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Differentiation of Phytoplasmas Infecting *Zizyphus jujuba* and *Paulownia coreana* Using PCR-RFLP

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The relationships between the phytoplasmas infecting *Zizyphus jujuba* and *Paulownia coreana* were investigated by PCR-RFLP. The 16S rRNA genes of the phytoplasmas were analyzed and compared with each other after PCR amplification. The amplified bands 1.4 kb in size were analyzed by both restriction digestion and sequencing after cloning into a plasmid vector. In some cases, two different kinds of inserts were observed in the isolates that originated from a single plant. However, many of them appeared to be the amplification products of chloroplastic 16S rRNA gene of host plants. The phytoplasma gene could be differentiated from the chloroplastic gene by restriction digestion of the plasmids carrying the amplification products. Only the recombinant plasmids carrying phytoplasma 16S rRNA gene produced a 1.4 kb band when digested with the enzyme *Ban*II. Of the 52 recombinant plasmids analyzed, 42 appeared to contain inserts that originated from the chloroplastic 16S rRNA gene of the host plants. No variation was detected among 16S rRNA genes of nine phytoplasma isolates infecting *Z. jujuba*. However, the phytoplasmas infecting *Z. jujuba* were different from that infecting *P. coreana*.

Keywords **:** *Paulownia coreana*, PCR-RFLP, phytoplasma, 16S rRNA, *Zizyphus jujuba*.

More than 300 different phytoplasma strains have been reported in the world since their first discovery by Doi et al. (1967). In Korea, more than 20 plant diseases are currently recognized to be caused by phytoplasmas. Although there have been some indirect evidences that there may exist many different strains in phytoplasmas infecting different species, there is no clear picture on the diversity of phytoplasmas in Korea (Han et al., 1996; 1997). One of the major obstacles to the study of phytoplasma has been the lack of any culturing method, since the analysis of a certain microbiological species requires fairly good purity specimens (McCoy et al., 1989). However, with the aid of molecular techniques, it is now possible to study phytoplasma to the extent that different strains can be identified from infected plant tissues (Deng and Hiruki, 1991; Gundersen et al., 1994). PCR has been used to differentiate strains of pathogenic or symbiotic microorganisms in the infected plants (Davis et al., 1992; Donofrio et al., 1992).

In most of the cases, 16S rRNA genes of microorganisms have been used to probe the differences. The reason may be that the genes have been fairly well conserved during the evolution and yet contains enough sequence variation that enables the identification of even a small difference between eukaryotes and prokaryotes. Ahrens and Seemüller (1992) amplified the 16S rRNA gene of phytoplasma using the primers specific to prokaryotic 16S rRNA gene sequence. Analysis of the amplified products by RFLP led them to the differentiation of four distinct groups of phytoplasmas infecting different host species. Lee et al. (1993) also took the same approach to analyze 40 different phytoplasma isolates collected from Asia, Europe, and North America, and divided them into 9 groups and 14 subgroups. The same approach was applied in Korea (Han et al., 1996; Han, 1998). Studying with phytoplasma-infected woody species in Korea, Lee and Yea (1993) reported that three groups could be identified by RFLP. Han et al. (1997) also reported that the phytoplasma infecting jujubes and sesame plants were of different strains.

Zizyphus jujuba and *Paulownia coreana* are very popular trees in Korea. Both have been widely planted by farmers as cash crop trees. However, the plantations of both tree species have been devastated by phytoplasma infection throughout the country. For the past 20 years, attempts have been made to find out the vectors that transmit the disease and the diversity of the phytoplasmas infecting different host plants.

In the present study, the diversity of phytoplasmas infecting *Z. jujuba* was examined and compared with the phytoplasma isolated from a different host plant, *P. coreana*.

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Materials and Methods

Experimental plants. The plants used in the present study were *Zizyphus jujuba* and *Paulownia coreana* showing witches' brooms. Plant tissues of 40 *Z. jujuba* and one *P. coreana* were collected from eight provinces in Korea. These plant tissues were stored at -70°C until use.

PCR amplification of 16S rRNA gene. DNA extraction and PCR amplification were done as described by Namba et al. (1993). The primers for the amplification of 16S rRNA gene of phytoplasma were SN910601: 5'-GTT TGA TCC TGG CTC AGG ATT-3' and SN910502: 5'-AAC CCC GAG AAC GTA TTC ACC-3'.

Restriction mapping and sequencing. The recombinant plasmids were analyzed by single or double digestions with eight different enzymes. These are *Apa*I, *Pvu*II, *Ban*II, *Sac*II, *Taq*1, *Sal*I/*Eco*RI, *Sal*I/*Stu*I, and *Sal*I/*Tth*III1. As references, 16S rRNA gene of the phytoplasma infecting *Oenothera hookeri* (Lim and Sears, 1989) and that of *Nicotiana tabacum* chloroplast (GeneBank Accession No. V00165 J01453) were used. The recombinant plasmids containing the PCR products were extracted by a commercial plasmid extraction kit (Wizard Miniprep., Promega Co., USA). The purified plasmids were used for PCR sequencing with an automated DNA sequencer (Li-Cor ; Model 4000, USA).

Results and Discussion

PCR amplification and restriction analysis of phytoplasma 16S rRNA gene. PCR amplification with the primer set SN910502 and SN910601 resulted in a 1.4 kb band in all the plants examined (Fig. 1). The size of the amplification products was in accordance with previous reports (Kim, 1992; Namba et al., 1993; Han et al., 1996, 1997). The amplified fragments were cloned into a cloning vector and then analyzed by restriction digestion. With the availability of *Oenothera* restriction map (Lim and Sears, 1989), it was possible to determine the orientation of the inserts in the vector. The *Sac*II site was especially helpful since it is well conserved among different taxa (Lim and Sears, 1989). In this way, it was possible to identify 52 recombinant clones having 5' end of the 16S rRNA gene near the SP6 promoter in the vector. The restriction digestion of the plasmids with the enzymes *Eco*RI, *Sac*II, *Taq*I, *Stu*I, and *Tth*III1 produced an approximate restriction map for each plasmid. Surprisingly, all the isolates were basically divided into two groups. Figure 2 shows one of such patterns when the plasmids were digested with the enzymes *Apa*I and *Sac*I. As references, the sequence from 16S rRNA gene of tobacco chloroplast and that of phytoplasma from *O. hookeri* was used. One group of isolates showed the same pattern that was obtained by the restriction digestion of chloroplastic gene. The results showed that only 9 out of 52 isolates from *Z. jujuba* and one from *P. coreana* turned out to have phytoplasma gene. The remaining isolates appeared to be chloroplastic genes of the host plants.

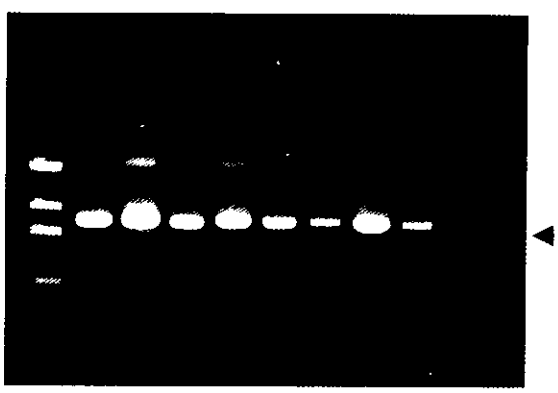

Fig. 1. Agarose gel (1%) electrophoresis of PCR amplified 16S rRNA gene from total DNA samples using the primer set SN910601 and SN910502. The 16S rRNA gene is indicated by an arrowhead. Lane 1: pGEM DNA marker, lanes 2-8: *Zizyphus jujuba*, lane 9: *Paulownia coreana*, lane 10: healthy *Ligustrum obtusifolium*, and lane 11: blank.

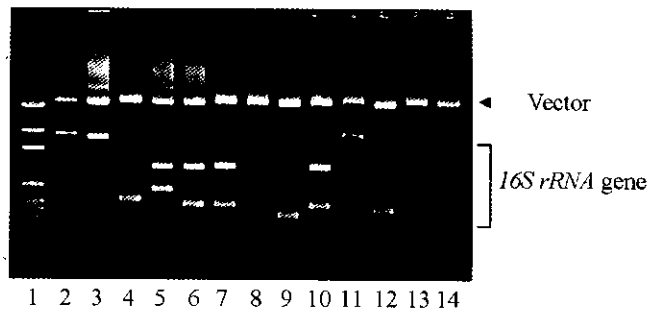

Fig. 2. Identification of the insertion of phytoplasma 16S rRNA gene in pGEM-T vector with the restriction enzymes *Apa*I and *Sac*I. The colonies lacking inserted DNA show a single band (vector). Those carrying the insert show multiple bands. Lane 1: pGEM DNA marker, lanes 2-14: *Zizyphus jujuba* isolates.

Difference of 16S rRNA gene between phytoplasma and plant chlroplast. Two different types of inserts were obtained from the isolates of Yangyang 2, Chungju 2, Puyo 2, and Sangju 2. Two primer sets that have been reported to be phytoplasma-specific (Ahrens and Seemüller, 1992; Namba et al., 1993) were tried. In both cases, the amplification of the same bands in both infected and healthy samples was observed. The sequence comparison revealed that many of them originated from the chloroplast gene. When the restriction patterns of PCR products were compared with those of *Oenothera hookeri* phytoplasma and tobacco chloroplast 16S rRNA, a *Sac*II site appeared to be present in all the samples at a similar position. In the case of *Eco*R1 site, the isolates of *Z. jujuba* and *P. coreana*, and *P. oenothera hookeri* appeared to have the same pattern. On the other hand, the chloroplast gene showed a different pat-

tern. Other enzyme sites found only in chloroplastic gene were *Apa*I and *Pvu*II. The same pattern was observed in *Taq*I sites where only chloroplast gene showed a different restriction profile. The enzyme *Ban*II produced a 1.4 kb band only in the isolates of *Z. jujuba* and *P. coreana*. There are three *Ban*II sites in the cloning vector. Two of them are seperated by multiple cloning sites of the plasmid (Promega Co., USA). Thus, the restriction of the recombinant plasmids with the enzyme will produce the whole length of the inserts. In the case of the phytoplasma isolates, no *Ban*II site was observed within the amplified fragments. On the other hand, the 16S rRNA gene of chloroplast did not produce the same band upon digestion, suggesting that they contain *Ban*II sites within the fragment. Two other enzyme sites, *Apa*I and *Pvu*II, appeared to be present only in the chloroplast gene.

The sequencing results (GeneBank Accession # 279271 and 279272) were compared with those already published (Fig. 3). Sequence comparison showed that while the homology between the 16S rRNA genes of both the phytoplasmas infecting *Z. jujuba* and *O. hookeri* was 90.2%, the homology between *P. coreana* phytoplasma and *O. hookeri* was 99.5%. The homology between tobacco chloroplast and phytoplasma 16S rRNA genes was 73.5%, suggesting that the chloroplast gene is distantly related to the phytoplasma genes. The conserved nature of the 16S rRNA genes may represent their stability during evolution. However, this presents a difficulty in differentiating phytoplasma genes from those in organelles. Since it is not possible to culture phytoplasmas *in vitro*, it is very difficult to isolate phytoplasma genes. To avoid the contamination of host plant DNA, it is necessary to design primers that are specific to phytoplasma DNA.

Alma et al. (1996) detected two strains belonging to different subgroups of phytoplasmas in grapevine. Marcone and Ragozzino (1996) speculated that the frequency of real double infections might be much higher than what had been reported. Ahrens and Seemüller (1992) also reported the same results. However, they were able to differentiate phytoplasma DNA from plant DNA by RFLP. On the other hand, Namba et al. (1993) observed only a 1.4 kb fragment from the amplification of infected plants, and did not detect

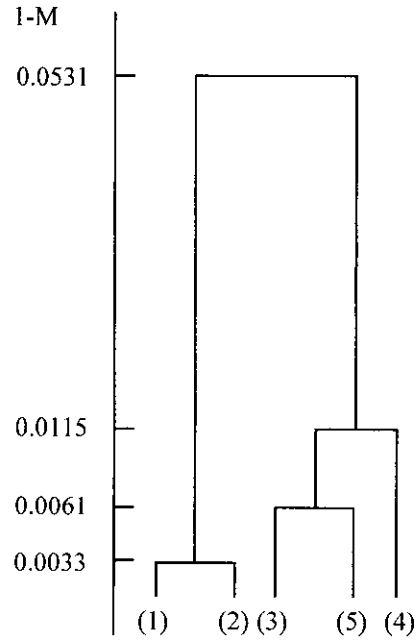

Fig. 3. Relatedness of phytoplasmas estimated by phytoplasma 16S rRNA sequence of (1) jujube witches' broom, (2) jujube witches' broom (Han, 1998), (3) paulownia witches' broom, (4) paulownia witches' broom (Han, 1998), and (5) *Oenothera hookeri* (Lim and Sears, 1989). Cluster analysis was performed using 1-M scores and UPGMA. M=NAB/(NA+NB), where NAB is the number of bands matched, and NA and NB are the number of bands observed in samples A and B, respectively.

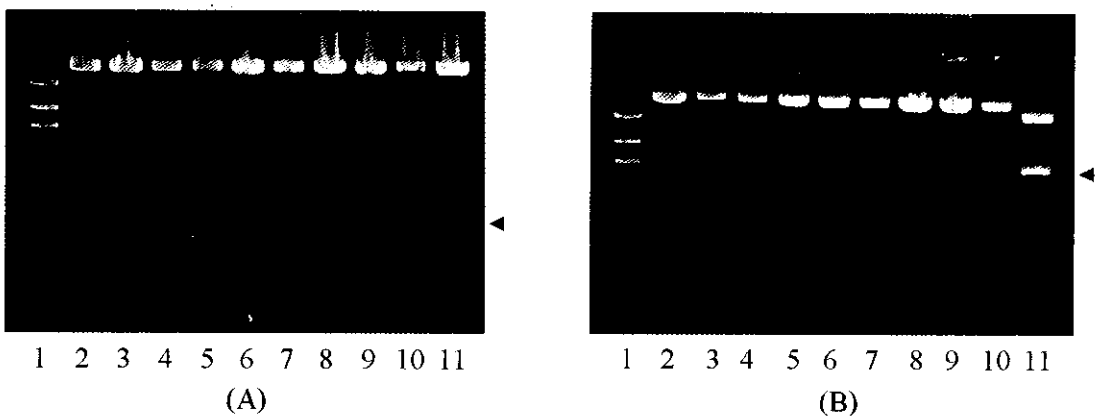

Fig. 4. Restriction profiles of phytoplasma 16S rRNA gene cloned in pGEM-T vector. (A) Digestion with the enzymes *Sal*I and *Stu*I, and (B) with *Sal*I and *Tth*III1. All the *Zizyphus jujuba* isolates lanes 2-10 were identical. However, *Paulownia coreana* isolate showed a different pattern at lane 11. Lane 1: pGEM DNA marker.

any other products with healthy plant samples. However, in the present study, the fairly large portion of the cases might be the results of contamination of the DNA from microorganisms, mitochondria, or chloroplasts. Thus, caution should be taken to select primers to amplify specific sequence that are ubiquitous to all organisms. The amplified fragments should also be examined by restriction mapping analysis with many different enzymes. The contradicting results obtained from different laboratories with the same primer set could be explained in two ways. First, since the host plant species in the studies were different, the annealing site in the 16S rRNA gene may have different sequences. Second, only the phytoplasma gene was accidentally amplified. However, as shown in the present study, cloning is an absolute step to detect double infections or false amplification of the similar sequences since amplification products may contain different kinds of DNAs.

Difference between phytoplasmas from *Z. jujuba* and *P. coreana* in 16S rRNA gene. Restriction digestion patterns of the 16S rRNA genes of phytoplasma isolated from both *Z. jujuba* and *P. coreana* were compared with four different enzymes. The pattern was the same when digested with the enzymes *Eco*RI and *Taq*I (data not shown). However, when compared by the digestion with the enzymes *Tth*III1 and *Stu*I (Fig. 4), they could be differentiated. In each case, a unique band was observed only in *P. coreana* phytoplasma. The markers that could differentiate the phytoplasmas of *Z. jujuba* from those of *P. coreana* were a 330bp *Tth*III1 fragment and a 350bp *Stu*I fragment, respectively.

Restriction mapping. The restriction maps for 16S rRNA genes were constructed as shown in Figure 5 by the fragment size appearing in the gel. It shows that phytoplasmas from both *Z. jujuba* and *P. coreana* have a unique restriction site for *Sac*II and *Eco*RI, and three sites for *Taq*I. However, the phytoplasma from *P. coreana* had a *Stu*I site and *Tth*III1 that were not present in *Z. jujuba* phytoplasma. No variation was observed among phytoplasmas that originated from *Z. jujuba* found in Korea. However, the sequence of phytoplasma 16S rRNA gene identified in the present study was found to contain six base difference from that reported by Han (1998). In the case of phytoplasma from *P. coreana*, seven bases were different from that reported by Han (1998).

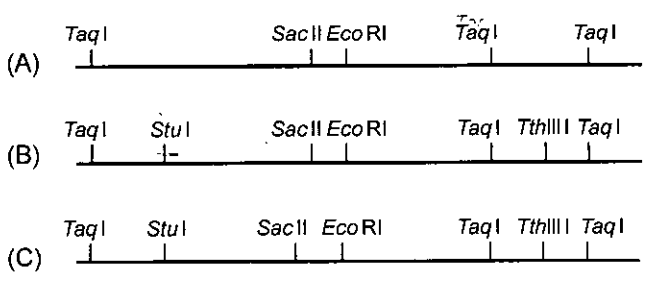

Fig. 5. Physical map of phytoplasma 16S rRNA gene obtained by restriction digestion of the recombinant plasmid pGEM-phyto clones with the enzymes *Taq*I, *Sac*II, *Eco*RI, *Stu*I, and *Tth*111I. (A) *Phytoplasma zizyphi*, (B) *Phytoplasma paulownia* in the present study, and (C) *Phytoplasma oenothera hookeri* (Lim and Sears, 1989).

In the present study, no variation was observed among the isolates of *Z. jujuba* by RFLP. Therefore, it was concluded that all the phytoplasma isolates from *Z. jujuba* were of the same strain. However, they were different from the strain infecting *P. coreana*. Also, the sequences identified in this study were different from those reported by Han (1998). This suggests that there may exist some minor sequence variations that cannot be detected by RFLP. This can be clarified by sequencing more isolates in the future. A drawback of the present study was the failure of amplification of the genes of other strains of phytoplasma infecting other woody species due to the limited amounts of tissues. Another problem in this approach was the use of the 16S rRNA gene sequence. The conserved nature of the gene could become a disadvantage in the study of sequence differentiation among closely related species or clones. If the spacer between 16S rRNA gene and 23S rRNA gene that are believed to contain more variation than the 16S rRNA gene could be amplified, differentiation of strains, if any, of phytoplasmas infecting Z. *jujuba* may be possible.

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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. *Phytopathology* 82:828-832.

Alma, A., Davis, R. E., Vibio, M., Danielli, A., Bosco, D., Arzone, A. and Bertaccini, A. 1996. Mixed infection of grapevines in north italy by Phytoplasmas including 16S rRNA RFLP subgroup 16Sr I-B strains previously unreported in this host. *Plant Dis.* 80:418-421.

Davis, R. E., Prince, J. P., Hammond, R. W., Dally, E. L. and Lee, I. M. 1992. Polymerase chain reaction detection of Italian periwinkle virenscence mycoplsmalike organism (MLO) and evidence for relatedness with aster yellows MLOs. *Petria* 2:184-193.

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

Doi, Y., Teranaka, M., Yora, K. and Asuyama, H. 1967. Mycoplasma- or PLT group-like microorganisms found in the

phloem elements of plants infected with mulberry dwarf, potato witches' broom, aster yellows or paulownia witches' broom. *Ann. Phytopath. Soc. Japan* 33:259-266.

Donofrio, M. A., Coonrod, J. D., Davidson, J. N. and Betts, R. F. 1992. Detection of Influenza A and B in respiratory secretions with the polymerase chain reaction. In: *PCR Methods and Application*, pp. 263-268. Cold Springer Harbor Laboratory Press.

Gundersen, D. E., Lee, I.-M., Rehner, S. A., Davis, R. E. and Kingsbury, D. T. 1994. Phylogeny of mycoplasmalike organism (Phytoplasmas): a basis for their classification. *J. Bacteriol.* 176:5244-5254.

Han, M. S., Noh, E. W., Lee, J. S., Chung, H. S., Youn, Y. and Yun, J. K. 1996. Differentiation of phytoplasmas by PCR-RFLP techniques in woody plants. *Res. Rep. For. Res. Inst. Korea* 32:72-79.

Han, M. S., Noh, E. W. and Yun, J. K. 1997. Occurrence of sesame phyllody disease in Korea and detection of its phytoplasma. *Korean J. Plant Pathol.* 13:239-243.

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 Univ. 91 pp.

Kim, Y. H. 1992. Detection of MLO-DNA from sumac witches broom mycoplasma by polymerase chain reaction. *Korean J. Mycoplasmol.* 3:54-58.

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

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

Lim, P.-O. and Sears, B. B. 1989. 16S rRNA sequence indicates that plant-pathogenic mycoplasmalike organisms are evolutionarily distinct from animal mycoplasmas. *J. Bacteriol.* 171:5901-5906.

Marcone, C. and Ragozzino, A. 1996. Genetic characterization and classification of two phytoplasmas associated with spartium witches' broom disease. *Plant Dis.* 80:365-371.

McCoy, R. E., Caudwell, A., Chang, C. J. et al. 1989. Plant diseases associated with mycoplasma-like organisms. In: *The Mycoplasmas. Vol. 5. Spiroplasmas, Acholeplasmas, and Mycoplasmas of Plants and Arthropods*, ed. by R. F. Whitcomb and J. G. Tully, pp. 545-640, Academic Press, San Diego, CA, USA.

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.