European Journal of Plant Pathology **103:** 137–145, 1997.

DNA probes and PCR primers for the detection of a phytoplasma associated with peanut witches'-broom

Miaw-Fan Chen[1,2] and Chan-Pin Lin[1*]
[1] *Department of Plant Pathology and Entomology, National Taiwan University, Taipei, Taiwan 107, R.O.C.;*
[2] *Present address: Taiwan Agricultural Chemicals and Toxic Substances Research Institute, Wufeng, Taichung, Taiwan 413, R.O.C.; *Author for correspondence (Fax: 886-2-366-1980)*

Accepted 30 September 1996

Key words: DNA probe, polymerase chain reaction (PCR)

Abstract

*Eco*RI restriction fragments of genomic DNA from the phytoplasma associated with peanut witches'-broom (PNWB) were cloned in plasmid pGEM-3Zf(+). Cloned inserts from seven PNWB-phytoplasma-specific recombinant plasmids and two subcloned plasmids were excised with restriction enzymes, labeled with digoxigenin, and used as probes. Probe PNWB281 and its derivative subclones PNWB281-4 and PNWB281-5 hybridized with DNA from PNWB-phytoplasma infected peanut and periwinkle specifically but not with DNA from healthy plants or plants infected with phytoplasmas associated with sweetpotato witches'-broom (SPWB), loofah, *Ipomoea obscura*, and paulownia witches'-broom, elm and aster yellows, rice yellow dwarf, and bamboo little leaf disease. Six other probes hybridized with DNA derived from PNWB and SPWB-phytoplasma-affected periwinkle but not with DNA from healthy plants or plants infected with other phytoplasmas mentioned. In Southern hybridizations, four of the nine cloned and subcloned probes could differentiate the PNWB-phytoplasma from SPWB-phytoplasma. Three primer pairs for PCR were synthesized according to the partial sequences at both ends of the cloned inserts and were able to distinguish PNWB-phytoplasma from SPWB-phytoplasma by using PCR for the first time. A minimum of 1 pg and 10 pg of total DNA from diseased periwinkle and peanut, respectively, was sufficient to amplify the specific PNWB-phytoplasma PCR fragments, allowing the detection of PNWB-phytoplasma DNA from healthy-looking periwinkle plants two weeks after graft inoculation.

Introduction

In Taiwan, peanut (*Arachis hypogaea* L.) witches'-broom (PNWB), a disease associated with a phytoplasma (formerly mycoplasmalike organism), was first discovered in a geographically isolated area, the Penghu Islands, in 1975 (Yang, 1975). The disease agents are transmitted by the insect vector *Orosius orientalis*, but not transmitted by seed (Yang, 1975).

Like other phytopathogenic phytoplasmas, PNWB-phytoplasma remains unculturable, thus limiting the study of phytoplasmas by traditional techniques. However, procedures enabling extraction and partial purification of phytoplasmas from host tissue were developed (Jiang and Chen, 1987). Antigen preparations from infected plants were also applied to produce polyclonal and monoclonal antibodies for the serological detection of phytoplasmas in our lab (Chang et al., 1995; Lin and Chen, 1985; Shen and Lin, 1993). In addition, recombinant DNA technology has also opened a new approach for the sensitive detection of phytoplasmas, and for the study of interrelationships between various phytoplasmas and other members of the class *Mollicutes* (Bonnet et al., 1990; Davis et al., 1988; Harrison et al., 1991; Kirkpatrick et al., 1987; Lee and Davis, 1988; Lim and Sears, 1991, 1992). Recently, polymerase chain reaction technology is also used to sensitively detect nucleic acids of phytoplasmas (Ahrens and Seemüller, 1992; Deng and Hiruki, 1990; Firrao et al., 1993; Schaff, 1992) and used to analyse the genetic relatedness between different phytoplasmas (Davis and Lee, 1993; Deng and Hiru-

ki, 1991; Gundersen et al., 1996; Pollini et al., 1995; Prince et al., 1993; Schneider et al., 1993; Seemüller et al., 1994; Zreiket al., 1995). On the basis of the results of restriction fragment length polymorphism (RFLP) analyses of 16S rDNA from representative type strains of phytoplasmas, 11 distinct phytoplasma 16S rRNA groups and more than 25 subgroups have been identified (Gundersen et al., 1994; Lee et al., 1993). PNWB-phytoplasma was thus classified in 16S rRNA group II (Lee et al., 1993).

In our previous studies, monoclonal antibodies and cloned DNA probes for sweetpotato witches'-broom (SPWB)-phytoplasma were developed and applied in the detection and differentiation of phytoplasmas. Phytoplasmas associated with SPWB and PNWB were proved to be serologically and genetically related and were coexist in three alternate weed hosts in fields (Ko and Lin, 1994; Shen and Lin, 1993). PCR primer pairs designed for SPWB-phytoplasmas in the previous study were unable to amplify distinguishable DNA fragment with templates prepared from SPWB- and PNWB-phytoplasma-infected plants (Ko and Lin, 1994). In phylogenetic studies, PNWB-phytoplasma and SPWB-phytoplasma were shown to be closely related and were placed in the same phylogenetic subclade according to the phylogenetic tree constructed by parsimony analysis of full 16 S rRNA gene sequences of 19 phytoplasmas and 46 members of the class *Mollicutes* (Gundersen et al., 1994). In our lab, PNWB-phytoplasma and SPWB-phytoplasma were also shown to be closely related and placed in the same phylogenetic subclade according to the phylogenetic tree constructed by parsimony analysis of full 16S-23S rRNA intergenetic spacer sequences of 11 phytoplasmas (C. P. Lin, unpublished data).

The objective of the present study was to develop the PNWB-phytoplasma-specific DNA probes and PCR primer pairs that can differentiate PNWB-phytoplasma from other phytoplasmas including the undistinguishable SPWB-phytoplasma. In addition, the PCR primers designed in this study were used for the rapid and sensitive amplification of PNWB-phytoplasma DNA in presymptomatic plants.

Materials and methods

Plant material. Peanut plants naturally infected with PNWB phytoplasma were collected from fields on the Penghu Islands. Periwinkle plant (*Catharanthus roseus* (L.) G. Don) infected with PNWB phytoplasma originally obtained by transmission through dodder (*Cuscuta austualis* R. Brown) was provided by I. L. Yang (Taiwan Agricultural Research Institute, Wufeng) and was maintained in periwinkle by side grafting. Plants affected by several other phytoplasma-associated diseases were maintained by periodic grafting to periwinkle plants in the green house as described previously (Shen and Lin, 1993). These included sweetpotato witches'-broom (SPWB), loofah witches'-broom, *Ipomoea obscura* witches'-broom, paulownia witches'-broom, aster yellows, and elm yellows. Rice with yellow dwarf was provided by C. C. Chen (Taichung District Agricultural Improvement Station, Changhua, Taiwan), and bamboo with bamboo little leaf diseases was provided by N. S. Lin (Institute of Botany, Academia Sinica, Taipei, Taiwan).

Isolation of total DNA. The procedure reported by Kollar et al. (1990) was used with minor modifications to isolate total DNA from healthy and diseased plants (Ko and Lin, 1994; Wu et al., 1993). Fifty grams of PNWB-phytoplasma-infected periwinkle or peanut were ground to fine powder in liquid nitrogen with a mortar and pestle. The frozen powder was then suspended in DNA extraction buffer (100 mm Tris-HCl, pH 8.0; 10 mm EDTA; 500 mm NaCl; 0.01% proteinase K; 1% *N*-lauroyl sarcosine; and 60 mm 2-mercaptoethanol) (Wu et al., 1993) to extract nucleic acids. Crude nucleic acids were further clarified by repeated precipitations with ethanol and extractions with phenol and chloroform as described previously (Sambrook et al., 1989; Wu et al., 1993). Nucleic acid preparation was finally resuspended in 5 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

Separation of PNWB-phytoplasma DNA from host DNA. Cesium chloride (CsCl)-bisbenzimide density gradient centrifugation was used to separate phytoplasma DNA from host plant DNA as described previously (Ko and Lin, 1994). After centrifugation, PNWB-phytoplasma DNA was visualized under ultraviolet (UV) light as a unique band uppermost in gradients of DNA from infected plants and was collected with a syringe. This DNA fraction was recentrifuged two more times, as previously described (Ko and Lin, 1994). After final centrifugation, the phytoplasma DNA band was extracted five times with equal volumes of NaCl-saturated isopropanol. The DNA was precipitated for 2 h at –20 °C after the addition of three volumes of sterile distilled water and eight volumes of cold ethanol. Upon collection by centrifugation at

13,000 g for 20 min, the DNA pellet was rinsed with 70% ethanol, dried, resuspended in TE buffer (pH 8.0), and quantified (Sambrook et al., 1989).

Molecular cloning of phytoplasma DNA and screening of recombinants. The procedures used for molecular cloning of PNWB-phytoplasma DNA and screening of recombinants was similar to those described previously (Ko and Lin, 1994). CsCl-purified PNWB-phytoplasma DNA was digested with *Eco*RI restriction endonuclease (Boehringer Mannheim GmbH, Mannheim, Germany) and ligated with *Eco*RI-digested and dephosphorylated pGEM3Zf(+) (Promega Corp., Madison, WI). The ligated DNA was used to transform *Escherichia coli* JM 109 according to the procedures described by Sambrook et al (1989). White colony transformants that grew on a Luria-Bertani medium containing ampicillin (100 μg/ml), X-gal (5-bromo-4-chloro-3-indoyl-β-galactoside, 0.004%), and IPTG (isopropyl-β-D-thiogalacto-pyranoside, 0.5 mM) were subcultured. To tentatively identify recombinants containing cloned fragments of PNWB-phytoplasma DNA, plasmid DNA was extracted from white colonies by alkaline lysis method and screened by dot hybridizations using digoxigenin-11-dUTP (Boehringer Mannheim) labeled total DNA extracts from healthy or PNWB-phytoplasma-infected periwinkle and peanut plants. Random priming procedure for labeling DNA with digoxigenin was performed in the present study according to the manufacture's instructions.

Each plasmid DNA preparation was denatured by boiling for 10 min and spotted onto nylon membranes (Micron Seps. Inc., Westboro, MA) using a micropipette. Membranes were then air-dried and cross-linked with the blotted plasmid DNA with a UV-crosslinker (Spectronics Corporation, Westburg, NY). The procedure used for hybridization and moderate stringency washes was described by Ko and Lin (1994).

Selected recombinant plasmids that hybridized with digoxigenin-labeled DNA from PNWB-phytoplasma-infected plants, but not with labeled DNA from healthy plants, were examined further for use as probes. Sizes of the cloned inserts in each selected recombinant were determined by agarose gel electrophoresis (0.8%) after *Eco*RI digestion. Cloned inserts selected for use as probes were separated by electrophoresis in 0.8% low-melting-temperature agarose gels, isolated from gel slices with Glass Max DNA isolation matrix system (Gibco BRL Life Technologies Inc., Grand Island, NY) and labeled with digoxigenin.

Specificity and sensitivity of cloned DNA probe. Dot hybridizations were also used to evaluate the specificity and detection sensitivity of each selected probe. To determine probe specificity, membranes were spotted with undigested DNA samples (200 ng of total DNA extracts per spot) from healthy periwinkle and peanut plants, PNWB-phytoplasma-infected periwinkle and peanut, and plants with eight other previously mentioned phytoplasma-associated diseases. Two membranes were used for hybridizing with each of the cloned PNWB-phytoplasma DNA probes. One membrane was washed under moderate stringency (55 °C), and the other was washed under high stringency (washed with 0.1 × SSC containing 0.1% SDS at 68 °C instead of 55 °C) as described previously (Ko and Lin, 1994).

To determine the detection sensitivity of the cloned PNWB-phytoplasma DNA probes, individual digoxigenin-labeled PNWB-phytoplasma DNA probes were hybridized with blots consisting of total DNA from PNWB-phytoplasma-infected periwinkle and peanut plants. Each sample DNA was applied to membranes as serial twofold dilutions in TE buffer (pH 8.0) ranging from 0.20 ng to 200 ng per dot (2 μl/dot). Membranes were evaluated under high stringency (68 °C wash).

Southern hybridization. To compare the selologically related PNWB-phytoplasma and SPWB-phytoplasma, Southern hybridization analyses were conducted. For each blot, 2 μg of total DNA from PNWB- and SPWB-infected periwinkle plants was digested with *Eco*RI, electrophoresed in 0.8% agarose gels, alkaline-denatured (0.5 M NaOH in 1.5 M NaCl for 45 min), neutralized (1 M Tris, pH 7.4, 1.5 M NaCl for 15 min), and transferred to the nylon membranes by following the procedures described by Southern (Southern, 1975). The membranes were cross-linked, prehybridized, hybridized with digoxigenin-labeled PNWB-phytoplasma DNA probes, and washed under high stringency, as previously described (Ko and Lin, 1994).

DNA sequencing. Recombinant plasmid pPNWB281, containing a PNWB-phytoplasma DNA fragment of 6.5 kb (PNWB281), was digested with *Hin*dIII restriction endonuclease and subcloned into plasmid pGEM3Zf(+) for the sequencing purpose. Two recombinant plasmids pPNWB281-4 and pPNWB281-

5 were selected for sequencing. Partial sequences at both ends of the inserts from the recombinant plasmids pPNWB19, pPNWB281-4, and pPNWB281-5 were determined with the Silver Sequence DNA Sequencing Systems (Promega Corporation, Madison, WI) according to the manufacturer's instructions using SP6 and pUC/M13 forward primers (Promega).

Polymerase chain reaction. According to the partial sequences of the cloned inserts from the three recombinant plasmids mentioned above, six oligonucleotide primers were synthesized as follows: 19a, 5′-GCCTTCATCATCAATTTTA-3′; 19b, 5′-TGGTTTAGGTATTAGATTA-3′; 281-4a, 5′-ACGGATTTGGTTAGTTTGATG-3′; 281-4b, 5′-ACTGGTAACTTGAACATCCCT-3′; 281-5a, 5′-AACCACTTCTTACACACTTTA-3′; 281-5b, 5′-ATCAATCACCACAATCTCTAT-3′. For PCR amplification, total DNA extracts from healthy or phytoplasma-infected plants were diluted in TE buffer (100 mM Tris, 0.1 M EDTA, pH 8.0) to give the final concentration of 100 ng/ μl. The reactions were performed in a 100-μl reaction mixture containing 5 μl of the dilute plant DNA template, 10 μl of 10 $\times$ Taq reaction buffer (500 mM KCl, 200 mM Tris-HCl, pH 8.4), 1.5 mM $MgCl_2$, 8 μl of 2.5 mM dNTP mixture, 10 μm of each primer-pair combination, and 2.5 units of Taq DNA polymerase (Gibco BRL Life Technologies Inc.). PCR was conducted for 30 PCR cycles in an automated thermal cycler (cycler 480, Perkin-Elmer Corp., Norwalk, CT) under the following conditions: denaturation for 25 s (1 min for first cycle) at 94 °C, annealing for 30 s at 46 °C (for primer pairs 19a + 19b), 50 °C (for primer pairs 281-4a + 281-4b) or 47 °C (for primer pairs 281-5a + 281-5b), and primer extension for 30 s (for primer pairs 19a + 19b, 281-4a + 281-4b) or 50 s (for primer pairs 281-5a + 281-5b) at 72 °C. After amplification, a 20 μl aliquot from each PCR reaction mixture was analyzed by electrophoresis in a 1.5% agarose gel and visualized by staining with ethidium bromide and UV illumination.

Detection of PNWB-phytoplasma by PCR analysis. Tenfold, serially diluted total DNA extracts from PNWB-phytoplasma-infected periwinkle and peanut, ranging from 0.1 pg to 10 ng, were used as PCR template to determine the minimum amount of template DNA needed to effectively amplify the PNWB-phytoplasma DNA fragment. PCR-amplified PNWB-phytoplasma DNA fragments were analyzed by 1.5% agarose gel electrophoresis.

PCR-based detection of PNWB-phytoplasma in presymptomatic plants. Periwinkle plants were grown to a height of approximately 25 cm and side-grafted with scions infected with PNWB-phytoplasma. Each scion consisted of a 5-cm segment of symptomatic periwinkle shoots. Test tissues were sampled at 7, 14, 21, 28, 35, 42, 49, and 56 days after graft inoculation. At the first two sampling times, tissue samples were removed from the original shoots below the grafted shoots. When side shoots began to emerge from the grafted shoots, these were also sampled (at 21 days and afterward) according to the method described by Kuske and Kirkpatrick (1992). Total DNA was extracted from the 5-10 g samples, consisting of leaves, petioles, shoots, and used as the template (100 ng/ μl, 5 μl for each amplification) for PCR amplification as described above.

Results

Cloning of PNWB-phytoplasma DNA fragments. A total of 145 and 370 transformant colonies were obtained in the cloning of PNWB-phytoplasma DNA extracted from diseased peanut and periwinkle, respectively. Ninety-nine recombinant plasmids were identified by dot hybridization to react specifically with labeled total DNA from PNWB-phytoplasma-infected peanut and periwinkle but not with those from healthy plants. Among these, only recombinant pPNWB95 was obtained by cloning DNA from PNWB-phytoplasma-infected peanut. Besides pPNWB95, six other recombinant plasmids pPNWB19, pPNWB55, pPNWB58, pPNWB105, pPNWB281, and pPNWB288 were selected for further characterization. Two recombinants pPNWB281-4 and pPNWB281-5 were further obtained by subcloning the recombinant pNWB281. Both clones were also identified to hybridized specifically with labeled total DNA from PNWB-phytoplasma-infected peanut and periwinkle but not with those from healthy plants. Digoxigenin-labeled cloned inserts of these recombinants were used as DNA probes, PNWB19, 55, 58, 95, 105, 281, 281–4, 281–5, and 288 (Table 1).

Specificity and sensitivity of probe. Probes PNWB281, PNWB281-4 and PNWB281-5 hybridized with DNA from PNWB-phytoplasma infected peanut and periwinkle specifically but not with DNA from healthy plants and plants infected with any of the other eight phytoplasmas examined under both high- and

Table 1. Results from dot hybridization of digoxigenin-labeled cloned peanut witches'-broom phytoplasma DNA probes to total DNA preparations from healthy plants or plants affected by various phytoplasmas[1]

Probes	Insert size(kb)	Hybridization with indicated DNA preparation[2]										
		H	PNWB	PNWB1	SPWB	PLWB	LWB	IOWB	AY	EY	RYD	BLLD
PNWB19	0.95	–	+	+	+	–	–	–	–	–	–	–
PNWB55	2.6	–	+	+	+	–	–	–	–	–	–	–
PNWB58	3.0	–	+	+	+	–	–	–	–	–	–	–
PNWB95	2.7	–	+	+	+	–	–	–	–	–	–	–
PNWB105	2.0	–	+	+	+	–	–	–	–	–	–	–
PNWB281	6.5	–	+	+	–	–	–	–	–	–	–	–
PNWB281–4	0.9	–	+	+	–	–	–	–	–	–	–	–
PNWB281–5	1.5	–	+	+	–	–	–	–	–	–	–	–
PNWB288	4.4	–	+	+	+	–	–	–	–	–	–	–

[1] +, Positive hybridization signal; –, no hybridization signal. Results are the same from both high- and moderate-stringency wash conditions.

[2] Each probe hybridized to DNA preparations from: H, healthy host plants including periwinkle, peanut, sweetpotato, rice, and bamboo; PNWB, peanut with peanut witches'-broom; PNWB1, periwinkle with peanut witches'-broom; SPWB, periwinkle with sweetpotato witches'-broom; PLWB, periwinkle with paulownia witches'-broom; LWB, periwinkle with loofah witches'-broom; IOWB, periwinkle with *Ipomoea obscura* witches'-broom; AY, periwinkle with aster yellows (NJ. strain); EY, periwinkle with elm yellows; RYD, rice with rice yellow dwarf; BLLD, bamboo with bamboo little leaf disease.

moderate-stringency wash conditions. The remaining six probes hybridized with DNA from PNWB-phytoplasma infected periwinkle and peanut and with DNA from SPWB-phytoplasma infected periwinkle but not with DNA from healthy plants and diseased plants infected by any of the other seven phytoplasmas under both high- and moderate-stringency wash conditions (Table 1).

In sensitivity tests with dot hybridizations under high-stringency wash conditions, PNWB-phytoplasma-specific probes detected the presence of PNWB-phytoplasma DNA in as little as 1.56 ng of total DNA from PNWB-infected periwinkle and in 6.25 ng of DNA from diseased peanut (result not shown).

Southern hybridization analysis. In Southern hybridization analyses, all cloned DNA probes hybridized with one or more fragments of *Eco*RI-digested total DNA extracted from PNWB-phytoplasma-infected periwinkle. PNWB-phytoplasma-specific probes PNWB281, 281-4, and 281-5 hybridized with one band at 6.5 kb, 0.9 kb, and 1.5 kb of *Eco*RI-digested total DNA from PNWB-phytoplasma-infected periwinkle, respectively, but not with that from SPWB-phytoplasma-infected periwinkle. Probes PNWB55, 58, and 105 gave the different hybridization band patterns with both DNA extracts from PNWB-phytoplasma- and SPWB-phytoplasma-infected periwinkle plants. The other three probes PNWB19, 95, and 288 gave the same hybridization band patterns with both DNA from PNWB-phytoplasma- and SPWB-phytoplasma-infected periwinkle. Representative hybridization results using probes PNWB281, 105, and 288 to total DNA from PNWB-phytoplasma- and SPWB-phytoplasma-infected periwinkle plants are shown in Figure 1.

PCR amplification of PNWB-phytoplasma DNA. After conducting 30 PCR cycles, a distinct DNA product of about 0.75 kb was obtained using primer pair 19a + 19b with total DNA from PNWB-phytoplasma-infected periwinkle and peanut or SPWB-phytoplasma-infected periwinkle as templates (Figure 2A). DNA fragments of 0.39 kb and 1.15 kb were amplified using primer pairs 281-4a + 281-4b and 281-5a + 281-5b, respectively, with total DNA templates from PNWB-phytoplasma-infected periwinkle and peanut, but not with DNA templates from diseased plants affected with serologically related SPWB-phytoplasma (Figures 2B & 2C). No specific PCR product was obtained in all other PCR reactions using the DNA templates from healthy plants and from other phytoplasma-affected plants.

In sensitivity tests with PCR, a minimum of 1 pg and 10 pg of total DNA extracts from diseased periwinkle and peanut, respectively was needed to effectively amplify the PNWB-phytoplasma-specific PCR product. Representative results of the PCR amplifica-

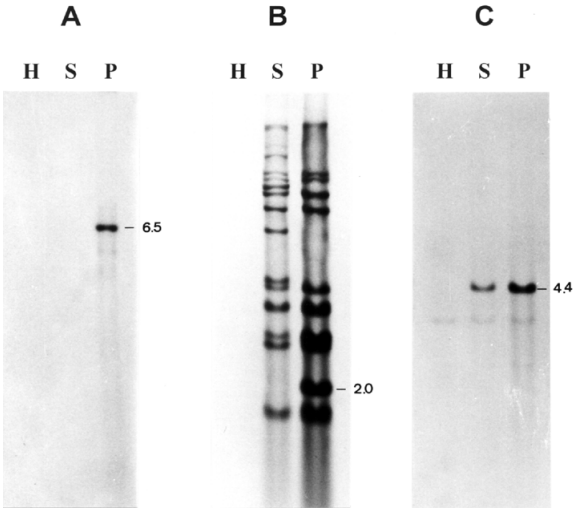

Figure 1. Southern hybridization analyses of *Eco*RI-digested total DNA prepared from healthy periwinkle plant (lanes H), sweetpotato witches'-broom phytoplasma (SPWB-phytoplasma)-infected periwinkle (lanes S) and peanut witches'-broom phytoplasma (PNWB-phytoplasma)-infected periwinkle (lanes P) hybridized with digoxigenin-labeled PNWB-phytoplasma DNA probes A, PNWB281 (6.5 kb); B, PNWB105 (2.0 kb); C, PNWB288 (4.4 kb). The sizes (in kilobases) of cloned inserts are shown in the margins, and were determined from fragments of lambda DNA digested with *Hin*dIII.

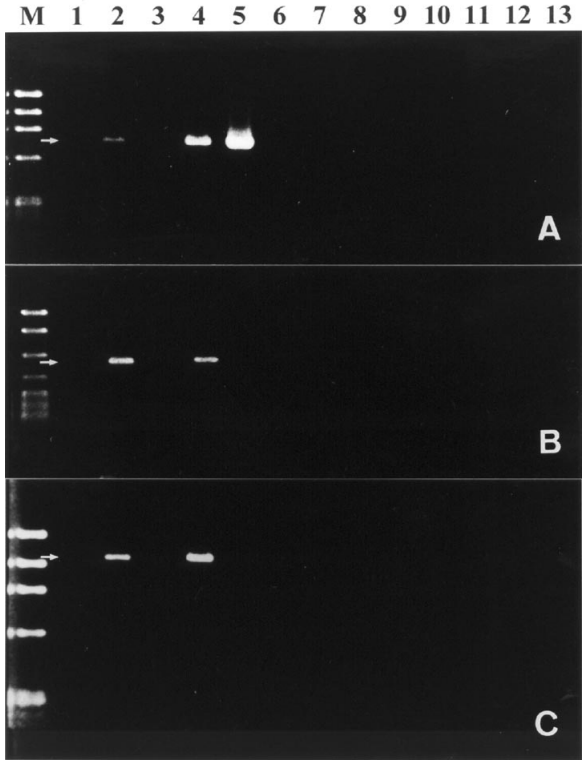

Figure 2. Amplified phytoplasma DNA from polymerase chain reaction (PCR) using primer pairs 19a + 19b (A), 281–4a + 281–4b (B), and 281–5a + 281–5b (C). The DNA templates were extracted from: lane 1, healthy peanut; lane 2, peanut with peanut witches'-broom; lane 3, healthy periwinkle; lane 4, periwinkle with peanut witches'-broom; lane 5, sweetpotato with sweetpotato witches'-broom; lane 6, periwinkle with loofah witches'-broom; lane 7, periwinkle with *Ipomoea obscura* witches'-broom; lane 8, periwinkle with paulownia witches'-broom; lane 9, periwinkle with elm yellows; lane 10, periwinkle with aster yellows (NJ strain); lane 11, rice with rice yellow dwarf; lane 12, bamboo with bamboo little leaf disease. Lane 13, water as negative control. M, molecular weight standards: A and C, $\varphi \times$ 174 DNA cut by *Hae*III, from top band to bottom band (in base pairs): 1353, 1078, 872, 603, 310; B, pBR322 DNA digested with *Msp*I, from top band to bottom band (in base pairs): 622, 527, 404, 307, 242/238, 217, 201. Arrows indicate molecular weight of 750 bp (A), 390 bp (B), and 1150 bp (C) amplification fragments.

tion with primer pair 19a + 19b are illustrated in Figure 3.

PCR-based detection of PNWB-phytoplasma in presymptomatic plants. Specific PNWB-phytoplasma-DNA fragments were effectively amplified using DNA templates extracted from presymptomatic or symptomatic periwinkles plants grafted with diseased scions for two weeks and thereafter (Figure 4). Typical witches'-broomed symptoms did not appear until the 5th week after graft inoculation under the conditions described.

Discussion

The cloned PNWB-phytoplasma DNA probe PNWB 281 and the subcloned probes PNWB281-4 and PNWB281-5 developed in this investigation were able to differentiate PNWB-phytoplasma from SPWB-phytoplasma by using hybridization analyses, and moreover, the PCR primers designed in this study could distinguished PNWB-phytoplasma from SPWB-phytoplasma effectively by using PCR-based detection for the first time. Before this, serological approaches had been applied in the detection of PNWB-phytoplasma and SPWB-phytoplasma (Hobbs et al., 1987; Shen and Lin 1993). Monoclonal antibodies developed for SPWB-phytoplasma developed in our previous study did show that these two phytoplasmas are closely related and undistinguishable (Shen and Lin 1993). Besides monoclonal antibodies, the DNA probes against SPWB-phytoplasma could differentiate SPWB-phytoplasma from PNWB phytoplasma only by Southern hybridization (Ko and Lin, 1994). Where-

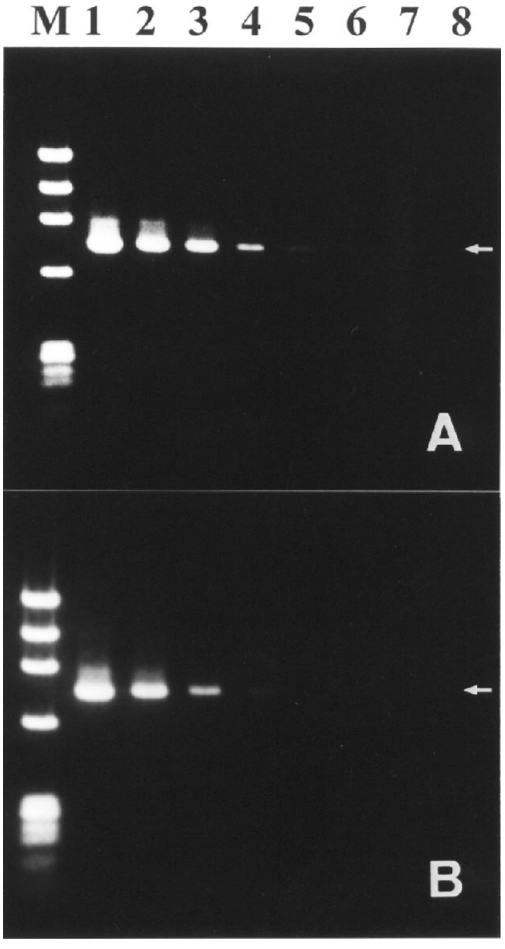

Figure 3. Sensitivity of the polymerase chain reaction (PCR) using DNA template from PNWB-phytoplasma infected periwinkle (A) and peanut (B). Thirty PCR cycles were conducted with primer pair 19a + 19b. Lanes 1 to 6, 10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 0.1 pg of total DNA were used as templates. Lnae 7, 10 ng of DNA from healthy periwinkle (A) and peanut (B) were used as templates. Lane 8, water as negative control. M, molecular weight standards $\varphi \times 174$ DNA cut by *Hae*III. From top band to bottom band (in base pairs): 1353, 1078, 872, 603, 310. Arrow indicates molecular weight of 750 bp amplification PCR products.

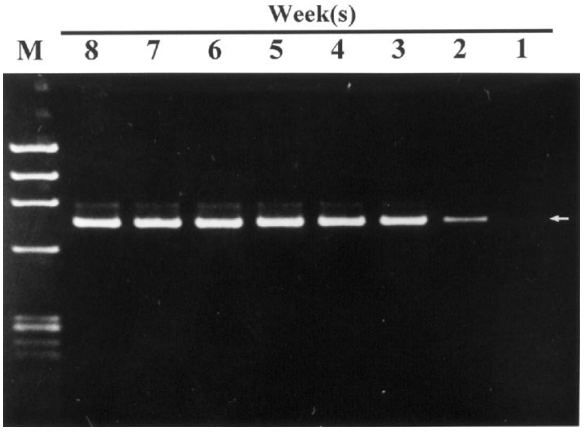

Figure 4. Amplification products from polymerase chain reaction (PCR) using primer pairs 19a + 19b after 30 PCR cycles. Phytoplasma DNA templates were prepared from grafted periwinkle 1, 2, 3, 4, 5, 6, 7, 8 wk after being grafted with PNWB-phytoplasma infected scions. Products were analyzed by 1.5% agarose gel and stained with ethidium bromide. M, molecular weight standards $\varphi \times 174$ DNA cut by *Hae*III. From top band to bottom band (in base pairs): 1353, 1078, 872, 603, 310. Arrow indicates molecular weight of 750 bp amplification PCR products.

as, PCR primers designed in that study could not distinguish SPWB-phytoplasma from PNWB-phytoplasma by using PCR-based detection. Therefore, DNA probes and PCR primers obtained in this study will be valuable for accurately monitoring these two agents in fields.

It should be pointed out that the total DNA (including the host plant DNA and phytoplasma DNA) extracted from diseased peanut was sticky and pigmented, and it was very difficult to further purify the phytoplasma DNA from the total DNA for cloning purpose. In addition, given the same amount of total DNA, the concentration of phytoplasma DNA from peanut and periwinkle could be very different because the titer of phytoplasmas from different plants could vary. For these reasons, we could only obtained one PNWB-phytoplasma-specific recombinant pPNWB95 by cloning DNA from PNWB-phytoplasma infected peanut. Detection sensitivity of the cloned DNA probes with phytoplasma DNA from greenhouse-grown periwinkle was higher than that from field-grown infected peanut both in PCR and hybridization analyses.

Polymerase chain reaction technology has recently been widely applied in the detection and identification of various phytoplasmas (Ahrens and Seemüller, 1992; Davis and Lee, 1993; Deng and Hiruki, 1990; Ko and Lin, 1994; Prince et al., 1993; Schaff et al., 1992; Zreik et al., 1995). In this study, PCR was also found effective in detecting the PNWB-phytoplasma in presymptomatic plants. Phytoplasma DNA could not be detected in the periwinkle in the first week after grafting with PNWB-phytoplasma infected scions in the present study, suggesting that the multiplication of phytoplasma had not yet reached a level sufficient for detection in ungrafted shoots by PCR The primer pairs 281–4a + 281–4b and 281–5a + 281–5b developed in this study were able to specifically amplify the PNWB-phytoplasma DNA fragment with the DNA templates from PNWB-phytoplasma-infected plants, and thus provided a reliable and rapid method to

differentiate the PNWB phytoplasma from other phytoplasmas especially from the closely related SPWB-phytoplasma.

Acknowledgements

We thank Y. L. Yu for critical review of the manuscript. This research was supported in part by a grant NSC84-2321-B002-125 from the National Science Council, Republic of China.

References

Ahrens U and Seemüller E (1992) Detection of DNA of plant pathogenic mycoplasmalike organisms by a polymerase chain reaction that amplifies a sequence of the 16S rRNA gene. Phytopathology 82: 828–832

Bonnet F, Saillard C, Kollar A, Seemüller E and Bove JM (1990) Detection and differentiation of the mycoplasmalike organism associated with apple proliferation disease using cloned DNA probes. Mol Plant-Microbe Interact 3: 438–443

Chang FL, Chen CC and Lin CP (1995) Monoclonal antibody for the detection and identification of a phytoplasma associated with rice yellow dwarf. European Journal of Plant Pathology 101: 511–518

Davis RE and Lee I-M (1993) Cluster-specific polymerase chain reaction amplification of 16S rDNA sequences for detection and identification of mycoplasmalike organisms. Phytopathology 83: 1008–1011

Davis MJ, Tsai JH, Cox RL, McDaniel LL and Harrison NA (1988) Cloning of chromosomal and extrachromosomal DNA of the mycoplasmalike organism that causes maize bushy stunt disease. Mol Plant-Microbe Interact 1: 295–302

Deng S and Hiruki C (1990) Enhanced detection of a plant pathogenic mycoplasma-like organism by polymerase chain reaction. Proc Jpn Acad 66(B): 140–144

Deng S and Hiruki C (1991) Genetic relatedness between two nonculturable mycoplasmalike organisms revealed by nucleic acid hybridization and polymerase chain reaction. Phytopathology 81: 1475–1479

Firrao G, Gobbi E and Locci R (1993) Use of polymerase chain reaction to produce oligonucleotide probes for mycoplasmalike organisms. Phytopathology 83: 602–607

Gundersen DE, Lee I-M, Rehner SA, Davis RE and Kingsbury DT (1994) Phylogeny of mycoplasmalike organisms (phytoplasmas): a basis for their classification. J Bacteriol 176: 5244–5254

Gundersen DE, Lee I-M, Schaff DA, Harrison NA, Chang CJ, Davis RE and Kingsbury DT (1996) Genomic diversity and differentiation among phytoplasma strains in 16S rRNA group I (aster yellows and related phytoplasmas) and III (X-disease and related phytoplasmas). Int J Syst Bacteriol 46: 64–75

Harrison NA, Tsai JH, Bourne CM and Richardson PA (1991) Molecular cloning and detection of chromosomal and extrachromosomal DNA of mycoplasmalike organisms associated with witches'-broom disease of pigeon pea in Florida. Mol Plant-Microbe Interact 4: 300–307

Hobbs HA, Reddy DVR and Reddy AS (1987) Detection of a mycoplasma-like organism in peanut plants with witches' broom using indirect enzyme-linked immunosorbent assay (ELISA). Plant Pathology 36: 164–167

Jiang YP and Chen TA (1987) Purification of mycoplasma-like organism from lettuce with aster yellows disease. Phytopathology 77: 949–953

Kirkpatrick BC, Stenger DC, Morris TJ and Purcell AH (1987) Cloning and detection of DNA from a nonculturable plant pathogenic mycoplasmalike organism. Science 238: 197–200

Ko HC and Lin CP (1994) Development and application of cloned DNA probes for a mycoplasmalike organism associated with sweetpotato witches'-broom. Phytopathology 84: 468–473

Kollar A, Seemüller E, Bonnet F, Saillard C and Bove JM (1990) Isolation of the DNA of various plant pathogenic mycoplasmalike organisms from infected plants. Photopathology 80: 233–237

Kuske ER and Kirkpatrick BC (1992) Distribution and multiplication of western aster yellows mycoplasmalike organisms in *Catharanthus roseus* as determined by DNA hybridization analysis. Phytopathology 82: 457–462

Lee I-M and Davis RE (1988) Detection and investigation of genetic relatedness among aster yellows and other mycoplasmalike organisms by using cloned DNA and RNA probes. Mol Plant-Microbe Interact 1: 303–310

Lee I-M, Hammond RW, Davis RE and Gundersen DE (1993) Universal amplification and analysis of pathogen 16S rDNA for classification and identification of mycoplasmalike organisms. Phytopathology 83: 834–842

Lim PO and Sears BB (1991) DNA sequence of the ribosomal protein gene rp12 and rps19 from a plant-pathogenic mycoplasmalike organism. FEMS Microbiol Lett 84: 71–74

Lim PO and Sears BB (1992) Evolutionary relationships of a plant-pathogenic mycoplasmalike organism and *Acholeplasma laidlawii* deduced from two ribosomal protein gene sequences. J Bacteriol 174: 2606–2611

Lin CP and Chen TA (1985) Monoclonal antibodies against the aster yellows agent. Science 227: 1233–1235

Pollini CP, Firrao G and Giunchedi L (1995) High 16S rDNA sequence homology between phytoplasmas from peach and apple trees affected by apple proliferation. Acta Hortic 386: 500–502

Prince JP, Davis RE, Wolf TK, Lee I-M and Mogen BD (1993) Molecular detection of diverse mycoplasmalike organisms (MLOs) associated with grapevine yellows and their classification with aster yellows, X-disease, and Elm yellows MLOs. Phytopathology 83: 1130–1137

Sambrook J, Fritsch EF and Maniatis TA (1989) Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Schaff DA, Lee I-M and Davis RE (1992) Sensitive detection and identification of mycoplasmalike organisms by polymerase chain reactions. Biochem Biophys Res Commun 186: 1503–1509

Schneider B, Ahrens U, Kirkpatrick BC and Seemüller E (1993) Classification of plant-pathogenic mycoplasma-like organisms using restriction-site analysis of PCR-amplified 16S rDNA. J Gen Microbiol 139: 519–527

Seemüller E, Schneider B, Mäurer R, Ahrens U, Daire X, Kison H, Lorenz KH, Firrao G, Avinent L, Sears BB and Stackebrandt E (1994) Phylogenetic classification of phytopathogenic mollicutes by sequence analysis of 16S ribosomal DNA. Int J Syst Bacteriol 44: 440–446

Shen WC and Lin CP (1993) Production of monoclonal antibodies against a mycoplasmalike organism associated with sweetpotato witches' broom. Phytopathology 83: 671–675

Southern EM (1975) Detection of specific sequences among DNA fragments separated by electrophoresis. J Mol Biol 98: 503–517

Wu FY, Lin CP and Chen CC (1993) Development of cloned DNA probes for a mycoplasmalike organism associated with rice yellow dwarf. Plant Pathol Bull (Taiwan, R.O.C.) 2: 128–135

Yang I-L (1975) Studies on the peanut witches' broom disease in Taiwan. T'ai-wan-Nung-Yeh-Chi-k'an 11: 93–108 (in Chinese)

Zreik L, Carle P, Bové JM and Garnier M (1995) Characterization of the mycoplasmalike organism associated with witches'-broom disease of lime and proposition of a *Candidatus* taxon for the organism, '*Candidatus* Phytoplasma aurantifolia'. Int J Syst Bacteriol 45: 449–453