Plant Pathol. J. 18(6) : 308-312 (2002)

The Plant Pathology Journal
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Genetic Differentiation of Phytoplasma Isolates by DNA Heteroduplex Mobility Assay and Single-Strand Conformation Polymorphism Analysis

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(Received on November 29, 2002)

Heteroduplex mobility assay (HMA) and single-strand conformation polymorphism (SSCP) analyses combined with PCR were developed for genetic differentiation of various phytoplasma isolates. In the HMA and SSCP analyses, differences in the mobility shifts and the SSCP band patterns identified three distinct types of phytoplasmas: Type I, jujube witches'-broom (JWB) and ligustrum witches'-broom (LiWB); Type II, mulberry dwarf (MD) and sumac witches'-broom (SuWB); and Type III, paulownia witches'-broom (PaWB). Results of the sequence analyses revealed that phytoplasmas of JWB and MD had 100% homology with LiWB and SuWB, respectively. On the other hand, PaWB phytoplasma had 97.8% homology with MD phytoplasma. The PCR-HMA and SSCP techniques were very useful in determining variations in sequence among several isolates of phytoplasmas. Furthermore, the methods were rapid, economical, highly sensitive, and easy to handle with the gels.

Keywords : heteroduplex, PCR, phytoplasma, polymorphism, SSCP.

Phytoplasmas, formerly called mycoplasma-like organisms, are wall-less prokaryotes associated with diseases in several hundreds of plant species in the world (McCoy et al., 1989). In Korea, phytoplasma diseases have been reported in various economically important perennial and vegetatively propagated plants, as well as, in annual crops grown from true seeds. Among the numerous phytoplasma diseases known in Korea, jujube witches'-broom (JWB) (Kim et al., 1968), paulownia witches'-broom (PaWB) (La, 1968), sumac witches'-broom (SuWB) (Kim, 1980), mulberry dwarf (MD) (Chang et al., 1971), and ligustrum witches'-broom (LiWB) (Choi et al., 1987) have been regarded as the most important. However, very little is known on the genetic diversity of phytoplasmas in Korea, because they have not been cultivated *in vitro*.

During the past decade, the development of molecular techniques has afforded many available methods for the identification of unculturable phytoplasmas. Heteroduplex mobility assay (HMA) is a new method in detecting and estimating genetic divergence among different genotypes and their strains without large-scale DNA sequencing, which is laborious and expensive (Delwart et al., 1993). This method is based on the observation that the structural deformations in double-stranded DNA that resulted from mismatches and nucleotide insertions or deletions cause a reduction in the electrophoretic mobility of these fragments in polyacrylamide gel electrophoresis. HMA has been evaluated as a reliable method for the classification of phytoplasmas (Cousin et al., 1998; Wang et al., 1999; Han et al., 1999).

PCR-based single-strand conformation polymorphism (SSCP) analysis was developed as a new technique for the detection of genetic variations (Orita et al., 1989). The technique was based on the principle that single-stranded molecules change into sequence-based secondary structures, and migrate at different rates in non-denaturing polyacrylamide gels.

Generally, there are more variations in the nucleotide sequences of phytoplasmas in the intergenic space (ITS) region between the 16S and 23S rRNA genes, than in the 16S rRNA gene (Kirkpatrick et al., 1994).

In this study, phytoplasma isolates were differentiated by HMA and SSCP analyses. DNA sequences of JWB, PaWB, MD, SuWB, and LiWB phytoplasmas were used to compare the ability of each method to detect sequence diversity.

Materials and Methods

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

DNA extraction. DNA was extracted following the method of

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Kollar et al. (1990) with minor modifications. One (1) gram of 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] polyvinylpyrrolidone-10 [Sigma, USA]), 1% (W/V) Cetavlon (hexadecyltrimethylammonium bromide), 0.5 M Tris-HCl (pH 8.0), 0.25 M EDTA (pH 8.0), and 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 for 5 minutes at 1,200×g. The aqueous phase was mixed with 0.7 vol. of isopropanol, and was left standing for 5 minutes at room temperature. The resulting pellet was washed with 70% ethanol and centrifuged for 5 minutes at 1,200×g, then dried with a vacuum for 10 minutes and re-suspended by 150 μl of distilled water. The concentration of DNA in a sample was calculated with a spectrophotometer at 260 nm.

Primers and PCR. Primer pairs P3 and P7 (Schneider et al., 1995) were used to amplify the 16S-23S rRNA ITS (intergenic spacer) region. The DNA was amplified in a 50 μM reaction volume containing 20-30 ng/μl DNA; 150 μM of each dNTP; 2.5 mM $MgCl_2$; 0.5 μl of each primer; 1X PCR buffer; and 1 U of AmpliTaq Gold DNA polymerase (Perkin-Elmer). 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 and stained with ethidium bromide. DNA bands were visualized using UV transilluminator.

Heteroduplex mobility analysis. Each 2 μl of PCR products of phytoplasma isolates was combined with an equal volume of PCR product of JWB phytoplasma, then added with 2 μl of 10X annealing buffer (1 M NaCl, 100 mM Tris-HCl [pH 7.8], 20 mM EDTA). DNA was denatured at 94°C for 2 minutes and re-natured by rapid cooling on ice for 10 minutes. The DNA fragments were separated in 8% polyacrylamide gel (acrylamide: bis=29:1) in 1X TBE buffer at 150 V for 5 hours, and silver stained following the manufacturer's (Bioneer, Korea) recommendations.

SSCP analysis. One μl of PCR product was mixed with 10 μl of loading buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol) and heated with boiling water for 6 minutes, then chilled immediately on ice for 10 minutes. The polyacrylamide gel (8%) electrophoresis was carried out at 4°C for 10 hours at 150 V with 0.5X TBE buffer and stained with silver nitrate.

Sequencing of PCR product. PCR products were purified with the gel clean kit (Qiagen) and ligated into the pCR2.1 vector. The ligation product was 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 a white colony by blue-white screening method. Each white colony picked from culture plates was added to 2 ml LB medium with ampicillin 0.1 mg/l and grown overnight at 37°C. Plasmid DNAs were prepared with the Miniprep (QIAprep Spin, Qiagen). 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).

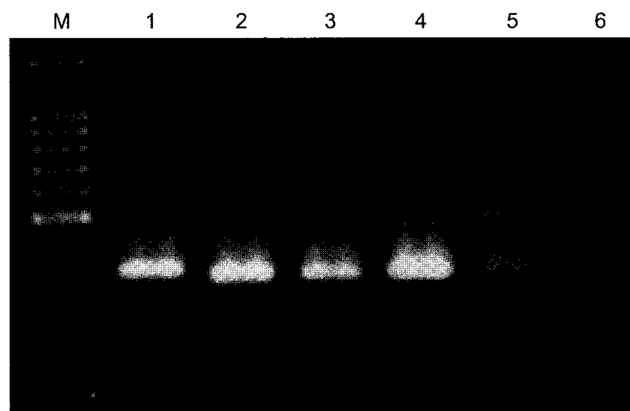

Fig. 1. PCR of DNA samples extracted from leaves of various phytoplasma-infected trees and healthy periwinkle plant using P3/P7 primer pair. Lanes M, 100 bp DNA marker; 1, JWB; 2, PaWB; 3, MD; 4, SuWB; 5, LiWB; 6, healthy periwinkle.

Results

Amplification of the 16S-23S rRNA intergenic spacer region gene. The primer pair P3/P7 amplified a fragment of about 320 bp that contains the 16S-23S intergenic spacer regions of JWB, PaWB, MD, SuWB, and LiWB phytoplasmas (Fig. 1). Approximately 340 bp was amplified from JWB and LiWB phytoplasma, whereas, from PaWB, MD and SuWB phytoplasmas, approximately 320 bp was amplified (Fig. 1). No DNA was amplified from healthy plants.

Heteroduplex mobility assay. The corresponding fragments containing 16S-23S rDNA space region were amplified using P3/P7 primer pair from a number of phytoplasma isolates, which represent a variety of epidemiological origins. Denaturing and reannealing mixtures of amplified DNA fragment from divergent phytoplasma isolates formed heteroduplexes. Pair-wise combinations of amplified products resulted in the formation of heteroduplexes between JWB (used as a standard) and PaWB, and between MD and SuWB. However, no heteroduplex was formed between JWB and LiWB (Fig. 2).

Single-strand conformation polymorphism analysis. All PCR products were analyzed with SSCP to subject variant sequence. The SSCP band patterns identified three distinct types of phytoplasmas: Type I (JWB and LiWB); Type II (MD and SuWB); and Type III (PaWB) (Fig. 3).

Sequence analysis. Using the 16S-23S rRNA gene primer pair, P3 and P7, the nucleotide sequences of JWB, PaWB, MD, SuWB, and LiWB phytoplasmas were determined.

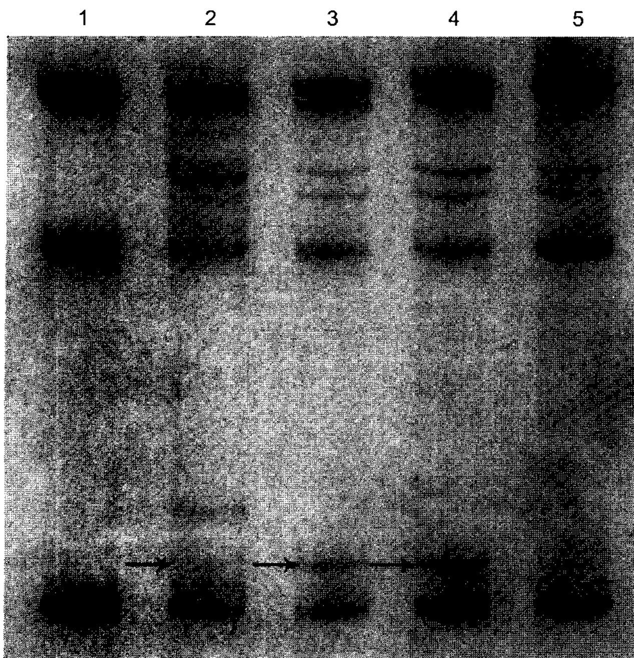

Fig. 2. Heteroduplex mobility of PCR products of the 16S/23S rRNA gene from different phytoplasma isolates. A product of JWB phytoplasma isolate, which was used as an internal standard, was mixed with each amplified DNA of phytoplasma isolates. Lanes 1, JWB; 2, JWB+PaWB; 3, JWB+SuWB; 4, JWB+MD; 5, JWB+LiWB. Arrows indicate heteroduplex.

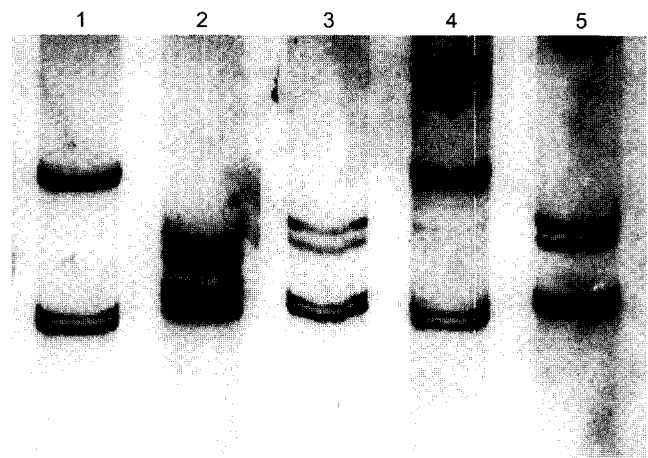

Fig. 3. Single-strand conformation polymorphism patterns of the 16S/23S rRNA genes from different phytoplasma isolates. Lanes 1, JWB; 2, PaWB; 3, MD; 4, LiWB; 5, SuWB.

The nucleotide sequences were 341 bp in length for JWB and LiWB; 321 bp for MD and SuWB; and 320 bp for PaWB. The size of 16S-23S spacer region DNA sequence was 263 bp for JWB and LiWB, 243 bp for MD and SuWB, and 242 bp for PaWB. In the sequence alignment, JWB and MD phytoplasma showed 100% homology with LiWB and SuWB phytoplasma, respectively. On the other hand, PaWB phytoplasma had 65.4% homology with JWB and LiWB phytoplasma, and 97.8% homology with MD and SuWB phytoplasmas (Figs. 4 and 5).

Discussion

Recent development on PCR-based assays provides a convenient means of obtaining phytoplasmas and other mollicutes without removing eukaryotic and organelle genomic DNAs (Deng and Hiruki, 1990, 1991a, 1991b; Ahrens and Seemuller, 1992; Davis and Lee, 1993; Lee et al., 1998). In this study, a PCR-based procedure using a primer pair for 16S/23S intergenic space region could be employed effectively in the detection and identification of a broad array of either known or unknown phytoplasmas from various host plants. Phytoplasmas were detected from all five trees showing phytoplasma symptoms. No PCR products were obtained from healthy plants.

The HMA (Delwart et al., 1993) and SSCP analyses (Orita et al., 1989), based on sequence differences between a known and a reference sequence, are particularly suitable for the comparison of genetic differences in rapidly evolving genetic relationships. The band(s) of a heteroduplex(es) could be segregated easily in the position expected on the gel, because even single 3-base pair gap can induce noticeable mobility retardation in the heteroduplex(es) formed (Delwart et al., 1993). In this study, a rapid and sensitive assay was described to differentiate intra-type of phytoplasmas based on mobility shifts of heteroduplexes and different band patterns of polymorphism of PCR products.

In the HMA and SSCP analyses, PaWB phytoplasma was clearly distinguished from the others, and comparisons in the nucleotide sequence of PaWB with MD phytoplasma showed two-base difference and one-base deletion. These results indicate that the HMA and SSCP analyses could detect even slight differences in nucleotide sequences among phytoplasma isolates.

The degree of mobility retardation of heteroduplexes in polyacrylamide gel was in proportion with the degree of genetic divergence among phytoplasma isolates. The major advantage of HMA is in the high accuracy of the procedure to differentiate phytoplasma isolates.

The SSCP analysis has been applied to detect point mutations in DNA fragment using long gels for more than 8 hours of running in electrophoresis. However, this method is affected by a number of physical factors, including gel composition such as acrylamide concentration, glycerol content, gel matrix type, and gel thickness, and electrophoresis conditions such as temperature, electric field and power, and buffer concentration (Orita et al., 1989; Prosser, 1993; Sheffield et al., 1993). In the SSCP analysis, the best

JWB	1	GGATGGATCA	CCTCCTTTCT	AAGGACATAC	ATATAAAAAT	CATCATCTTC	50
LiWB	1	**********	**********	**********	**********	**********	50
MD	1	**********	**********	*****A*C*A	T***C*TCT*	**GTT*TGAG	50
SuWB	1	**********	**********	*****A*C*A	T***C*TCT*	**GTT*TGAG	50
PaWB	1	**********	**********	*****A*C*A	T***C*TCT*	**GTT*TGAG	50
JWB	51	AGTTTTGAAA	GACTTAGGTT	AAAATATAAG	TTTTTCTTTT	TACAAAAAAA	100
LiWB	51	**********	**********	**********	**********	**********	100
MD	51	**AC**A*G*	A*G**---**	TC*T*G***C	**GC*TGCAA	ATTGT*TTTG	100
SuWB	51	**AC**A*G*	A*G**---**	TC*T*G***C	**GC*TGCAA	ATTGT*TTTG	100
PaWB	51	**AC**A*G*	A*G**---**	TC*T*G***C	**GC*TGCAA	ATTGT*TTTT	100
JWB	101	AGTGTTTCTC	TTATATAAAA	GACCAAAGGG	CCTATAGCTC	AGTTGGTTAG	150
LiWB	101	**********	**********	**********	**********	**********	150
MD	101	CAACA**T*A	A*C*T*TT**	**TT**-***	**********	**********	150
SuWB	101	CAACA**T*A	A*C*T*TT**	**TT**-***	**********	**********	150
PaWB	101	CAACA**T*A	A*C*T*TT**	**TT**-***	**********	**********	150
JWB	151	AGCACACGCC	TGATAAGCGT	GAGGTCGGTG	GTTCGAGTCC	ACTTAGGCCC	200
LiWB	151	**********	**********	**********	**********	**********	200
MD	151	**********	**********	**********	****A*****	*T********	200
SuWB	151	******C***	**********	**********	****A*****	*T********	200
PaWB	151	******T***	**********	**********	****A*****	*T*******-	200
JWB	201	ACCAATTTTA	TATCAGGAAA	TTATTTACTT	CGAAGAAAGT	TCTTTGAAAA	250
LiWB	201	**********	**********	**********	**********	**********	250
MD	201	****TA----	-*C**CA**T	AGGCAA*A*C	TT**A****C	**********	250
SuWB	201	****TA----	-*C**CA**T	AGGCAA*A*C	TT**A****C	**********	250
PaWB	201	****TA----	-*C**CA**T	AGGCAA*A*C	TT**A****C	**********	250
JWB	251	GTAGATAAAC	ATGATTTGCT	TAATTCAATT	AAAGTTGAAG	AAAGTAAGGG	300
LiWB	251	**********	**********	**********	**********	**********	300
MD	251	**********	GAAGG**AA-	----------	***A*CA***	G**C******	300
SuWB	251	**********	GAAGG**AA-	----------	***A*CA***	G**C******	300
PaWB	251	**********	GAAGG**AA-	----------	***A*CA***	G**C******	300
JWB	301	CGTACAGTGA	ATGCCTTGGC	ACTAAGAGCC	GATGAAGGAC	G---------	350
LiWB	301	**********	**********	**********	**********	G---------	350
MD	301	**C******G	**********	**********	**********	G---------	350
SuWB	301	**C******G	**********	**********	**********	G---------	350
PaWB	301	**C******G	**********	**********	**********	G---------	350

Fig. 4. Nucleotide sequences of 16S-23S rRNA intergenic space region of JWB, PaWB, MD, SuWB, and LiWB phytoplasma isolates amplified by PCR with a primer pair P3/P7.

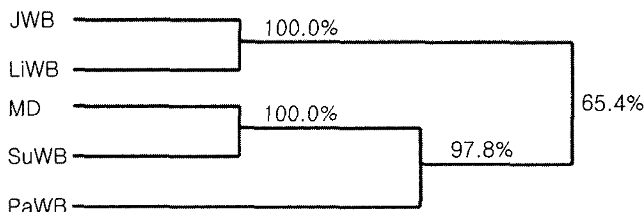

Fig. 5. Similarity of phytoplasmas in 16S-23S rRNA intergenic space region.

condition for electrophoresis was as follows: 8% polyacrylmide gel, 0.5X TBE buffer, and 150V for 10 hours in cold room temperature. On the other hand, the best condition for HMA was 8% polyacrylamide gel with 1X TBE buffer at 150V for 5 hours in room temperature. The sensitivity of HMA and SSCP analyses, and the possibility of applying them in the screening of variants before sequencing, makes them very powerful procedures in distinguishing phytoplasma isolates. HMA and SSCP analyses have proven to be very simple, fast, and reliable methods for the detection and differentiation of different strains and groups of phytoplasmas.

Acknowledgment

This research was supported in part by the International Cooperation Research Program of the Ministry of Science & Technology (01-H02-00-050-00) and the Korea Research Foundation Grant (KRF-2001-037-GA0010).

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