26. Genetic Differentiation of Phytoplasma Isolates Determined by a DNA Heteroduplex Mobility Assay

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Abstract: Regions representing about 80% of the 16S rDNA sequences of nine phytoplasma isolates were amplified by polymerase chain reaction (PCR). These partial 16S rDNA sequences amplified from the various phytoplasmas were used in DNA heteroduplex mobility assays (HMA). Based on DNA distances derived from HMA analysis, the nine phytoplasma isolates may be classified into four distinct groups: group I, paulownia witches'-broom (China), potato purple top (France); group II, tomato stolbur (France); group III, eastern aster yellows (U.S.A.), faba bean phyllody (Sudan), vinca phyllody (Sudan), tomato big bud (Australia); group IV, crotalaria witches'-broom (Thailand), clover proliferation (Canada).

Key words: Mollicutes; 16S rDNA; PCR; phylogeny; molecular diagnosis.

Introduction. Phytoplasmas are associated with diseases in a few hundred plant species,⁶⁾ but the inability thus far to isolate phytoplasmas in pure culture has slowed progress toward their identification and characterization. In classical plant mollicutes studies, disease or pathogen identification and classification have relied primarily on comparative studies and their biological properties such as symptoms induced in diseased hosts, identity of plant species susceptible to infection, and specificity of phytoplasma transmission by insect vectors.¹⁴⁾ The development of phytoplasma-specific molecular probes for serological and DNA hybridization assays has provided a rapid and reliable means of phytoplasma diagnosis and classification.⁵⁾ To date molecular differentiation of similar phytoplasma isolates has been studied mainly on the basis of DNA sequence homologies and DNA polymorphisms.^{3), 6)–12), 14)–15)} Currently, two common schemes for phytoplasma classification are in use; one based on polymorphisms in 16S rDNA and the other on homology of random fragments of chromosomal DNA. The two schemes have yielded very similar results for several dozen phytoplasma isolates.^{3), 7)–13)} Although applications of these methods are providing useful information for a new understanding of relatedness among a variety of phytoplasmas, such procedures are time-consuming and complicated. Furthermore, their usefulness is limited in studies involving differentiation of very closely related phytoplasmas. A new method named DNA heteroduplex mobility assay (HMA), developed to differentiate human immunodeficiency virus (HIV) strains,²⁾ may help overcome some of these problems. This method is based on the use of DNA heteroduplexes formed between related sequences, which reduce their mobilities in polyacrylamide gels proportional to their degree of divergence. The present study demonstrates that HMA is a reliable, simple and accurate method for the classification of phytoplasmas.

Materials and methods. Source of Phytoplasmas. Nine phytoplasma isolates from North America, Asia, Africa, Australia and Europe were used in this study. Clover

proliferation (CP) and eastern aster yellows (EAY, a New York isolate) were the same as in the previous study.¹⁾ The diseased paulownia plants infected by paulownia witches'-broom (PaWB) were provided by Keri Wang, Paulownia Research Center, China; potato purple top (PPT, France), tomato stolbur (TS, France), faba bean phyllody (FBP, Sudan), vinca phyllody (VP, Sudan), tomato big bud (TBB, Australia) and croton witches'-broom (CWB, Thailand) were provided by M.T. Cousin, Station de Pathologie végétale, France. With the exception of PaWB, which was maintained in paulownia, all phytoplasmas were maintained on *Catharanthus roseus* in a greenhouse at 22°C±2°C.

Nucleic acid extraction. Samples from healthy plants and plants infected with phytoplasmas were prepared by a modified procedure of Lee *et al.*⁸⁾ Each sample, about 1.0 g of whole leaf midribs cut from the plants, was pulverized in liquid nitrogen in a mortar. The powder was triturated in 10 ml of DNA extraction buffer (100 mM Tris-HCl [pH 8.0], 100 mM EDTA, 250 mM NaCl, 100 µg of proteinase K per ml). To this sample, 1 ml of 10% sodium dodecyl sulfate (SDS) and 0.5 ml of 20% sarkosyl were added. The sample was incubated at 55°C for 1 hr and then centrifuged at 3,000 g for 10 min to separate debris. Crude DNA was precipitated from the supernatant by adding 2 volumes of 95% ethanol and placing at -20°C overnight. After centrifugation at 16,000 g for 15 min, the pellet was resuspended in 1 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]) containing 100 µg of proteinase K per ml and 0.5% SDS, and incubated at 37°C for 1 hr, followed by the addition of 175 µl of 5 M NaCl and 140 µl of 10% cetyltrimethylammonium bromide (CTAB) in 0.7 M NaCl. The mixture was incubated at 65°C for 10 min, then extracted once with an equal volume of Tris-saturated phenol and an equal volume of chloroform-isoamyl alcohol (24:1). The sample was then extracted at least twice with 2 volumes of chloroform-isoamyl alcohol (24:1). Two volumes of 95% ethanol and 1/10 volume of 3M sodium acetate were added to the aqueous phase and placed at -70°C for at least 1 hr. The DNA was pelleted by centrifugation at 16,000 g for 15 min. The pellet was then washed with 70% ethanol, dried under vacuum, and resuspended in 200 µl of TE buffer.

PCR amplification. The universal primer pair R16F2/R2 previously designed on the basis of an AY phytoplasma⁹⁾ was used in this study to amplify a 16S rDNA fragment from phytoplasma DNA. The DNA fragments were amplified from 10 µl of 5-fold diluted DNA samples extracted from phytoplasma-infected plants. The amplification was performed in a 100 µl PCR reaction mixture containing 20 mM each of dATP, dCTP, dGTP, and dTTP, 1 mM each of upstream and downstream primer, 10 µl of 10x PCR reaction buffer and 2.5 mM MgCl₂, 2.5 U Taq DNA polymerase (Promega Corp.), and 100 µl of mineral oil. The PCR was carried out for 45 cycles in a Biooven (BioTherm, Virginia) under the following conditions: 1st cycle, denaturation 5 min at 94°C; annealing 2 min at 50°C; extension 3 min at 72°C; the next 43 cycles, denaturation 1 min at 94°C; the final cycle, extension for 7 min at 72°C. Denaturation and annealing conditions were the same as for the first cycle. PCR products were analyzed by electrophoresis in 1.5% agarose gel followed by staining in ethidium bromide and visualization of DNA bands using a UV transilluminator.

Heteroduplex mobility analysis. A sample of 10 µl of PCR samples from each of the phytoplasma isolates was combined with 8 µl of PaWB PCR product, then 2 µl of 10x annealing buffer added (1 M NaCl, 100 mM tris-HCl [pH 7.8], 20 mM EDTA). DNA was denatured at 94°C for 2 min and annealed by rapid cooling on ice. The DNA fragments were separated in 5% polyacrylamide gel (30:0.8 acrylamide: bis) in TBE buffer (0.088 M tris-borate, 0.089 M boric acid, 0.002 M EDTA) at 250 V for 3 hr, followed by staining in ethidium bromide and visualization of DNA bands using a UV transilluminator. Heteroduplex mobilities were calculated as the average distance of migration of the heteroduplex bands divided by the distance of migration of the homoduplex bands. DNA distances between phytoplasmas can be calculated from the following equation: DNA

distance = $-\ln [(mobility-0.045)/1.14]/13.6$.²⁾

Results. The corresponding 1.2-kb fragments of 16S rDNA gene were amplified from each of the nine phytoplasma isolates (Fig. 1), which represent a variety of epidemiological origins. Heteroduplexes were formed by pairwise combination of amplified products from PaWB and each of the other eight phytoplasma isolates. The relative mobilities of heteroduplexes to homoduplexes were obtained by subjecting these heteroduplexes to polyacrylamide gel electrophoresis. Three groupings were observed by mobility analysis: large mobility shifts and divergence were observed for heteroduplexes formed with CWB and CP phytoplasma isolates; intermediate shifts and divergence were observed for heteroduplexes formed with EAY, FBP, VP, and TBB phytoplasma isolates; and the smallest shifts and divergence were observed for heteroduplexes formed with TS and PPT phytoplasma isolates (Fig. 2).

Fig. 1. Polymerase chain reaction (PCR) amplification of a 16S rDNA sequence from various phytoplasma. Lanes: M, 123-bp DNA ladder; 1, CP; 2, CWB; 3, EAY; 4, FBP; 5, PaWB; 6, PPT; 7, TBB; 8, TS; 9, VP.

Fig. 2. Ethidium bromide-stained polyacrylamide gel showing the range of heteroduplex mobilities determined with different phytoplasma isolates. Lanes: 1, VP; 2, TBB; 3, TS; 4, FBP; 5, PPT; 6, PaWB; 7, EAY; 8, CWB; 9, CP.

Estimates of genetic distance between paulownia witches'-broom and other eight phytoplasma isolates were obtained by pairwise heteroduplex mobility analysis. Based on the DNA distances (Table I), a phylogenetic tree was constructed from deduced DNA distances (Fig. 3). The nine phytoplasma isolates could be easily differentiated from each

Table I. DNA distances in percentages derived from heteroduplex mobility assay of 16S rDNA sequences of various phytoplasmas

	PPT	TS	FBP	EAY	TBB	VP	CP	CWB
PaWB	0	19.20	34.71	35.97	37.13	38.97	44.08	45.62

Fig. 3. Phylogenetic tree based on DNA distances inferred from heteroduplex mobility analysis of 9 phytoplasmas 16S rDNA.

other and four groups have been observed: group I, PaWB, and PPT; group II, TS; group III, FBP, EAY, TBB, and VP; group IV, CP and CWB.

Discussion. In the present work, we have investigated the use of DNA HMA to differentiate among phytoplasma isolates from different geographical regions of the world. Our results indicate the potential of HMA as an important new method for the classification of phytoplasma isolates; we have demonstrated that HMA can be employed for differentiation of phytoplasma isolates as closely interrelated as those in the AY phytoplasma strain clusters. Previous PCR-RFLP analysis had shown that EAY and TBB phytoplasmas are members of Type I of an AY phytoplasma strain cluster, but it was not possible to distinguish between them.¹¹⁾

Based on DNA distances derived from HMA analysis, the phylogenetic relationships among EAY, PaWB, TBB, and CP have now been determined. The order of phylogenetic relationship among these four phytoplasma isolates is: PaWB, EAY, TBB and CP, which is in agreement with that previously reported on the basis of 16S rDNA RFLP analysis.¹¹⁾

Five phytoplasma isolates from different regions, PPT (France), TS (France), FBP (Sudan), VP (Sudan), and CWB (Thailand) were used for HMA analysis and their phylogenetic relationships with four other phytoplasma isolates determined. The nine phytoplasma isolates were tentatively classified into four groups. Some discrepancies were found between our results and those of the previous work,^{6),13)} in particular in the position of CWB which is reportedly related to FBP and TBB.¹³⁾ Specific reason(s) for these differences must be clarified in future studies. Unlike HIV,²⁾ phytoplasma classification appears not to be region-specific and symptom-specific; this characteristic makes it more difficult to differentiate among phytoplasmas. It suggests that a large number of phytoplasmas must be analyzed before any conclusion regarding phylogenetic insights is reached.

The fact that the distance between DNAs from PaWB and PPT inferred from HMA analysis is zero could be due to factors such as insufficient denaturation of the 16S rDNA

fragments or to problems in formation and detection of heteroduplexes in polyacrylamide gel. However, these alternatives are not applicable, because repeated experiments have yielded the same results. It follows, then, that there are no detectable sequence differences between the 16S rDNA fragments of these two phytoplasma isolates.

A major advantage of HMA resides in the high accuracy of the procedure to differentiate among different phytoplasma isolates. RFLP-based phytoplasma differentiation uses different restriction enzymes to screen differences among phytoplasma-DNA sequences, the prerequisite for RFLP analysis being that the enzyme-recognition sequence should exist in the phytoplasma DNA sequence. HMA analysis is performed on the basis that DNA heteroduplexes formed between related sequences have a reduced mobility in polyacrylamide gels proportional to their degree of divergence.²⁾ Ganguly *et al.*⁴⁾ reported that under suitable conditions, even a single-base difference in DNA heteroduplexes can be discerned in polyacrylamide gel. Our results suggest that HMA will prove important in phytoplasma differentiation when other methods, e.g. RFLP, are not readily applicable to differentiation of very close phytoplasmas.

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References

- 1) da Rocha, A., Ohki, S. T., and Hiruki, C. (1986): *Phytopathology*, **76**, 864–868.
- 2) Delwart, E. L. *et al.* (1993): *Science*, **262**, 1257–1261.
- 3) Deng, S., and Hiruki, C. (1991): *Phytopathology*, **81**, 1475–1479.
- 4) Ganguly, A., Rock, M. J., and Prockop, D. J. (1993): *Proc. Natl. Acad. Sci. U.S.A.*, **90**, 10325–10329.
- 5) Hiruki, C. (1992): *Korean J. Mycoplasmaology*, **3**, 19–28.
- 6) Kirkpatrick, B. *et al.* (1994): *IOM Letters*, **3**, 228–229.
- 7) Kuske, C. R., Kirkpatrick, B. C., and Seemüller, E. (1991): *J. Gen. Microbiol.*, **137**, 153–159.
- 8) Lee, I.-M., Davis, R. E., and Hiruki, C. (1991): *Appl. Environ. Microbiol.*, **57**, 3565–3569.
- 9) Lee, I.-M. *et al.* (1993): *Phytopathology*, **83**, 829–833.
- 10) Lee, I.-M. *et al.* (1994): *ibid.*, **84**, 559–566.
- 11) Lee, I.-M. *et al.* (1993): *ibid.*, **83**, 834–842.
- 12) Namba, S. *et al.* (1993): *ibid.*, **83**, 786–791.
- 13) Saeed, E. *et al.* (1994): *IOM Letters*, **3**, 268.
- 14) Schaff, D., Lee, I.-M., and Davis, R. E. (1992): *Biochem. Biophys. Res. Commun.*, **186**, 1503–1509.
- 15) Schneider, B. *et al.* (1993): *J. Gen. Microbiol.*, **139**, 519–527.
- 16) Sears, B. B., and Kirkpatrick, B. C. (1994): *Amer. Soc. Microbiol. News*, **60**, 307–312.