Development and Application of Cloned DNA Probes for a Mycoplasmalike Organism Associated with Sweetpotato Witches'-broom

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ABSTRACT

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*Eco*RI restriction fragments of genomic DNA from the mycoplasmalike organism (MLO) associated with sweetpotato witches'-broom (SPWB) were cloned in plasmid pGEM3Zf(+). Cloned inserts from 14 SPWB-MLO-specific recombinant plasmids were *Eco*RI-excised, labeled with digoxigenin, and used as probes. Probes hybridized with DNA derived from SPWB-MLO-affected periwinkle and sweetpotato but not with DNA from healthy plants or plants infected with MLOs associated with loofah, paulownia, and *Ipomoea obscura* witches'-broom, elm and aster yellows, rice yellow dwarf, and bamboo little leaf disease. Twelve of 14 probes also hybridized with the serologically related peanut witches'-broom (PNWB) MLO. In Southern hybridizations, nine of 12 probes cross-reacted to PNWB MLO but could be used to differentiate the SPWB MLO from PNWB MLO readily according to the different band patterns. The probes could detect SPWB MLO DNA with as little as 0.10 ng and 0.39 ng of DNA from periwinkle and sweetpotato, respectively. SPWB MLO was also detected by hybridizing diseased-tissue blots on nitrocellulose membrane with a digoxigenin-labeled probe. A minimum of 10 pg of total DNA from diseased periwinkle and sweetpotato was needed as template to amplify SPWB MLO DNA by the polymerase chain reaction using either of two primer pairs synthesized according to the sequence of a cloned 1.4-kb insert.

Sweetpotato (*Ipomoea batatas* (L.) Lam.) is an important food crop in Taiwan, especially in the Penghu Islands. Sweetpotato witches'-broom (SPWB), a disease associated with a nonculturable mycoplasmalike organism (MLO), was first reported on Aguni Island of the Ryukyu Islands (29) and has been recorded in many areas and countries (28). In Taiwan, SPWB disease was first discovered in a geographically isolated area, the Penghu Islands, in 1969 (31).

SPWB MLO, like other MLOs, has not been axenically cultured. Recently, monoclonal antibodies against SPWB MLO were developed and applied in the detection and differentiation of SPWB MLO (28). Besides monoclonal antibodies, cloned DNA probes also were used to detect MLOs in plant and insect tissue by using nucleic acid hybridization assays. Probe hybridizations were used to differentiate MLOs, to monitor their distribution within a host plant, and to provide information concerning genetic relatedness among MLOs from various hosts and geographic locales (2–4,6–8,11,13,15,16,18–20,23,26).

The in vitro enzymatic amplification of DNA, known as polymerase chain reaction (PCR), is able to copy a single molecule of DNA and to specifically amplify a single-copy gene sequence from complex genomic DNA. This technology has provided a breakthrough in the detection of DNA and RNA molecules and has recently been used to amplify fragments of MLO DNA from host DNA extracts for detection purposes (1,9,12,25).

In this paper, isolation and molecular cloning of SPWB-MLO DNA from diseased plants, characterization of SPWB-MLO-specific DNA probes, and application of these probes for detection and differentiation of SPWB MLO are reported. A tissue-print method (5,22) combined with a nucleic acid hybridization assay was also applied for the detection and localization of SPWB MLO in sweetpotato tissue. In addition, the sequence data of one cloned SPWB-MLO DNA fragment were used to design PCR primers for the amplification of SPWB-MLO DNA.

MATERIALS AND METHODS

Plant material. Sweetpotato naturally infected with SPWB MLO was collected from fields on the Penghu Islands. Madagascar periwinkle (*Catharanthus roseus* (L.) G. Don) infected with

SPWB MLO originally obtained by transmission through dodder (*Cuscuta australis* R. Brown) was provided by I. L. Yang (Taiwan Agricultural Research Institute, Wufang) and was maintained and propagated in periwinkle by side grafting. Plants affected by several other MLO-associated diseases were maintained in periwinkle plants or collected from fields as described previously (28). These included periwinkle with peanut witches'-broom (PNWB), loofah witches'-broom, paulownia witches'-broom, *Ipomoea obscura* 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 disease was provided by N. S. Lin (Institute of Botany, Academia Sinica, Taipei, Taiwan).

DNA extraction. The procedure used for extraction of total DNA from plants was a modification of the protocol described by Kollar et al (17) and was routinely practiced in our lab (30). In brief, leaves, petioles, stems, or roots (50 g) of SPWB MLO-infected sweetpotatoes 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; 100 mM EDTA; 250 mM NaCl; 0.01% proteinase K; and 1% *N*-lauroyl sarcosine) (30) to extract nucleic acids. Crude nucleic acids were further clarified by repeated precipitations with ethanol and extractions with phenol and chloroform as described previously (24,30). Nucleic acid preparation was finally resuspended in 5 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

Separation of SPWB-MLO DNA from host DNA. Cesium chloride (CsCl)-bisbenzimide density gradient centrifugation was used to separate MLO DNA from host plant DNA (3,14,17,27,30). Approximately 1 mg of DNA sample was added to each 13.5-ml quick-seal polyallomer gradient tube containing 1 g of CsCl per milliliter of TE buffer (pH 8.0) and 1 mg of bisbenzimide per tube. The gradients were adjusted to a final density of 1.57 g/ml with additional TE buffer and centrifuged to equilibrium at 150,000 *g* (48,000 rpm) for 26 h at 20 C in a Beckman 70.1 Ti rotor (Beckman Instruments, Inc., Fullerton, CA). SPWB-MLO 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, except that only 100 μg of bisbenzimide was added per tube. After final centrifugation, the MLO 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 (24).

Molecular cloning of MLO DNA and screening of recombinants. Approximately 1 μg of CsCl-purified SPWB-MLO DNA was digested with *Eco*RI restriction endonuclease (Boehringer Mannheim GmbH, Mannheim, Germany). Resulting fragments were ligated with *Eco*RI-digested and dephosphorylated pGEM3Zf(+) (Promega Corp., Madison, WI) and used to transform competent cells of *Escherichia coli* JM 109 according to the procedures described by Sambrook et al (24). White colony transformants that grew on a Luria-Bertani medium (24) containing ampicillin (100 μg/ml), X-gal (5-bromo-4-chloro-3-indoyl-β-galactoside, 0.004%), and IPTG (isopropyl-β-D-thiogalactopyranoside, 0.5 mM) were subcultured. To tentatively identify recombinants containing cloned fragments of MLO DNA, plasmid DNA was extracted from 255 randomly selected white colonies by alkaline lysis method (24) and screened by dot hybridizations using digoxigenin-11-dUTP (Boehringer Mannheim) labeled total DNA extracts from healthy or SPWB-MLO-infected periwinkle as probes. Probe DNA was labeled by a random priming procedure with digoxigenin according to the manufacturer's instructions.

Each plasmid DNA preparation was denatured by boiling for 10 min, immediately cooled on ice for 5 min, and then spotted onto Magna Graph nylon membranes (Micron Seps., Westboro, MA) using a micropipette. Membranes were air-dried and cross-linked with the blotted DNA with a UV-crosslinker (Spectronics Corporation, Westburg, NY). Filters were prehybridized for 2 h at 68 C in hybridization buffer containing 0.01% SDS, 0.1% *N*-lauroyl sarcosine, 5× SSC solution (1× SSC = 0.15 M sodium chloride, 15 mM sodium citrate, pH 7.0), and 0.5% blocking reagent (Boehringer Mannheim). For hybridization, digoxigenin-labeled DNA from healthy periwinkle or SPWB-MLO-infected periwinkle was added to the hybridization buffer (25 ng/ml), and incubation was continued with agitation at 68 C for 16 h.

Membranes were given two moderate stringency washes (30 min each) at room temperature in 2× SSC containing 0.1% SDS, and twice again at 55 C with 0.1× SSC containing 0.1% SDS. After the final wash, membranes were air-dried, and signal detection was performed using digoxigenin nucleic acid detection kit (Boehringer Mannheim) following supplier's instructions. The blots were visualized by incubating the membranes in the dark in a solution containing colorimetric substrate, nitroblue tetrazolium, and 5-bromo-4-chloro-3-indolyl phosphate.

Selected recombinant plasmids that hybridized with digoxigenin-labeled DNA from SPWB-MLO-infected periwinkle, but not with labeled DNA from healthy periwinkle, 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 eluted from gel slices (24) and labeled with digoxigenin, as described previously.

Specificity and sensitivity of cloned DNA probe. Dot hybridizations were also used to evaluate the specificity and sensitivity of each selected probe. To determine probe specificity, two sets of membranes were tested as replicates. One membrane was washed under moderate stringency (described previously), and the other was washed under high stringency (last two washes with 0.1× SSC containing 0.1% SDS at 68 C instead of 55 C). Each membrane was spotted with undigested DNA samples (200 ng of total DNA extracts per spot) from healthy periwinkle and sweetpotato plants, SPWB-MLO-infected periwinkle and sweetpotato, and plants with eight other previously mentioned MLO-associated diseases, and then hybridized with each of the cloned SPWB-MLO DNA probes, as previously described.

To determine the detection sensitivity of the cloned SPWB-MLO DNA probes, individual digoxigenin-labeled SPWB-MLO DNA probes were hybridized with blots consisting of total DNA extracts from SPWB-MLO-infected periwinkle and sweetpotato. Each sample DNA was applied to membranes as serial twofold dilutions in TE buffer (pH 8.0) ranging from 0.1 ng to 100 ng per dot (2 μl/dot). Membranes were evaluated under high stringency (68 C wash).

Southern hybridization. Southern hybridization analysis was used to compare the serologically related SPWB MLO and PNWB MLO. For each blot, 2 μg of total DNA from SPWB- and PNWB-infected periwinkle plants was digested with *Eco*RI, electrophoresed in 1.0% agarose gels, alkaline-denatured (0.5 M NaOH in 1.5 M NaCl for 10 min), and transferred with a Trans Vac apparatus (Hoefer Scientific Instruments, San Francisco, CA) to nylon membranes according to manufacturer's instructions. Membranes were cross-linked, prehybridized, hybridized with digoxigenin-labeled SPWB-MLO DNA probes, and washed under high stringency, as previously described.

Tissue-print hybridization. Tissue-print hybridization was carried out by a modification of the method described by Chia et al (5). Two pieces of Whatman 3-mm filter papers were placed below a piece of dry nitrocellulose membrane (Micron Seps. Inc.). Cross sections of stem samples from healthy and SPWB-MLO-infected periwinkle and sweetpotato were hand-printed onto the nitrocellulose membrane for 3 s with the cut surface of the tissue in direct contact with the membranes. After air-drying, membranes were cross-linked, prehybridized, hybridized with digoxigenin-labeled-DNA probes, and washed under high stringency, as previously described. Upon development, blots were examined under a stereo microscope.

DNA sequencing. Recombinant plasmid pSPWB54, contain-

ing a SPWB-MLO DNA fragment of 1.4 kb (SPWB54) in pGEM3Zf(+), was first purified with a Qiagen-tip20 column (Qiagen Inc., Chatsworth, CA). Partial sequences at both ends of the insert were determined with the Sequenase DNA sequencing kit (US Biochemical, Cleveland, OH) according to the manufacturer's instructions with SP6 and T7 primers (Promega).

PCR amplification. From the partial sequence of cloned insert SPWB54, four regions were selected to synthesize primers for PCR assays (TIB Molbiol, Berlin, Germany). The primer pairs were designed to direct the amplification of approximate 1.0 or 1.1-kb fragment of SPWB-MLO DNA. For PCR amplification, total DNA extracts from healthy or MLO-infected plants were diluted in TE buffer (50 mM Tris-HCl, 5 mM EDTA, pH 8.0) to give a final concentration of 20 ng/μl. The reactions were performed in a 100-μl PCR reaction mixture containing 100 ng of plant DNA sample, 8 μl of 25 mM dNTP mixture, 0.5 μM of each primer-pair combination, 2 units of Super Taq DNA polymerase (HT Biotechnology Ltd., Cambridge, England), and 10 μl of 10× Taq reaction buffer (500 mM KCl, 100 mM Tris-HCl, 15 mM $MgCl_2$, 0.1% [w/v] gelatin, pH 9.0, 1% Triton X-100). PCR was conducted for 25, 30, 35, and 40 PCR cycles in an automated thermal cycler (The Perkin-Elmer Corp., Norwalk, CT) under the following conditions: denaturation for 25 s (1 min for first cycle) at 94 C, annealing for 20 s at 46 or 52 C, and primer extension for 25 s at 72 C. After amplification, a 10-μl aliquot from each PCR reaction mixture was analyzed by electrophoresis in a 1.5% agarose gel. PCR products were stained with ethidium bromide and visualized by UV transillumination.

PCR-based detection of SPWB-MLO DNA. Tenfold, serially diluted total DNA extracts from SPWB-MLO-infected periwinkle and sweetpotato, ranging from 0.01 pg to 10 ng, were used as PCR templates to determine the minimum amount of DNA needed to effectively amplify the SPWB-MLO DNA fragment. PCR-amplified SPWB-MLO DNA fragments were analyzed by 1.5% agarose gel electrophoresis after conducting 35 PCR cycles with the adopted primer pair and an annealing temperature of 46 C.

RESULTS

Cloned DNA probe. A total of 255 transformant colonies of *E. coli* was obtained in the cloning of SPWB-MLO DNA. Sixty-four recombinant plasmids were identified by dot hybridization to react specifically with labeled total DNA from SPWB-MLO-infected periwinkle but not with that from healthy periwinkle DNA. Among these, 14 recombinant plasmids were selected for further characterization based on *Eco*RI restriction analysis and size of the cloned DNA inserts. The cloned inserts of these 14 plasmids were labeled with digoxigenin and used as the cloned DNA probes (Table 1).

Specificity and sensitivity of probe. A summary of results from dot hybridization is given in Table 1. No dot hybridization signal was observed between any probe and DNA from healthy host plants that included periwinkle, sweetpotato, peanut, rice, and bamboo. Two of the 14 probes, SPWB16 and SPWB27, hybridized with DNA extracts from SPWB-MLO-infected periwinkle and sweetpotato specifically but not with DNA extracts from plants infected with any of the other eight MLOs that were examined. The remaining 12 probes only hybridized with DNA extracts from SPWB-MLO-infected periwinkle and sweetpotato and with DNA extracts from PNWB-MLO-infected periwinkle and peanut under both high- and moderate-stringency wash conditions (Table 1).

In sensitivity tests with dot hybridizations under high-stringency wash conditions, SPWB-MLO-specific probes detected presence of MLO DNA in as little as 0.10 ng of total DNA extracted from SPWB-infected periwinkle and in 0.39 ng of DNA from diseased sweetpotato. Representative hybridization results from this type of experiment are illustrated in Figure 1.

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 SPWB-MLO-infected periwinkle. At moderate stringency, SPWB16 and SPWB27 hybridized with one band at 4.1 kb and two bands at 3.2 kb and 3.5 kb, respectively. Neither probe hybridized to *Eco*RI-digested DNAs from PNWB-infected periwinkle. The other three probes, SPWB36, 54, and 59, gave only one hybridization band with both DNA extracts from SPWB-MLO- and PNWB-MLO-infected periwinkle plants corresponding to the sizes of inserts at 5.3, 1.4, and 3.0, respectively. The remaining nine DNA probes SPWB5, 8, 12, 15, 25, 28, 62, 73, and 99, hybridized both with blots of total DNA extracts from SPWB-MLO-infected plants and from PNWB-MLO-infected periwinkle but with different band patterns. When hybridized to total DNA extracts from SPWB-MLO-infected periwinkle, these nine probes all gave multiple hybridization bands (3 to 20 bands), including a band at the size of cloned inserts. This indicated that reiterative sequences were contained in the *Eco*RI-digested random fragments of SPWB-MLO and PNWB-MLO DNA preparations. Representative results of the Southern hybridization analyses using cloned DNA probes SPWB16, 25, and 54 to total DNA extracts from SPWB-MLO- and PNWB-MLO-infected periwin-

TABLE 1. Summary of results from dot hybridization of digoxigenin-labeled cloned sweetpotato witches'-broom mycoplasmalike organism (MLO) DNA probes to DNA preparations from healthy plants or plants affected by various MLO-associated diseases

Probe	Insert size (kb)	Hybridization with DNA extracts from[a,b]										
		HP	SPWB	SPWBP	PNWB	PLWB	LWB	IOWB	AY	EY	RYD	BLLD
SPWB5	2.0	−	+	+	+	−	−	−	−	−	−	−
SPWB8	2.5	−	+	+	+	−	−	−	−	−	−	−
SPWB12	4.3	−	+	+	+	−	−	−	−	−	−	−
SPWB15	1.6	−	+	+	+	−	−	−	−	−	−	−
SPWB16	4.1	−	+	+	−	−	−	−	−	−	−	−
SPWB25	2.8	−	+	+	+	−	−	−	−	−	−	−
SPWB27	3.5	−	+	+	−	−	−	−	−	−	−	−
SPWB28	1.1	−	+	+	+	−	−	−	−	−	−	−
SPWB36	5.3	−	+	+	+	−	−	−	−	−	−	−
SPWB54	1.4	−	+	+	+	−	−	−	−	−	−	−
SPWB59	3.0	−	+	+	+	−	−	−	−	−	−	−
SPWB62	5.2	−	+	+	+	−	−	−	−	−	−	−
SPWB73	3.0	−	+	+	+	−	−	−	−	−	−	−
SPWB99	2.3	−	+	+	+	−	−	−	−	−	−	−

[a] +, Positive hybridization signal; −, no hybridization signal.

[b] HP, healthy plants. Each probe hybridized to DNA preparations from healthy host plants including periwinkle, sweetpotato, peanut, rice, and bamboo; SPWB, sweetpotato with sweetpotato witches'-broom; SPWBP, periwinkle with sweetpotato witches'-broom; PNWB, peanut with peanut 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; EY, periwinkle with elm yellows; RYD, rice with yellow dwarf; BLLD, bamboo with little leaf disease.

kle plants are shown in Figure 2.

Detection of SPWB MLO by tissue-print hybridization. In the tissue-print hybridization assay, both stem-tissue blots printed by the SPWB-MLO-affected sweetpotato and periwinkle showed strong hybridization signals in the areas corresponding to the phloem tissues when hybridized with the digoxigenin-labeled DNA probes. The micrograph in Figure 3 indicates that SPWB MLO could be readily detected in the phloem tissues of stem. On the other hand, there was no color development in stem-tissue blots from uninfected sweetpotato (Fig. 3).

PCR amplification of SPWB-MLO DNA. According to the partial sequence data, the sequences of oligonucleotide primers were synthesized as follows: 1a, 5′-TGTCGGAATTACCTGGAA CA-3′ (SP6 end); 2b, 5′-TCTATTCGTCGGAAACCCAA-3′ (T7 end); 3a, 5′-GGAACATCATTAGCAACGCAAC-3′ (SP6 end); 4b, 5′-TCGAGTAGTTTATTTTTATCTT-3′ (T7 end). After conducting 25 to 40 PCR cycles with primer pairs 1a+4b and 3a+4b, a distinct DNA fragment of about 1.1 kb was produced using total DNA from SPWB-MLO-infected periwinkle and sweetpotato and DNA from PNWB-MLO-infected periwinkle as templates. Primer pairs 1a+2b and 3a+2b failed to amplify the expected 1.0-kb or any other DNA fragment at both annealing temperatures and were not used in further studies. The best results were obtained by conducting 35 and 40 PCR cycles with the primer pairs 1a+4b and 3a+4b at an annealing temperature of 46 C. These parameters were now adopted for the PCR assays routinely used in our lab to amplify the 1.1-kb SPWB-MLO DNA from field-collected sweetpotato samples and to screen the SPWB-MLO-free propagating tissues of sweetpotato. No specific PCR product was obtained in all other PCR reactions with the DNA templates from healthy plants and from other MLO-affected plants. A minimum of 10 pg of total DNA extracts from diseased periwinkle and sweetpotato was needed to effectively amplify the 1.1-kb PCR product for electrophoresis when 35 PCR cycles were conducted (Fig. 4).

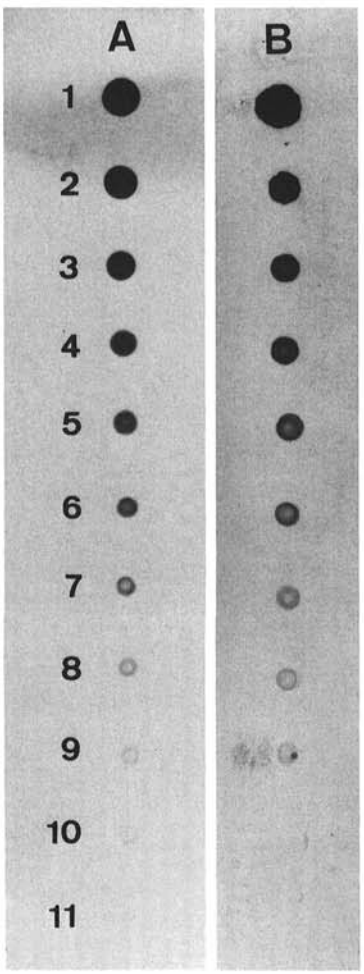

Fig. 1. Dot hybridization of the digoxigenin-labeled probe SPWB16 (4.1 kb) to twofold serial dilutions of total DNA extracted from either **A,** periwinkle or **B,** sweetpotato infected with the sweetpotato witches'-broom mycoplasmalike organism: 1, 100 ng; 2, 50 ng; 3, 25 ng; 4, 12.5 ng; 5, 6.25 ng; 6, 3.13 ng; 7, 1.56 ng; 8, 0.78 ng; 9, 0.39 ng; 10, 0.20 ng; 11, 0.10 ng.

DISCUSSION

DNA probes against SPWB MLO developed in this study were useful in detecting the SPWB disease and also in differentiating MLOs associated with other diseases. The monoclonal antibodies produced in the previous study (28) are currently used along with the DNA probes developed in this study to investigate the distribution of SPWB MLO in various parts of infected plants with tissue-printing techniques (W. C. Shen and C. P. Lin, *unpublished data*). All monoclonal antibodies developed recognized preparations from plants infected with PNWB MLO, showing that these two MLOs are closely related (28).

Our primary concern in developing DNA probes for the detection of the SPWB MLO was their relative sensitivity and specificity. Detection sensitivity varied according to the plant host tested. This was particularly evident after screening field-grown infected sweetpotato and greenhouse-grown periwinkle. Results from these probes indicated that they were able to detect SPWB-MLO DNA consistently to 0.10 ng of DNA from periwinkle and 0.39 ng of DNA from sweetpotato with dot hybridization. Variations between samples were interpreted to reflect the differences in MLO titers in different infected host tissues. In specificity tests, we found that most probes cross-hybridized with DNA preparations from PNWB-MLO-infected plants in a manner similar to the results of previous serological assays (28). In this study, South-

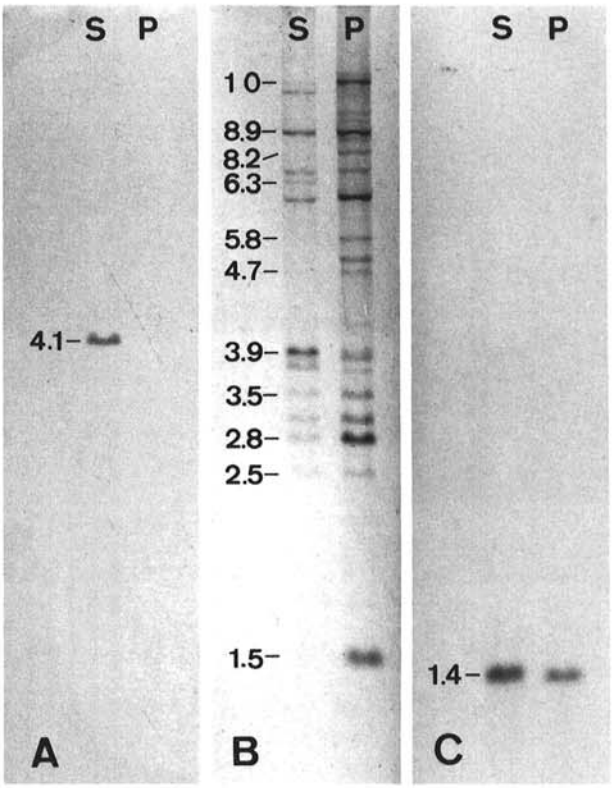

Fig. 2. Southern hybridization analyses of *Eco*RI-digested total DNA prepared from sweetpotato witches'-broom mycoplasmalike organism (SPWB MLO)-infected periwinkle (lanes S) and peanut witches'-broom MLO-infected periwinkle (lanes P) hybridized with digoxigenin-labeled cloned SPWB-MLO DNA probes **A,** SPWB16 (4.1 kb); **B,** SPWB25 (2.8 kb); and **C,** SPWB54 (1.4 kb). The sizes (in kilobases) are shown in the margins, and were determined from fragments of lambda DNA digested with *Hin*dIII and *Eco*RI.

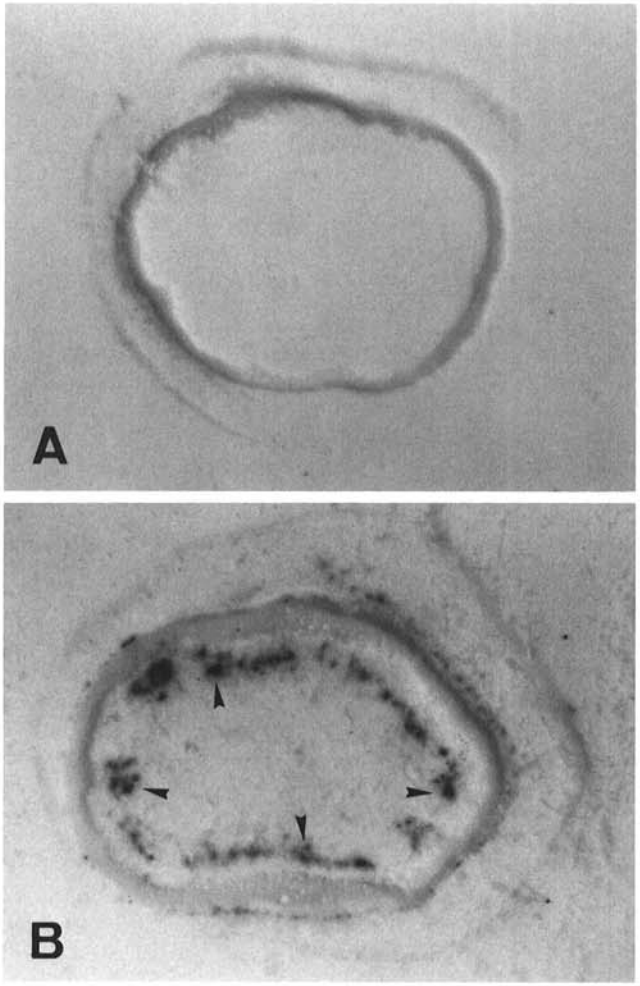

Fig. 3. Detection of sweetpotato witches'-broom mycoplasmalike organisms (SPWB MLO) in tissue blots of infected stems of sweetpotato on nitrocellulose membranes. The blots were reacted with digoxigenin-labeled SPWB-MLO DNA probe SPWB16. Stem cross sections of **A,** healthy sweetpotato and **B,** diseased sweetpotato showing localization of the specific blue-purple reaction product in areas on the blots (arrows) corresponding to the position of the phloem in sweetpotato stems (×18).

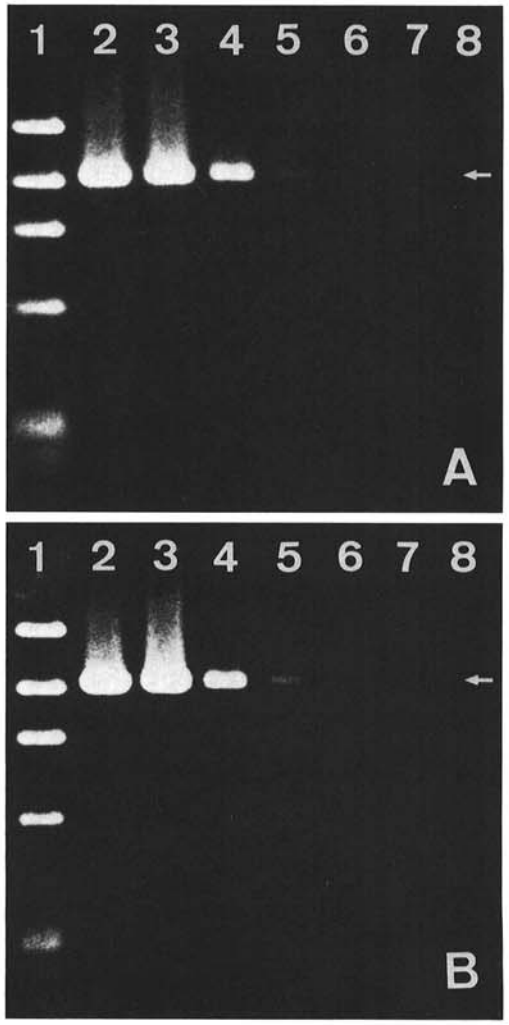

Fig. 4. Polymerase chain reaction (PCR)-amplified sweetpotato witches'-broom mycoplasmalike organism DNA analyzed by 1.5% agarose gel electrophoresis. The gel was stained with ethidium bromide, and the bands were visualized with ultraviolet light. Lane 1, molecular weight standards PhiX 174 RF DNA digested with *Hae*III. From top band to bottom band (in base pairs): 1,353, 1,078, 872, 603, 310, 281 + 271. Lanes 2 to 8: 10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 0.1 pg, and 0.01 pg of total DNA preparations from **A,** periwinkle and **B,** sweetpotato were used as templates for each PCR amplification of the 1.1-kb DNA fragment (arrows).

ern hybridization analyses provided evidence for differentiating SPWB MLO from PNWB MLO.

The recent development of the tissue-printing technique has been useful for determining the distribution and localization of some pathogens in a host (5,22). In this study, probe hybridizations were shown to be of sufficient sensitivity to detect SPWB-MLO DNA in sap expressed onto membranes from freshly cut sweetpotato tissues during the formation of tissue imprints. We have now combined the tissue-printing technique with the DNA hybridization assay to routinely detect SPWB MLO in sweetpotato. The tissue-print hybridization technique could be the most efficient and applicable method for detection of SPWB MLO in epidemiological studies. It allows a large number of samples to be processed simultaneously in a regular laboratory when using nonradioactive DNA probes.

Recently, PCR techniques have been applied in the detection or classification of MLO pathogens by developing primers from 16S rRNA sequence (1,10,21,25) or from sequences of specific cloned MLO DNA fragments (12). In our study, sequences of clones 16 and 27 were also analyzed, and many primer pairs were designed to amplify SPWB-MLO DNA according to the partial sequence data. Those primer pairs all amplified an inconstant number of nonspecific DNA fragments when DNA templates from SPWB-MLO- or PNWB-MLO-infected plants were used. Those primers were not suitable for further applications. The PCR primers adopted in the study were designed according to the sequence of the recombinant pSPWB54 and can efficiently amplify a 1.1-kb SPWB-MLO DNA fragment using DNA templates prepared from SPWB-infected periwinkle and sweetpotato. PCR amplification along with electrophoretic analysis of the 1.1-kb DNA fragment is now routinely applied in ecological studies and in the screening of SPWB-MLO-free propagating tissues.

LITERATURE CITED

1. 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.
2. Bertaccini, A., Davis, R. E., Lee, I.-M., Conti, M., Dally, E. L., and Douglas, S. M. 1990. Detection of chrysanthemum yellows mycoplasmalike organism by dot hybridization and Southern blot analysis. Plant Dis. 74:40-43.
3. Bonnet, F., Saillard, C., Kollar, A., Seemüller, E., and Bove, J. M. 1990. Detection and differentiation of the mycoplasmalike organism associated with apple proliferation disease using cloned DNA probes. Mol. Plant-Microbe Interact. 3:438-443.
4. Chen, J., Chang, C. J., and Jarret, R. L. 1992. DNA probes as molecular markers to monitor the seasonal occurrence of walnut

witches'-broom mycoplasmalike organism. Plant Dis. 76:1116-1119.
5. Chia, T.-F., Chan, Y.-S., and Chua, N.-H. 1992. Detection and localization of viruses in orchids by tissue-print hybridization. Plant Pathol. 41:355-361.
6. Davis, M. J., Tsai, J. H., Cox, R. L., McDaniel, L. L., and Harrison, N. A. 1988. Cloning of chromosomal and extrachromosomal DNA of the mycoplasmalike organism that causes maize bushy stunt disease. Mol. Plant-Microbe Interact. 1:295-302.
7. Davis, R. E., Lee, I.-M., Douglas, S. M., and Dally, E. L. 1990. Molecular cloning and detection of chromosomal and extra-chromosomal DNA of the mycoplasmalike organism associated with little leaf disease in periwinkle (*Catharanthus roseus*). Phytopathology 80:789-793.
8. Davis, R. E., Sinclair, W. A., Lee, I.-M., and Dally, E. L. 1992. Cloned DNA probes specific for detection of a mycoplasmalike organism associated with ash yellows. Mol. Plant-Microbe Interact. 5:163-169.
9. 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.
10. Deng, S., and Hiruki, C. 1991. Amplification of 16S rRNA genes from culturable and nonculturable mollicutes. J. Microbiol. Methods 14:53-61.
11. Deng, S., and Hiruki, C. 1991. Localization of pathogenic mycoplasma-like organisms in plant tissue using in situ hybridization. Proc. Jpn. Acad. 67(B):197-202.
12. 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.
13. Harrison, N. A., Bourne, C. M., Cox, R. L., Tsai, J. H., and Richardson, P. A. 1992. DNA probes for detection of mycoplasmalike organisms associated with lethal yellowing disease of palms in Florida. Phytopathology 82:216-224.
14. Harrison, N. A., Tsai, J. H., Bourne, C. M., and Richardson, P. A. 1991. Molecular cloning and detection of chromosomal and extra-chromosomal DNA of mycoplasmalike organisms associated with witches'-broom disease of pigeon pea in Florida. Mol. Plant-Microbe Interact. 4:300-307.
15. Hibben, C. R., Sinclair, W. A., Davis, R. E. and Alexander, J. H., III. 1991. Relatedness of mycoplasmalike organisms associated with ash yellows and lilac witches'-broom. Plant Dis. 75:1227-1230.
16. Kirkpatrick, B. C., Stenger, D. C., Morris, T. J., and Purcell, A. H. 1987. Cloning and Detection of DNA from a nonculturable plant pathogenic mycoplasmalike organism. Science 238:197-200.
17. Kollar, A., Seemüller, E., Bonnet, F., Saillard, C., and Bove, J. M. 1990. Isolation of the DNA of various plant pathogenic mycoplasmalike organisms from infected plants. Phytopathology 80:233-237.
18. Kuske, C. R., and Kirkpatrick, B. C. 1992. Distribution and multiplication of western aster yellows mycoplasmalike organisms in *Catharanthus roseus* as determined by DNA hybridization analysis. Phytopathology 82:457-462.
19. Kuske, C. R., and Kirkpatrick, B. C., and Seemüller, E. 1991. Differentiation of virescence MLOs using western aster yellows mycoplasma-like organism chromosomal DNA probes and restriction fragment length polymorphism analysis. J. Gen. Microbiol. 137:153-159.
20. Lee, I.-M., and Davis, R. E. 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.
21. 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.
22. Lin, N. S., Hsu, Y. H., and Hsu, H. T. 1990. Immunological detection of plant viruses and a mycoplasmalike organism by direct tissue blotting on nitrocellulose membranes. Phytopathology 80:824-828.
23. Nakashima, K., Kato, S., Iwanami, S., and Murata, N. 1993. DNA probes reveal relatedness of rice yellow dwarf mycoplasmalike organisms (MLOs) and distinguish them from other MLOs. Appl. Environ. Microbiol. 59:1206-1212.
24. Sambrook, J., Fritsch, E. F., and Maniatis, T. A. 1989. Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
25. Schneider, B., Ahrens, U., Kirkpatrick, B. C., 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.
26. Schneider, B., Mäurer, R., Saillard, C., Kirkpatrick, B. C., and Seemüller, E. 1992. Occurrence and relatedness of extrachromosomal DNAs in plant pathogenic mycoplasmalike organisms. Mol. Plant-Microbe Interact. 5:489-495.
27. Sears, B. B., Lim, P.-O., Holland, N., Kirkpatrick, B. C., and Klomparens, K. L. 1989. Isolation and characterization of DNA from a mycoplasmalike organism. Mol. Plant-Microbe Interact. 2:175-180.
28. Shen, W. C., and Lin, C. P. 1993. Production of monoclonal antibodies against a mycoplasmalike organism associated with sweet-potato witches' broom. Phytopathology 83:671-675.
29. Shinkai, A. 1964. Studies on insect transmission of sweet potato witches' broom disease in the Ryukyu Islands. Pages 1-44 in: Spec. Res. Rep. Agric. Sec. Econ. Dep. Ryukyu Islands.
30. Wu, F. Y., Lin, C. P., and Chen, C. C. 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.
31. Yang, I. L. 1969. Studies on witches' broom of sweet potato in Taiwan (I). J. Taiwan Agric. Res. 18:50-60.