

Genetic Relatedness Between Two Nonculturable Mycoplasma-like Organisms Revealed by Nucleic Acid Hybridization and Polymerase Chain Reaction

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ABSTRACT

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Dot-blot hybridization, using nine recombinant plasmids containing clover proliferation (CP) mycoplasma-like organism (MLO) DNA segments, showed that the cloned CP MLO-specific DNA probes hybridized with DNA isolated from CP MLO and potato witches'-broom (PWB) MLO-infected periwinkle plants, but not with DNA extracted from healthy periwinkle plants nor from those infected by MLOs associated with clover phyllody (CPD), hydrangea virescence (HV), eastern aster yellows (EAY), and western aster yellows (AY27). Very similar restriction fragment length polymorphism (RFLP) of CP and PWB MLO chromosomal DNA was

observed by Southern-blot hybridization using four different CP MLO DNA probes. Similar DNA fragments were amplified when DNAs from CP or PWB MLO-infected plants were used as PCR templates, whereas no DNA fragments were amplified when DNAs from healthy CPD, HV, EAY, and AY27 MLO-infected plants were used as polymerase chain reaction templates. These results suggested that CP and PWB MLO isolates are genetically related, yet distinct from CPD, HV, EAY, and AY27 MLO isolates.

Additional keywords: genomic DNA, mollicutes, nonradioactive labeling.

Although mycoplasma-like organisms (MLOs) are known to cause hundreds of diseases in a variety of trees and crops of agricultural importance worldwide (15,32,33), little progress has been made on their classification due to the lack of effective techniques to detect and compare these pathogens. The major obstacle is the inability, so far, to culture these microorganisms *in vitro*. However, some advance has been made in detecting plant pathogenic MLOs using polyclonal (36-39) and monoclonal (19,29,30) antibodies. Recently, cloned chromosomal or extra-chromosomal MLO DNA from infected insect hosts or plants has been used for the detection and study of genetic relatedness among MLO isolates (2,8-13,21,23,25-28,35). Molecular cloning of chromosomal and extrachromosomal MLO DNA and their application in the detection and comparison of MLOs at the level of genetic material opens a new approach for the classification of these nonculturable mollicutes.

In Alberta, the report of plant diseases associated with MLO dates back to the early 1960s. Clover proliferation (CP), then known as a yellows-type virus disease of alsike clover (*Trifolium hybridum* L.), was reported to be distinct from clover phyllody (CPD) and aster yellows (AY27) on the basis of symptomatology and vector-pathogen-host plant relationships (6). Soon after the initial discovery of a variety of yellows-type diseases associated with plant pathogenic MLOs (14), CP was found constantly associated with a strain of nonculturable MLO (3,17).

In this investigation, we report the use of nine cloned CP MLO DNA fragments for dot-blot hybridization analyses of MLOs associated with six different plant diseases and of four cloned CP MLO DNA fragments for restriction fragment length polymorphism (RFLP)/Southern-blot analysis of CP and potato witches'-broom (PWB) MLOs. The sequence data of one cloned CP MLO DNA fragment was used to design two polymerase chain reaction (PCR) primer pairs for amplification of DNA *in vitro*.

MATERIALS AND METHODS

Plants and MLO isolates. MLO isolates were maintained in periwinkle (*Catharanthus roseus* (L.) G. Don.) plants. Eastern aster yellows (EAY, a New York isolate) in periwinkle was kindly provided by Dr. T. A. Chen, Rutgers University, NJ. CPD MLO (40) in clover was a gift from Dr. L. N. Chiykowski, Agriculture Canada, Ottawa. CP, PWB (3,17), western aster yellows (AY27) (a subculture of AP-1, Alberta isolate), and hydrangea virescence (HV) were originally collected in Alberta (4,5,16). MLOs, except HV, were transmitted by dodder (*Cuscuta subinclusa* Dur. & Hilg.) to periwinkle seedlings. All MLO isolates in periwinkle were maintained by grafting periodically to young periwinkle seedlings, and kept in the greenhouse.

Molecular cloning of CP MLO DNA and screening of recombinants. Enzyme-treated periwinkle midribs and petioles were used to enrich CP MLO (24). Molecular cloning of CP MLO DNA and screening of CP MLO-specific recombinants were as described previously (13).

Dot-blot hybridization. Crude nucleic acids were prepared from midribs and petioles of both healthy and MLO-infected periwinkle plants (12). The nucleic acid samples, denatured at 95 C for 10 min and chilled on ice, in fourfold serial dilutions in TE, were applied onto PhotoGene nylon membrane (BRL, Gaithersburg, MD) on a dot-blot apparatus (Tyler Research Corporation, Edmonton). Nine plasmid DNAs with specific MLO DNA inserts greater than 1 kb were labeled with biotin-14-dATP in a BioNick labeling system according to the manufacturer's instruction manual (BRL). The prehybridization, hybridization with biotinylated probes, and washing of the membrane were performed as described (13). Reprobing of the membrane with another recombinant plasmid was done by boiling the membrane for 20 min in 0.1× SSC (1× SSC = 0.15 M sodium chloride and 0.015 M sodium citrate, pH 7.0), 0.1% sodium dodecyl sulfate (SDS) to remove biotinylated DNA probes. Furthermore nine selected recombinant plasmids were denatured and spotted onto a nylon membrane and probed with labeled DNA extracted from various MLO-infected plants to test the genetic relatedness among MLOs associated with six different plant diseases.

RFLP/Southern-blot hybridization. DNAs isolated from healthy and CP MLO-infected plants were subjected to 0.7% agarose gel electrophoresis using TAE (0.04 M Tris-acetate, 0.002 M EDTA) buffer and transferred to nylon membranes, followed by probing with different cloned CP MLO DNA to determine if the cloned DNA fragment is chromosomal or extrachromosomal DNA of CP MLO. DNAs (5 μ g) isolated from healthy, CP MLO- and PWB MLO-infected periwinkle plants were digested with *Eco*RI and *Hind*III restriction endonucleases as described previously (13). The digested DNAs were subjected to 0.8% agarose gel electrophoresis using TAE buffer for RFLP analysis.

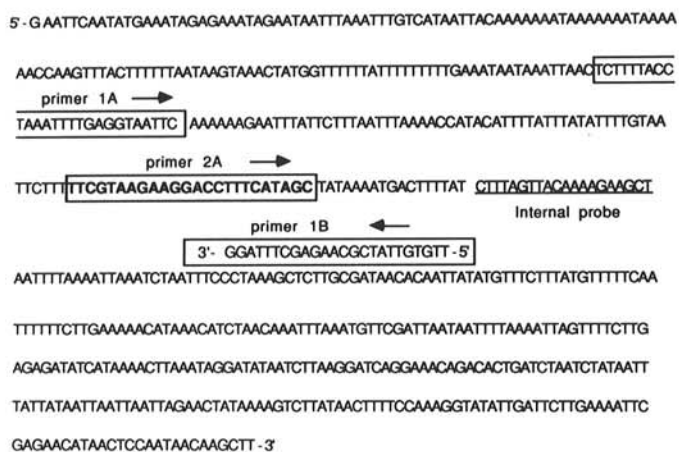


Fig. 1. Sequence of clover proliferation (CP) mycoplasmalike organism (MLO) DNA segment in pCP88, base composition 39% A (234), 41% T (246), 11% G (66), and 9% C (54). Position of polymerase chain reaction primers and internal probe are indicated.

The gel was then soaked in 0.25 N HCl for 15 min with gentle agitation to partially depurinate the DNA. The DNA was denatured and neutralized (1). The DNA was finally transferred onto a PhotoGene nylon membrane (41). The membrane was air-dried and baked at 80 C for 2 h. For the preparation of biotinylated probes containing four different recombinant plasmids and lambda DNA, prehybridization, hybridization, signal detection, and reprobing of the membrane were performed as described (13).

TABLE I. Dot-blot hybridization between nine cloned clover proliferation (CP) mycoplasmalike organism (MLO) DNA fragments and nucleic acids from healthy and various MLO-infected plants

Recombinant plasmids ^a	Insert size (kb)	Total nucleic acids ^b						
		H	CP	HV	CPD	EAY	AY27	PWB
pCP57	1.1	-	+	-	-	-	-	+
pCP32	1.3	-	+	-	-	-	-	+
pCP132	1.4	-	+	-	-	-	-	+
pCP92	1.8	-	+	-	-	-	-	+
pCP34	2.5	-	+	-	-	-	-	+
pCP16	2.9	-	+	-	-	-	-	+
pCP118	3.7	-	+	-	-	-	-	+
pCP5	4.1	-	+	-	-	-	-	+
pCP110	5.9	-	+	-	-	-	-	+

^aNucleic acids isolated from indicated sources were dot-blotted onto nylon membrane and probed against nine biotinylated cloned CP MLO DNA fragments.

^bNine cloned CP MLO DNA fragments were dot-blotted onto nylon membrane and probed against radioactive-labeled nucleic acids isolated from the source plants indicated. H = healthy periwinkle; CP = clover proliferation; HV = hydrangea virescence; CPD = clover phylloidy; EAY = eastern aster yellows; AY27 = western aster yellows; PWB = potato witches'-broom.

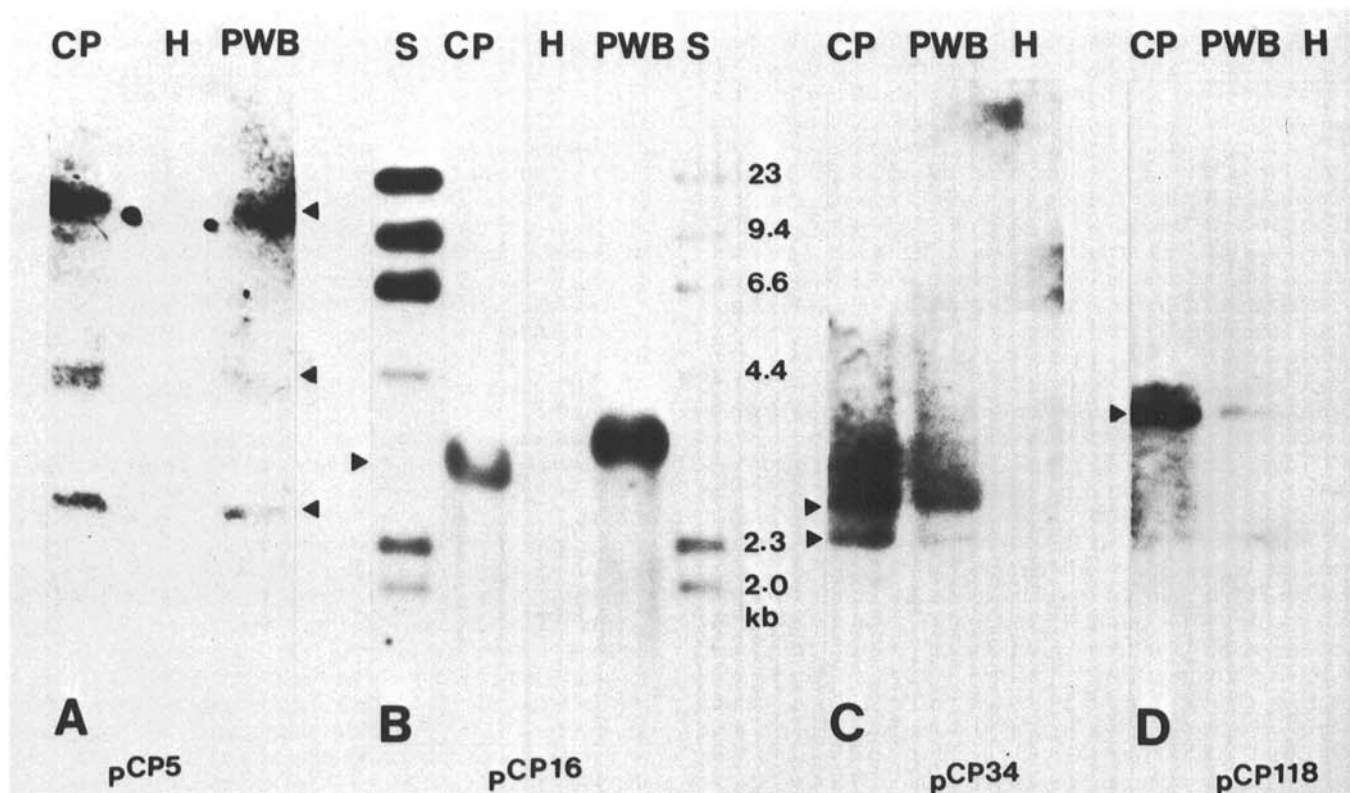


Fig. 2. Restriction fragment length polymorphism (RFLP)/Southern-blot hybridization using four biotinylated cloned clover proliferation (CP) mycoplasmalike organism (MLO) DNA fragments as probe. Nucleic acids from healthy, CP and potato witches'-broom (PWB) MLO-infected periwinkle plants were digested with *Eco*RI and *Hind*III, electrophoresed on 0.8% agarose, transferred onto PhotoGene nylon membrane, and probed with biotinylated pCP5, pCP16, pCP34, pCP118 respectively. S, lambda phage DNA digested with *Hind*III. H, nucleic acids from healthy periwinkle plants. CP, nucleic acids from CP MLO-infected periwinkle plants. PWB, nucleic acids from PWB MLO-infected periwinkle plants. Arrows indicate similar bands from both CP and PWB MLO DNAs.

DNA sequencing. CP MLO DNA of 600 base pairs (pCP88) in pUC19 was subcloned into plasmid pBluescript SK (\pm) (Stratagene, La Jolla, CA) (1). Enzymatic DNA sequencing (34) of both double- and single-stranded templates was carried out with SK and KS primers (Stratagene) and *Taq* or T7 DNA polymerases (Promega, Madison, WI) as described (11).

Polymerase chain reaction (PCR) amplification. Two PCR primer pairs were synthesized according to the sequence of the

TABLE 2. Amplification of DNA using two different primer pairs synthesized according to the sequence of pCP88 containing clover proliferation (CP) mycoplasma-like organism (MLO) DNA

PCR ^a primer pair	Size of amplified DNA (bp)	Nucleic acid templates ^b							
		pCP88	H	CP	PWB	HV	CPD	EAY	AY27
1A:1B	196	+	-	+	+	-	-	-	-
2A:1B	109	+	-	+	+	-	-	-	-

^aPolymerase chain reaction.

^bH = healthy periwinkle; CP = clover proliferation; PWB = potato witches'-broom; HV = hydrangea virescence; CPD = clover phyllody; EAY = eastern aster yellows; AY27 = western aster yellows.

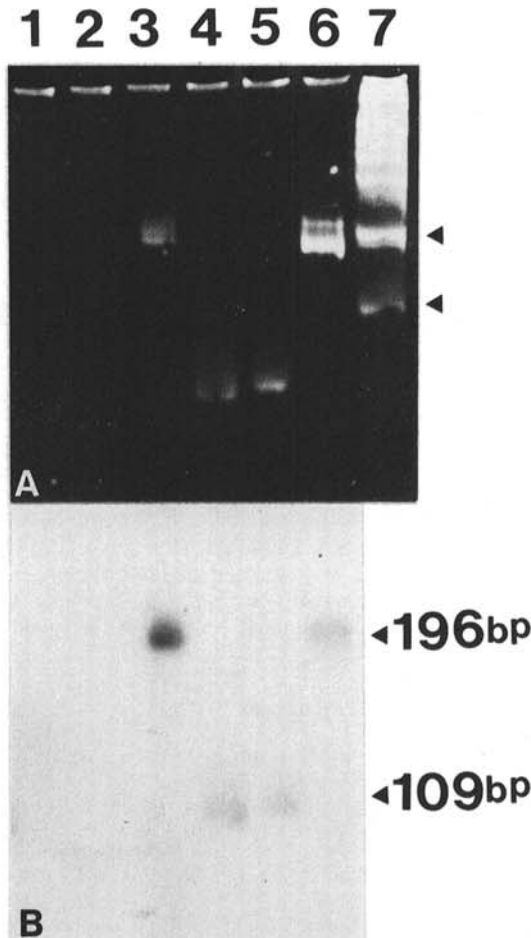


Fig. 3. Gel electrophoresis and solution hybridization of clover proliferation (CP) mycoplasma-like organism (MLO) and potato witches'-broom (PWB) MLO polymerase chain reaction (PCR) products. The amplified products were probed with labeled CP MLO-specific internal probe, fractionated in 8% acrylamide gel, stained with ethidium bromide (A), and autoradiographed (B). Nucleic acids from the following sources were used as PCR templates. Lanes 1 and 2, healthy periwinkle plants. Lanes 3 and 4, CP MLO-infected periwinkle plants. Lanes 5 and 6, PWB MLO-infected periwinkle plants. Lanes 1, 3, 6, amplifications of 196-bp DNA fragments. Lanes 2, 4, 5, amplifications of 109-bp DNA fragments. Lane 7, molecular weight standard. pBR322 digested with *Hin*I. Right-hand arrowheads indicate 154-bp and 220-bp DNA fragments in (A).

cloned CP MLO DNA (Fig. 1) (11). Nucleic acids were extracted from healthy plants and CP, PWB, CPD, HV, EAY, and AY27 MLO-infected plants (13). The nucleic acids were treated with proteinase K (31). Five microliters of 10-fold serially diluted proteinase K-treated DNA samples was used as PCR templates. The amplification was carried out in a 100 μ l of PCR reaction mixture according to the conditions described previously (11,18). PCR products were immediately placed in a -20 C freezer for 30 min. Mineral oil was removed from the top of the tube by micropipetting without chloroform extraction. One-tenth volume of PCR products was analyzed by 8% polyacrylamide gel electrophoresis to determine whether any DNA fragment was amplified.

Solution hybridization of PCR products was carried out using a deoxyoligonucleotide internal probe to distinguish specific or nonspecific amplification (11,20).

PCR-based detection of CP and PWB MLO DNA. Serial dilutions of nucleic acid samples from CP and PWB MLO-infected plants, and PCR products amplified from 10-fold serial dilutions of nucleic acid templates from CP and PWB MLO-infected plants were denatured and dot-blotted on nylon membrane. Then they were probed with a [³²P]-labeled internal probe (10^6 - 10^7 cpm/pmol). Prehybridization and hybridization conditions were the same as described previously (11).

RESULTS

Dot-blot hybridization. Differential hybridizations showed that sixteen recombinant plasmids contained CP MLO DNA segments. Southern-blot hybridizations indicated that each of the sixteen recombinant plasmids contains a chromosomal DNA fragment of CP MLO (13). From these sixteen CP MLO-specific recombinant plasmids, nine containing CP MLO-specific DNA fragments greater than 1 kb were selected for the construction of biotinylated DNA probes in this investigation. Hybridizations carried out either by the nine biotinylated recombinant plasmids or [³²P]-labeled DNA probes from various MLO-infected plants are summarized in Table 1. All the recombinant plasmids reacted with DNAs from CP or PWB MLO-infected plants but did not



Fig. 4. Polymerase chain reaction (PCR)-amplified clover proliferation (CP) mycoplasma-like organism (MLO) and potato witches'-broom (PWB) MLO DNAs analyzed by 8% acrylamide gel electrophoresis. The gel was stained with ethidium bromide and the bands were visualized with UV light. Lanes 2-4, 6×10^{-3} , 6×10^{-4} , 6×10^{-5} ng of nucleic acids from CP MLO-infected plants were used as PCR templates for the amplification of 196-bp DNA fragment (arrow). Lanes 5-7, 6×10^{-2} , 6×10^{-3} , 6×10^{-4} ng of nucleic acids from CP MLO-infected periwinkle plants were used as PCR amplification of the 109-bp CP MLO DNA (arrow). Lanes B-D, 6×10^{-3} , 6×10^{-4} , 6×10^{-5} ng of nucleic acids from PWB MLO-infected plants were used as PCR templates for the amplification of the large DNA fragment. Lanes E-G, 6×10^{-2} , 6×10^{-3} , 6×10^{-4} ng of nucleic acids from PWB MLO-infected periwinkle plants were used as PCR amplification of the smaller DNA fragment. One tenth of the PCR product was applied to each lane. Lane 1, molecular weight standards ϕ X174 RF DNA cut by *Hae*III, 72-bp and 118-bp DNA fragments were resolved on the gel. Lane A, 123-bp DNA ladder, 123-bp, 246-bp, and 369-bp DNA fragments were seen on the gel.

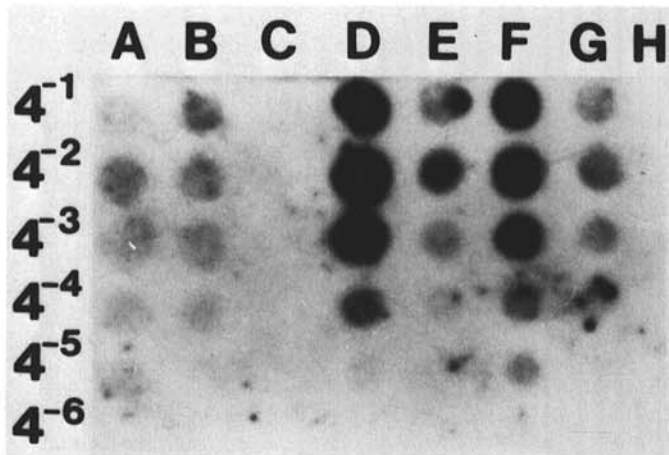


Fig. 5. Detection of clover proliferation (CP) and potato witches'-broom (PWB) mycoplasma-like organism (MLO) DNA fragments using [32 P]-end labeled CP MLO-specific internal probe. One tenth of each polymerase chain reaction (PCR)-amplified sample after fourfold serial dilution was denatured and spotted onto nylon membrane. A-C, 600 ng of nucleic acids from CP, PWB MLO-infected, and healthy periwinkle plants, respectively, were subjected to fourfold serial dilutions and dot-blotted to nylon membrane. A minimum of 2.5 ng of nucleic acid was needed to detect CP and PWB MLOs. D, PCR product of 196-bp DNA fragment with 6×10^{-5} ng of nucleic acid (containing CP MLO DNA) as template (from Lane 4 in Fig. 4). E, PCR product of 109-bp DNA fragment with 6×10^{-2} ng of nucleic acid (containing CP MLO DNA) as template (from Lane 5 in Fig. 4). F, PCR product of larger DNA fragment with 6×10^{-5} ng of nucleic acid (containing PWB MLO DNA) as template (from Lane D in Fig. 4). G, PCR product of small DNA fragment with 6×10^{-2} ng of nucleic acid (containing PWB MLO DNA) as template (from Lane E in Fig. 4). H, combination of one tenth each of PCR mixture for the amplification of 196-bp and 109-bp DNA fragments with 6×10^{-2} ng of nucleic acid from healthy periwinkle as PCR template.

react with DNAs from healthy plants or plants infected by the four other MLOs. The labeled DNAs from CP and PWB MLO-infected plants reacted with all the recombinant plasmids. None of the labeled DNAs from plants infected by the four other MLOs reacted with recombinant plasmids (Table 1).

RFLP/Southern-blot hybridization. From the nine recombinant plasmids used for dot-blot hybridization (Table 1), four with DNA inserts greater than 2 kb were selected for RFLP/Southern-blot studies of CP and PWB MLOs. Very similar RFLP patterns were obtained for CP and PWB MLOs when four different recombinant probes were used (Fig. 2).

PCR amplification of CP MLO and PWB MLO DNA fragments. The cloned CP MLO DNA fragment has an A + T content of 80% (Fig. 1), which agrees with a previous report (22). One 196-bp and one 109-bp DNA fragment were amplified from nucleic acids extracted from both CP and PWB MLO-infected plants. No DNA fragments were amplified from nucleic acids isolated from healthy periwinkle plants or CPD, HV, EAY, AY27 MLO-infected plants (Table 2). The amplified fragments hybridized to a CP MLO-specific internal probe, indicating specific amplification of MLO DNA from nucleic acids isolated from CP and PWB MLO-infected plants (Fig. 3). A 196-bp DNA fragment was observed on the gel that corresponded to 6×10^{-5} ng of template for PCR of not only CP but also PWB MLOs (Fig. 4, diluted 10^2 -fold). One-tenth volume of the PCR products for both CP and PWB MLOs was subjected to fourfold dilutions and positive hybridization signals were observed even after 4^4 -fold dilutions (Fig. 5). A 109-bp DNA fragment was observed on the acrylamide gel that corresponded to 6×10^{-2} ng of template (Fig. 4, diluted 10^2 -fold) for both CP and PWB. One-tenth volume of the PCR products for CP and PWB MLO were also subjected to fourfold serial dilutions, and positive signals were observed after 4^4 -fold dilutions (Fig. 5). When no PCR was applied, a minimum of 2.5 ng of nucleic acid samples from both CP and

PWB MLO-infected plants was needed to detect MLOs using a CP MLO-specific internal probe. Nucleic acids from healthy periwinkle, whether subjected to PCR or not, did not show hybridization signals (Fig. 5).

DISCUSSION

In the early 1960s, the pathogen of CP was considered to be distinct from those of CPD and AY27 on the basis of its characteristic flower proliferation, adventitious growth, and general growth habit of infected plants, together with the long incubation period and low acquisition efficiency of the vector (6,7). Since then, the inability to culture these MLOs in cell-free medium and their restrictive presence in extremely low concentrations in the phloem tissue have hindered research on the detection and characterization of these microorganisms. However, recombinant DNA technology has opened a new approach for more explicit studies of these prokaryotes by providing novel techniques for the detection and characterization of MLO in plants and insects (2,8-13,21,23,25-28,35).

The fact that the nine labeled probes did not hybridize with DNAs associated with CPD, EAY, and AY27 suggested that CP MLO is genetically distinct from CPD and AY MLOs, which confirms the earlier claim that CP MLO is distinct from AY MLO and CPD MLO (Table 1), although CP, CPD, and AY are transmitted by the same vector, *Macrostes fascifrons* (6,7,40). It is interesting to note that genetically different MLOs are transmitted by the same vector. In our investigation, periwinkle plants infected by PWB MLO show a greening symptom similar to that caused by CP MLO at the late stage of infection, suggesting the possibility that, in nature, PWB MLO was originally transmitted from clover associated with proliferation disease to potato. These MLO agents, however, caused seemingly different types of diseases in different natural hosts in Alberta. While RFLP/Southern-blot hybridizations gave rise to very similar RFLP patterns of CP MLO and PWB MLO DNAs (Fig. 2), this result further suggests that CP MLO and PWB MLO are genetically closely related. Similar detection sensitivities were obtained with both CP and PWB MLOs using PCR and a deoxyoligonucleotide internal probe (Fig. 5). In addition, that similar DNA fragments were amplified by PCR from both CP MLO and PWB MLO-infected plants (Figs. 3,4) further suggests that CP and PWB MLOs are genetically related because PCR primer pairs were synthesized according to the sequence of CP MLO DNA (Fig. 1). Otherwise, no DNA fragments or differently sized DNA fragments would have been amplified when nucleic acids from PWB MLO-infected plants were used as PCR templates. Thus, the results of this investigation strongly indicate that CP and PWB MLOs are genetically related, and are distinct from CPD, HV, EAY, and AY27 MLOs.

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