Use of Polymerase Chain Reaction to Produce Oligonucleotide Probes for Mycoplasmalike Organisms

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ABSTRACT

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Specific oligonucleotide probes for detecting apple proliferation and clover phyllody MLOs (mycoplasmalike organisms) in infected tissues were derived from a partial sequence of their 16S ribosomal RNA genes and were tested by dot blot hybridization against amplified DNA from both healthy plants and plants infected by MLOs. Probes were disease specific, with the exception of probes designed for the apple proliferation MLO, which reacted with the DNA extracted from a periwinkle infected by the plum leptonecrosis MLO. The sequenced DNAs were obtained by PCR (polymerase chain reaction) from crude nucleic acid extracts with primers that allowed the amplification of an 865-bp DNA fragment from infected but not from healthy periwinkles. The amplification of a similar fragment was obtained from all MLO-infected periwinkles examined.

Additional keywords: Mollicutes, ribosomal DNA.

Taxonomic and genetic relationships between prokaryotes colonizing the plant phloem, commonly known as mycoplasmalike organisms (MLOs), and already recognized mycoplasmas were anticipated based on morphological similarities. Estimation of genome size (30), DNA base composition (22,40), and isolation and sequencing of the 16S rRNA of the evening primrose MLO (29) and of the western aster yellows MLO (24) confirmed the hypothesis that MLOs belong to the class Mollicutes, although they are phylogenetically distinct from the genus *Mycoplasma*.

MLOs are believed to be causative agents of many, often serious, plant diseases affecting more than 300 plant species (32). Because methods for in vitro cultivation of MLOs are not available yet, their differentiation and characterization are difficult as a result of the lack of efficient diagnostic procedures. Techniques based on electron and fluorescence microscopy, symptomatology, host, and vector range supply useful basic information but offer little help in characterizing organisms and are inadequate for diagnostic purposes.

More recently, the use of molecular diagnostic reagents, such as monoclonal antibodies (MAbs) and DNA probes, have been helpful in detecting MLOs (5,8,10,11,21). Moreover, DNA probes have been useful tools for differentiating MLOs (25–27). The introduction of polymerase chain reaction (PCR) (33) has increased detection sensitivity (12,38). The development of cloned DNA probes or MAbs for MLOs is not an easy task, however, requiring laborious steps in purification and large numbers of screenings.

The methodology reported here was developed to amplify minimum amounts of MLO DNA by PCR, in the presence of large amounts of host DNA. The amplified DNA was sequenced to find short, pathogen-specific DNA regions used as oligonucleotide probes. The need for large amounts of infected tissue, the tedious and often inefficient separation procedure, and the time-consuming final screening are avoided. In addition, the methodology appears promising for producing disease-specific diagnostic probes without previously transferring the etiological agent to a herbaceous host.

MATERIALS AND METHODS

Sources of isolates. DNA was isolated from healthy and apple proliferation (AP)-infected apple (*Malus domestica* Borkh.) trees; from healthy and clover phyllody (CP) naturally infected clover (*Trifolium repens* L.) plants; from healthy periwinkles (*Catharanthus roseus* (L.) G. Don); and from periwinkles infected with one of the following diseases:

- Apple proliferation, Italian isolate (7). Except when otherwise specified, AP-infected periwinkle refers to this isolate;
- Clover phyllody (6);
- Plum leptonecrosis isolated from peach GF305;
- Tagetes (*Tagetes patula* L.) witches' broom found in Udine, Italy;
- Wood Sorrel (*Oxalis acetosella* L.) witches' broom found in Bergamo, Italy;
- Periwinkle virescence found in Udine, Italy;
- Apple proliferation (German isolate) and aster yellows, both kindly provided by E. Seemüller (Institut für Pflanzenschutz im Obstbau, Dossenheim, Germany) (31).

All diseases were transmitted to periwinkles by dodder in the greenhouse, except periwinkle virescence, which was isolated from an unidentified vector, and were maintained in greenhouse-grown periwinkle seedlings by grafting. All diseased samples were kindly provided by L. Carraro (University of Udine, Italy).

Mycoplasma mycoides ssp. *capri* strain PG3 (number 183 of the Laboratoire de Pathologie Bovine, CNEVA, Lyon, France; kindly supplied by F. De Simone, Istituto Zooprofilattico, Brescia, Italy) and *Escherichia coli* (strain DH5α, purchased from GIBCO BRL, Gaithersburg, MD) were used as references.

Primer design for PCR. 16S rRNA sequences of organisms belonging to all known genera of the class Mollicutes were recovered from the EMBL GenBank sequence data banks and were analyzed with sequence-analysis packages (14,17). Oligonucleotide sequences common to most mycoplasmas were selected by screening the database, and those present in organisms other than myco-

plasmas were discarded. A final selection of oligonucleotide sequences was synthesized (synthesis performed by Oligogene, Medicina, Italy) and used as PCR primers. Two microliters of a 3-day growth of bacteria (*M. m. capri* and *E. coli*) or of DNA extracted and diluted, as described below for periwinkles, was added to a PCR reaction containing 1× *Taq* reaction buffer (Boehringer, Mannheim, Germany), 0.1 mM each dNTP (Boehringer), and 400 ng each of primer and water to 98.5 μl, overlaid with three drops of mineral oil. PCR reactions were heated to 95 C for 10 min; the temperature was lowered to 80 C, and 1.5 μl of 1 U/μl of *Taq* polymerase (Boehringer) was added to each tube. Amplification was carried out in the PREM III machine (LEP Scientific, Milton Keynes, England), with 30 cycles of denaturation (94 C) for 30 s, annealing for 30 s, and extension (72 C) for 30 s. The final extension step was 10 min at 72 C. Annealing temperatures tested were 37, 42, 48, 55, 57, 60, and 65 C.

The sequences of primers chosen for further use were P1, 5′-CAGCAGYCGCGGTAATACATA-3′ (direct, positions 519–539 in *E. coli*), and P4, 5′-RMCCCGAGAAGCTATTCACCG-3′ (reverse, positions 1,389–1,369 in *E. coli*) (Y = C or T; R = A or G; M = A or C). The sequence of the primers contained restriction enzyme-recognition sites (*Sac*II in P1 and *Xmn*I in P4).

DNA extraction. DNA for generating probes was extracted separately from AP- and CP-infected periwinkles after a modification of the method of Rogers and Bendich (36). In each extraction, equal amounts of healthy and MLO-infected tissues were run in parallel. Leaves and stems were collected and surface-sterilized with 1% sodium hypochlorite. Twenty grams of fresh tissues was ground in liquid nitrogen in a mortar and pestle. Hot (60 C) CTAB buffer (2% CTAB; 100 mM Tris, pH 8; 20 mM EDTA, pH 8; 1.4 M NaCl; 1% PVP 40,000) was added at an equal volume to the fine powder, and the samples were incubated at 55 C for 25 min. The samples were extracted twice with chloroform and isoamyl alcohol (24:1, v/v); after the first extraction, one-tenth volume of a CTAB solution (0.7 M NaCl; 10% CTAB) was added to the aqueous phase. Nucleic acids were precipitated by adding two-thirds volume of cold isopropanol to the aqueous phase. The pellet was suspended in high-salt TE (10 mM Tris, pH 8; 1 mM EDTA, pH 8; 1 M NaCl) buffer. Nucleic acids were recovered by precipitation with 95% ethanol, followed by washing with 70% ethanol, and were dissolved in 1 ml of TE (10 mM Tris, pH 8; 1 mM EDTA, pH 8) buffer. Nucleic acid concentration was estimated by measuring absorbance at 255 nm: All preparations contained between 1 and 1.5 μg/μl of nucleic acids. Nucleic acid solutions were repeatedly diluted 10-fold, and 2 μl of each dilution was added to the PCR reactions.

Amplification, cloning, and sequencing of 16S rDNA. The serial dilution of the DNA extracted from CP-infected periwinkle was amplified, at a 57 C annealing temperature. Electrophoresis of 5 μl of the final solution was carried out on a 1.5% agarose gel in TAE buffer (40 mM Tris acetate; 1 mM EDTA) in the presence of ethidium bromide (0.5 μg/ml). The highest dilution that gave a plateau amplification was chosen, and the remaining 95 μl was run on a preparative, low-melting point, 1.5% agarose gel. The fluorescent 865-bp band was excised from the gel and purified with the Geneclean II kit (BIO 101, La Jolla, CA). The DNA fragment was digested with *Sac*II (Promega, Madison, WI) and *Xmn*I (Stratagene, La Jolla, CA), in accordance with the recommendations of the manufacturers. The resulting fragment was purified again by electrophoresis and ligated into the *Sma*I-*Sac*II sites of the vector pBluescript II (Stratagene). Transformation of competent *E. coli* DH5α cells, recombinant clones recovery, and further DNA manipulations were carried out according to standard procedures (3). The DNA fragment was subcloned using its two *Hin*fIII sites. Double-strand sequencing was carried out using the dideoxy method (37) on an automatic sequencer at CIB (Consorzio Interuniversitario Biotecnologie) laboratories (Trieste, Italy).

DNA extracted from AP-infected periwinkles was processed by the same method, except that amplification was carried out at a 42 C annealing temperature, and the subcloning was done on the two *Taq*I sites of the resulting DNA fragment.

Disease-specific oligonucleotide probes. Two oligonucleotide probes (24-mer) specific for AP and one specific for CP were chosen based on comparison to the EMBL GenBank sequence databases. Probe sequences AP1 5′-TAAGCGTTGAACTTATAC CATAGA-3′, AP2 5′-CTAAAACTCACGCTTCAGCTACTC-3′, and CP1 5′-TAACGACTGAAATTAAACTATAGA-3′ were used. Probes were labeled with digoxigenin using the DIG oligonucleotide 3′-end labeling kit (Boehringer), according to the manufacturer's guidelines.

Diagnostic assay. For dot blot hybridizations, small-scale extractions of DNA were made from 0.3 g of fresh leaves and stems of all healthy and diseased plants, following the protocol of Doyle and Doyle (15).

Nucleic acids from the small-scale extractions were dissolved in 500 μl of TE. Twenty microliters was run on an 0.7% agarose gel to check DNA amounts and quality; the results were comparable among the different samples. Two microliters was used in a PCR reaction, as described, at an annealing temperature of 42 C. After cycling, 4 μl was added to 46 μl of 2× SSC (1× SSC is 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0), and the resulting solution was vacuum filtered on nylon membranes (Boehringer) with a Milliblot 96-well dot-blot manifold (Millipore, Bedford, MA). When dried, the membrane was placed sequentially on 3MM paper sheets (Whatman, Maidstone, England) saturated with 1.5 M NaCl and 0.5 M NaOH (for 5 min), with 0.5 M Tris, pH 7.4 (for 30 s), and with 0.5 M Tris, pH 7.4, and 1.5 M NaCl (for 5 min). It was dried again and baked in an oven for 30 min at 120 C.

Prehybridization was carried out in 5× SSC, 0.1% N-lauroylsarcosine, 0.02% SDS (sodium dodecyl phosphate), and 1% blocking reagent (Boehringer) at 42 C. After 1 h, the prehybridization solution was discarded and replaced by 1 ml of a solution with the same composition, containing about 20 pmol of labeled oligonucleotide probe. The hybridization was allowed to proceed for 4 h at 42 C. Washings were 2 × 5 min in 2× SSC and 0.1% SDS, followed by 2 × 5 min in 0.1× SSC and 0.1% SDS. The temperature of the stringency washings was 42 C for AP1, 46 C for AP2, and 38 C for CP1. The enzymatic detection of the digoxigenin-labeled probe on the membrane was carried out according to the manufacturer's guidelines, with both chemiluminescent and colorimetric substrates (Boehringer).

RESULTS

Selection of primers for amplifying 16S rDNA. Primers P1 and P4 were used in PCR for amplifying, at a variety of annealing temperatures, DNA from 2 μl of liquid growth of *M. m. capri* and *E. coli* and from 2 μl of the 1 ml of DNA solution obtained from the extraction of 20 g of tissue from healthy and CP- and AP-infected periwinkles. A DNA fragment of approximately 865 bp was amplified from *M. m. capri* and from CP-infected periwinkle DNA when the PCR annealing temperature was 57 C (data not shown). The size of the amplified DNA fragment was consistent with the distance between the beginning of P1 and the end of P4 in the sequence of the evening primrose MLO rRNA (863 bp) (29). When the PCR annealing temperature was lowered to 42 C, a similar fragment was amplified from *E. coli* and from AP-infected periwinkle DNA, as shown in Figure 1. The 865-bp fragment was not amplified when PCR was performed on DNA extracted from healthy plant DNA at annealing temperatures from 37 to 60 C.

DNA preparations obtained from healthy and AP- and CP-infected periwinkles also were diluted prior to PCR. Plateau amplifications of the 865-bp DNA fragment were achieved up to 10^{-3} dilutions for CP-infected (annealing at 57 C) and AP-infected (annealing at 42 C) periwinkles. The 10^{-3} dilution was chosen for cloning in both cases.

At the PCR annealing temperature of 42 C, a fragment, approximately 865-bp, also was amplified from small-scale DNA extrac-

tions from periwinkles infected by other MLOs (Fig. 2).

In many amplifications carried out at an annealing temperature of 42 C, an additional amplified DNA fragment of approximately 225 bp was observed. When a second PCR was carried out under the same conditions, with the gel-purified 865 bp as a template, this additional product was no longer amplified.

Design of oligonucleotide probes. The 865-bp fragments amplified from DNA extracted from AP- and CP-infected periwinkles were cloned and sequenced. At least two regions of each sequence (starting approximately at positions 70 and 770 from the 5′ end, respectively) were specific for the particular organism when compared to other sequences in the databases. These regions correspond to variable regions named V7 and V9 (beginning in *E. coli* at positions 589 and 1,239, respectively) by Gray et al (18). Within these regions, three 24-mer oligonucleotides were chosen (Fig. 3) to be used as pathogen-specific probes. AP1, AP2, and CP1 were synthesized, labeled, and used in hybridizations.

Because two regions revealing high variability were close to the ends of the fragment, it was not necessary to subclone before sequencing. Subcloning was performed to obtain double reading of the sequence and to achieve phylogenetic information on the microorganisms examined.

Analysis of the AP-MLO 16S rDNA sequence showed the presence of a potential target site for the annealing of primer P1, 215 nt from the 3′ end. In the absence of a perfectly matching target, nonstringent conditions during annealing in the first PCR cycles may have caused the amplification of the additional product, as seen in Figure 2.

Validation of probes. Results of the hybridizations of probes AP1, AP2, and CP1 against the amplified DNA from various sources are shown in Figures 4 and 5. All samples were amplified at a 42 C annealing temperature, with the exception of the positive control for CP, which was amplified at a 57 C annealing temperature. The AP1 and AP2 probes hybridized with DNA amplified from the AP-infected periwinkles (both the Italian and German isolates), the AP-infected apple, and the plum leptonecrosis-infected periwinkle. CP1 hybridized only with the DNA amplified from CP-infected periwinkle and clover.

DISCUSSION

The conventional approach to the production of molecular diagnostic reagents for detecting MLOs in infected tissues requires collecting large amounts of infected tissue (often with the previous transfer of MLOs to a suitable herbaceous host plant from which they can be obtained in higher titer), processing infected tissue for recovering cells or their DNA, and separating and purifying antigens or DNA to be used in immunization or cloning. Antibodies or cloned DNA probes are produced and screened for specificity. Although the basic techniques have been improved by enrichment methods for MLO cells or DNA (8,21,23,27), they are still inefficient and time-consuming and require a herbaceous host and/or large amounts of infected tissue.

The detection of MLOs by nucleic acid hybridization was accomplished mainly with cloned probes complementary to genomic or extrachromosomal sequences. Because of the limited detection of this technique, several workers have recently introduced PCR into their diagnostic procedures, using primers derived from previously cloned DNA fragments (12,38).

The detection of MLOs by rRNA-derived probes has already been attempted. Nur et al (35) showed that cloned rRNA genes from animal mycoplasmas and plant spiroplasmas hybridized with chloroplast rRNA genes and were unsuitable as diagnostic probes in dot blot hybridization assays. Recently, the use of PCR has allowed the selective amplification of rDNA of different MLOs (1,2,13,28,39) and their differentiation by RFLP (restriction fragment length polymorphism) analysis (1,2,28,39). A small number of MLO groups sharing similar patterns was identified. The members of each group were not differentiated, however, and pathogen-specific probes were not devised. Although rRNAs of all microorganisms share most of their sequences, it has been shown that short rRNA sequences are characterized by high variability, and oligonucleotide probes derived from those regions may act as probes for identifying specific microorganisms; the possibility of designing specific probes for many bacterial pathogens, including mycoplasmas, has been demonstrated (4,16). The direct probing of ribosomal RNA, which is present in a large number of copies in every cell, may provide sensitive detection of microorganisms. The potential of oligonucleotide probes for sensitive detection of MLOs was the subject of a preliminary report by Kirkpatrick and Fraser (19).

Our results show that pathogen-specific sequences actually exist in MLO rRNA genes. Moreover, broad, group-specific PCR primers may be designed that do not amplify periwinkle DNA but are suitable for amplifying all tested MLOs. It is possible, therefore, to selectively amplify MLO rDNA and to select from the sequence of the amplified fragment suitable oligonucleotide probes potentially valuable for diagnosing specific diseases.

To demonstrate the general efficiency of the method, two MLO-caused diseases with different characteristics were chosen. Clover phyllody affects herbaceous plants; its agent is a "virescence" MLO (category 1 of Chiykowski and Sinha [9]); it induces virescence

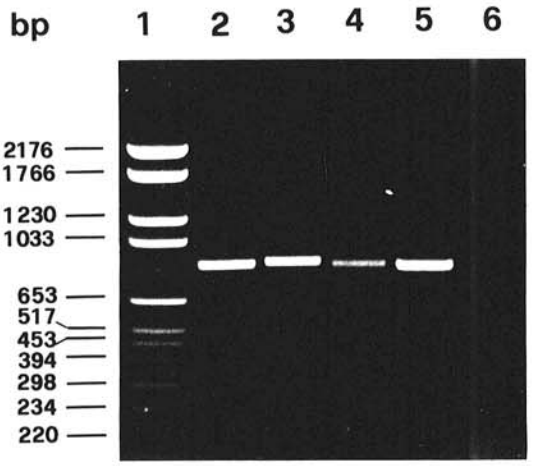

Fig. 1. Agarose gel electrophoresis of PCR products, amplified at an annealing temperature of 42 C, from DNA extracted from a variety of sources. Lane 1, molecular marker VI (Boehringer); lane 2, *M. m.. capri*; lane 3, *E. coli*; lane 4, clover phyllody-infected periwinkle; lane 5, apple proliferation-infected periwinkle; and lane 6, healthy periwinkle. (Lanes numbered from left to right.)

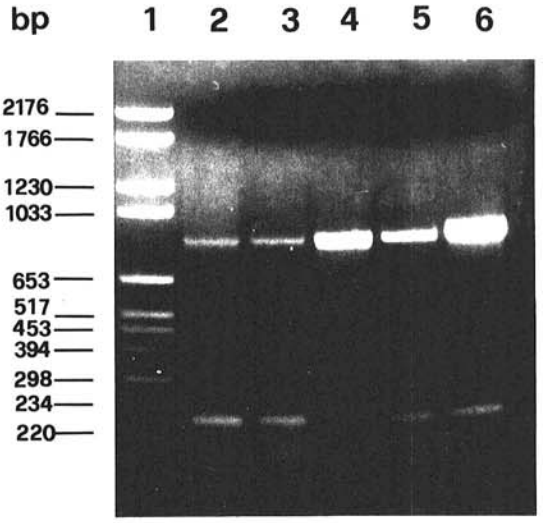

Fig. 2. Agarose gel electrophoresis of PCR products, amplified at an annealing temperature of 42 C, from small-scale DNA extractions from periwinkles infected with lane 2, aster yellows; lane 3, tagetes witches' broom; lane 4, wood sorrel witches' broom; lane 5, plum leptonecrosis; and lane 6, periwinkle virescence. Lane 1, Molecular Marker VI (Boehringer). (Lanes numbered from left to right.)

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REGION V7
Acholeplasma modicum   M23933   AGTTTGGGGTTTAAACACAGT.GCTCAACGC..TGTGGCGCTTCAAAA ACTGATTTAC
Evening primerose MLO  M30790   AGTTTATGGTCTAAGTGCAAT.GCTCAACAT..TGTGATGCTATAAAA ACTGTTTAGC
Apple prolif. MLO              AGTCTATGGTATAAGTTCAAC.GCTTAACG.T.TGTGATGCTATGGAA ACTGTTTGAC
Clover phyllody MLO            AGTCTATAGTTTAATTTCAGT.GCTTAACG.C.TGTTGTGCTATAGAA ACTGTTTTAC
Mycoplasma pirum       M23940   AGTCTGGTGTTAAAAACAACT.GCTTAACGGT.TGT ATGCATTGGAA ACTTCTAGTC
M. capricolum          X00921   AGTTTGAGGTT.AAAGTCCGGAGCTCAACTCC.GGTTC.GCCTTGAAG ACTGTTTTAC
Achol. entomophilum    M23931   AGTTTGAGGTT.AAAGNCCGGAGCTCAACTCC.NGTTC.GCCTTGAAA ACTGCGGGAC
Spiroplasma citri      M23942   AGTTTG.GGGTCAAATCCTGGAGCTCAACTCCAGGTTC.GCCTTGAAA ACTGTTAAGC
Clostridium innocuum   M23732   AGTCTG.TAGTAAAAGGCAATGGCTCAAC..CATTGTAAGCTATGGAA ACTGGTATGC

REGION V9
Acholeplasma modicum   M23933   CTGGAACAAAAGGAAGCGAAGGAGCGA.TCTGGAGCAAAACCT.CTAAAAA
Evening primerose MLO  M30790   CTGTTACAAAGGGTAGCTGAAGCGCAAGTTTTTGGCGAAT.CT.C.AAAAA
Apple prolif. MLO              CTGTTACAAAGAGTAGCTGAAGCGTGAGTTTTAG..CAAATCT.C.AAAAA
Clover phyllody MLO            TTGATACAAAGAGTAGCTGAAACGCGAGTTCTTAGCCAAT.CT.C.ACAAA
Mycoplasma pirum       M23940   CCATTACAAACAGTTGCGAATCCGCAAG.GTGGAGCTAAT.CTGC.AAAGA
M. capricolum          X00921   CTGGTACAAAGAGTTGCAATCCTGTGAA.GGGGAGCTAAT.CT.C.AAAAA
Achol. entomophilum    M23931   CCGATACAAAGAGTCGCAATCTCGCNNG.GGGGAGCTAAT.CT.C.AAAAA
Spiroplasma citri      M23942   TCGGTACAAACAGTTGCGATCTCGTAAG.AGGGAGCTAAT.CTG..AAAAA
Clostridium innocuum   M23732   CGACCACAAAGAGCAGCGACTTGGTGAC.AAGAAGCGAAT.CTCATAAAGA
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Fig. 3. Alignment of 16S rDNA variable regions V7 and V9 in members of the class Mollicutes and reference organisms. Sequences complementary to the oligonucleotide probes tested in this work are underlined (from the top, AP1, CP1, and AP2).

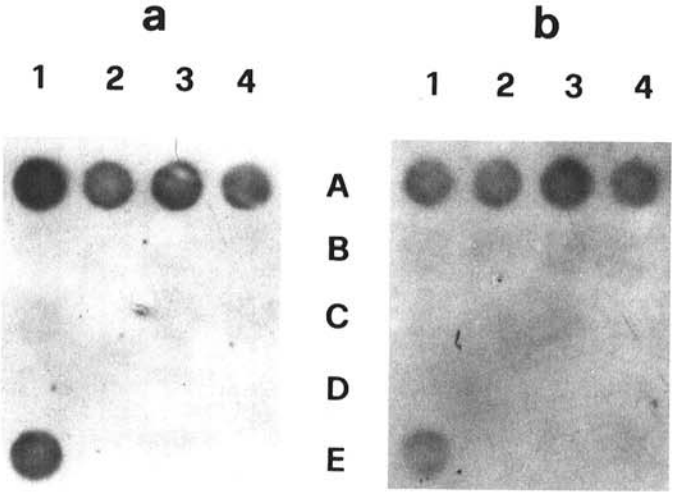

Fig. 4. Dot blot hybridization assay of probes **A,** AP1 and **B,** AP2 against DNA from a variety of sources amplified by PCR at an annealing temperature of 42 C (57 C for the positive control for probe CP1). Row A: lane 1, positive control for probes AP1 and AP2, DNA amplified and sequenced for development of probe; lane 2, apple proliferation (AP) on periwinkle (German isolate); lane 3, AP on periwinkle (Italian isolate); and lane 4, AP on apple. Row B: lane 1, positive control for probe CP1, DNA amplified and sequenced for development of probe; lane 2, clover phyllody (CP) on periwinkle; lane 3, CP on clover; and lane 4, aster yellows on periwinkle. Row C: lane 1, periwinkle virescence on periwinkle; lane 2, *E. coli*; lane 3, *M. m. capri*; and lane 4, wood sorrel witches' broom on periwinkle . Row D: lane 1, healthy apple; lane 2, healthy clover; lane 3, healthy periwinkle; and lane 4, tagetes witches' broom on periwinkle. Row E: lane 1, plum leptonecrosis on periwinkle; and lanes 2, 3, and 4, healthy periwinkles.

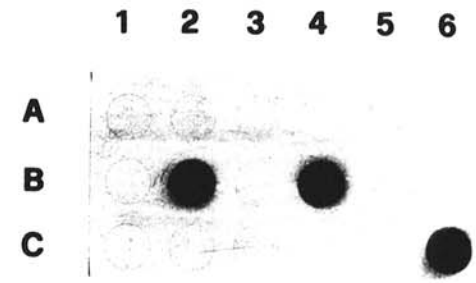

Fig. 5. Dot blot hybridization assay of probe CP1 against DNA from a variety of sources amplified by PCR at an annealing temperature of 42 C (57 C for the positive control for probe CP1, spot B1). Row A: lane 1, positive control for probes AP1 and AP2, DNA amplified and sequenced for development of probe; lane 2, apple proliferation (AP) on periwinkle (German isolate); lane 3, AP on periwinkle (Italian isolate); lane 4, AP on apple; lane 5, healthy apple; and lane 6, healthy periwinkle. Row B: lane 1, aster yellows on periwinkle; lane 2, positive control for probe CP1, DNA amplified and sequenced for development of probe; lane 3, periwinkle virescence on periwinkle; lane 4, clover phyllody (CP) on clover; lane 5, healthy clover; and lane 6, healthy periwinkle. Row C: lane 1, *E. coli*; lane 2, *M. m. capri*; lane 3, wood sorrel witches' broom on periwinkle; lane 4, tagetes witches' broom on periwinkle; lane 5, plum leptonecrosis on periwinkle; and lane 6, CP on periwinkle.

and phyllody in clover and in periwinkle (6). Numerous pleomorphic cells can be detected by electron microscopy in the phloem of CP-infected periwinkles (34). AP affects woody plants; its agent does not induce virescence or phyllody (category 2 of Chiykowski and Sinha [9]) in its natural host or when transferred to periwinkle (7). Relatively few, roundish cells can be detected by electron microscopy in the phloem of AP-infected periwinkles (34). Moreover, DNA extracted from AP-infected plants did not hybridize with cloned chromosomal DNA of severe strain of western aster yellows MLO (26) nor with its plasmids, DNA fragments shown to have sequence homologies with extrachromosomal DNA of virescence MLOs (25). Therefore, the AP MLO is not believed to be closely related to severe strain of western aster yellows MLO or other virescence-inducing MLOs (26).

Using P1 and P4 as primers, an 865-bp DNA fragment containing five variable regions, V7, V8, V3, V4, and V9 (18), was amplified by PCR from both CP and AP MLOs. However, the amplification of AP-MLO 16S rDNA was achieved only by lowering the annealing temperature to 42 C: A condition that allows the 16S rDNA of several prokaryotes to be amplified. Nevertheless, DNA dilution prior to PCR prevented the amplification of rDNA from contaminant microorganisms, because MLOs exhibit the highest prokaryote population in the artificially infected periwinkles.

Although both hybridizations against PCR amplified DNA and ribosomal RNA theoretically can be performed with the oligonucleotide probes developed as described in this paper, the use

of PCR is recommended, because of the difficulties related to handling RNA, the possible interference of the large amount of plant rRNA in the hybridization step, and the higher sensitivity achievable with PCR. A PCR-based diagnostic assay can be developed with at least two approaches. A disease-specific pair of PCR primers may be derived from the most suitable regions (V7 and V9); an internal probe may eventually be selected to increase the specificity. An alternative choice is the use of group-specific (instead of disease-specific) PCR primers in amplification, followed by hybridization with a disease-specific internal probe derived from one of the variable regions (V7 or V9). The latter alternative was investigated in this work, and the results demonstrate that this approach is effective, at least for CP- and AP-infected periwinkle, clover, and apple plants. Comparative field tests are in progress to determine the diagnostic efficiency of the two alternatives.

While this work was in progress, a number of methods for PCR-mediated amplification of MLOs' 16S rDNA were developed by Deng and Hiruki (13), Ahrens and Seemüller (1), Ahrens et al (2), Schneider et al (39), and Lee and Davis (28). The present availability of different PCR primer pairs for amplifying the 16S rRNA gene is expected to improve the reliability of the method described here by offering a way of checking amplified fragments prior to cloning. The sequencing of 16S rDNA of many MLOs for taxonomic purposes (20) also is envisaged to provide the data necessary for constructing further pathogen-specific probes.

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