant and in the case of low-density phytoplasma. As in these findings, diagnosing infection of phytoplasma at the same band in a specific position by amplifying DNA with universal and specific primers according to the PCR method was similar to that in other reports (Deng and Hiruki, 1981; Harrison et al., 1982; Kim, 1992; Lee and Yea, 1993; Marcone *et al*, 1997).

RFLP analysis of PCR products

The results of analysis of PCR products amplified by the universal primer with three kinds of restricted enzymes are as follows :

Analysis by restricted enzymes of Alu I

Upon analysis of phytoplasma-infected JW test materials by restricted enzymes of *Alu* I, all materials showed three bands, 720, 300 and 280 bp, but in case of No. 4 JW, they showed five bands, 980, 380, 300, 280 and 220 bp. In the case of PW, all materials showed the same band at 450, 390, 250 and 210 bp, SW at 760, 280 and 260 bp, and MD at 410, 340, 230. 210 and 110 bp (Fig. 3). According to Marcone et al. (1999), they reported that they performed PCR-RFLP with a

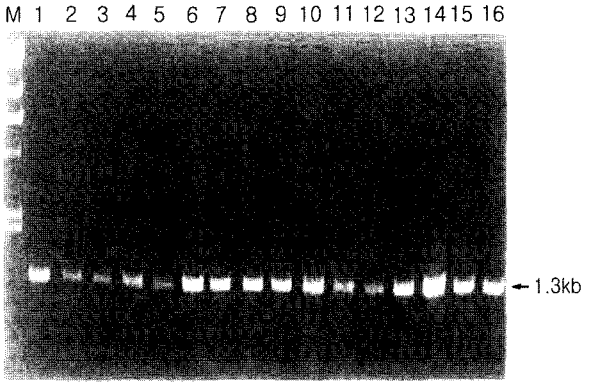

Fig. 1. Agarose-gel electrophoresis of polymerase chain reaction amplified 1.3kbp 16S rRNA gene fragments obtained using Mollicutes specific primer set(SN910601 and SN910502).M, γ/Hind Ⅲ ; Lane 1 to 4, jujube witches' -broom; lane 5 to 8, sumach witches' -broom; lane 8-12,

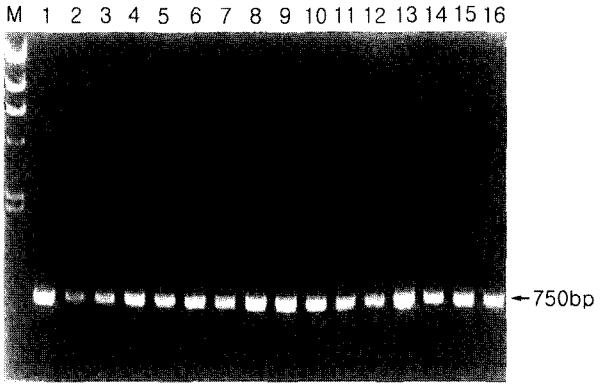

Fig. 2. Agarose-gel electrophoresis of polymerase chain reaction amplified 1.3kbp 16S rRNA gene fragments obtained using phytoplsama specific primer set(SN910601 and SN910204). Lane M, γ/Hind Ⅲ ; Lane 1 to 4, jujube witches' -broom; lane 5 to 8, sumach witches' -broom; lane 8-12, paulownia witches' -broom; lane 13-16, mulberry witches' -broom.

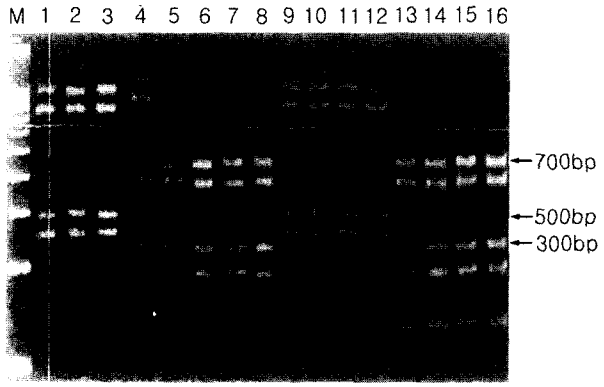

Fig. 3. RFLP analysis of 4 phytoplsama 16 S rDNA. DNA products were digested with Alu Ⅰ. M, DNA ladder; Lane 1 to 4, jujube witches' -broom; lane 5 to 8, sumach witches' -broom; lane 8-12, paulownia witches' -broom; lane 13-16, mulberry witches' -broom.

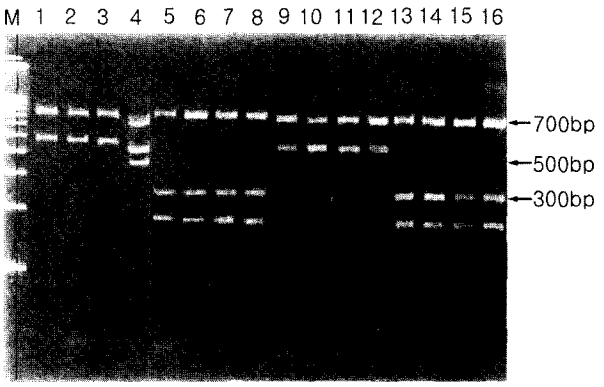

Fig. 4. RFLP analysis of 4 phytoplsama 16 S rDNA. DNA products were digested with Hpa Ⅱ. M, DNA ladder; Lane 1 to 4, jujube witches' -broom; lane 5 to 8, sumach witches' -broom; lane 8-12, paulownia witches' -broom; lane 13-16, mulberry witches' -broom.

universal primer for 16S rRNA genes of spartium witches' -broom symptoms and found two different phytoplasmas which existed in a plant of similar symptoms. In this test, JW-infected No. 4 material should be further examined, whether phytoplasmas of two origins were infected or a variable host was formed. Meanwhile, judging from these results, distinction of phytoplasmas between trees may be possible since there is no relation among JW, MD, PW and SW.

Analysis by restricted enzymes of Hpa Ⅱ

In analysis of phytoplasma by restricted enzymes of Hpa Ⅱ, JW and SW showed two bands, 800 and 500 bp, but JW No.4 material showed three bands, 580, 400 and 320 bp. Meanwhile, PW and MD was classified by three bands, 860, 250 and 190 bp. Distinction of phytoplasmas by treatment of *Hpa* Ⅱ enzymes was not available, but JW and SW, and PW and MD showed the same trends (Fig. 4). These were similar to the results that three bands occurred upon treatment of *Hpa* Ⅱ in the amplified 16S rRNA products of a jujube tree, as in the findings by Lee (1998) and Namba *et al.* (1993). However, distinction of phytoplasmas by treatment of *Hpa* Ⅱ enzymes may not be available. Meanwhile, JW No. 4 material showed a different pattern in this test, and it may require further examination.

Analysis by restricted enzymes of Sal Ⅰ

Analysis by restricted enzymes of Sal Ⅰ also showed that JW and SW, except JW No. 4 material, had two same patterns, 800 bp and 500 bp. Meanwhile, both PW and MD were classified as two patterns, 490 bp and 320 bp (Fig. 5). As in the findings by Lee *et al.*, treatment of Sal Ⅰ enzyme, like *Hpa* Ⅱ enzyme, may possibly distinguish between JW and SW groups, and between PW and MD groups, but hardly distinguish a phytoplasma group.

Analysis of the amplified PCR-products by restricted enzymes of *Alu* Ⅰ, Hpa Ⅱ and *Sal* Ⅰ showed that phytoplasmas were detected from JW, SW, PW and MD. Meanwhile, distinction of phytoplasmas by hosts was practicable by treatment of restricted enzymes of Alu Ⅰ. This was possible to diagnose each plant infected with phytoplasma by analysis of restricted enzymes, and there was a difference between plants and between phytoplasmas. Also, it was consistent with

other results showing that distinction between phytoplasma- origins may be available by this method (Marcone *et al.*, 1997; Namba *et al.*, 1983). Gibb (1996) *et al.* could detect phytoplasmas of different properties from several kinds of plants infected with phytoplasma, and reported that analysis of limited enzymes of *Alu* I, *Rsa* I and *Mse* I identified yellow crinkle, mosaic and dieback symptoms of phytoplasma of a papaya as infection by phytoplasmas of different origins and that analysis of limited enzymes showed the same electrophoresis although the kinds of plants were different. In this test, in case of a jujube tree, the results from infection of phytoplasma of two origins were identical with electrophoresis of the amplified PCR-products of 16S rRNA in JW and SW, and in PW and MD. Meanwhile, analysis of restricted enzymes of PW and MD showed the same results, which were consistent with classification of PW and MD into the same group among phytoplasma groups (Lee *et al.*, 1993; Namba *et al.*, 1983). In this test, analysis of limited enzymes of phytoplasmas being parasitic on paulownia and mulberry trees was in accord with the same results.

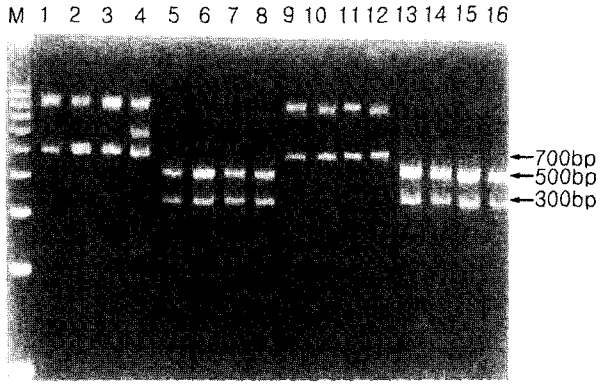

Fig. 5. RFLP analysis of 4 phytoplsama 16 S rDNA. DNA products were digested with Sal I. M, DNA ladder; Lane 1 to 4, jujube witches' -broom; lane 5 to 8, sumach witches' -broom; lane 8-12, paulownia witches' -broom; lane 13-16, mulberry witches' -broom.

Phytoplasmas of two origins doubly-infected in one plant were detected and reported (Gibbs, 1996), which was the same result that a jujube tree was infected with phytoplasmas of two origins in this test. Analysis of phytoplasmas of MD and cnidium, wild grapes, jujube witches' -broom by restricted enzymes in our country showed that they had specialize properties and relations between phytoplasmas. Meanwhile, phytoplasmas of different origins in MD and JW were reported (Hiruki, 1992). The results of this test showed that JW-infected No. 4 material was collected from farmhouses where used antibiotic substances, and therefore our study will be made of variability of phytoplasma due to treatment of antibiotics.

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