MOLECULAR PLANT-MICROBE INTERACTIONS
Vol. 3, No. 6, pp. 438-443, 1990

Detection and Differentiation of the Mycoplasmalike Organism Associated with Apple Proliferation Disease Using Cloned DNA Probes

F. Bonnet,[1] C. Saillard,[1] A. Kollar,[2] E. Seemüller,[2] and J. M. Bové[1]

[1]Laboratoire de Biologie Cellulaire et Moléculaire, Institut National de la Recherche Agronomique and Université de Bordeaux II, Domaine de la Grande Ferrade, 33883 Villenave d'Ornon Cedex, France. [2]Biologische Bundesanstalt, Institut für Pflanzenschutz im Obstbau, D-6915 Dossenheim, Federal Republic of Germany.
Received 22 June 1990. Accepted 3 August 1990.

An enriched preparation of chromosomal DNA of the mycoplasmalike organism (MLO) associated with apple proliferation (AP) disease was obtained by CsCl buoyant density gradient centrifugation of the total DNA extracted from periwinkle plants infected with AP. The MLO-enriched DNA was digested with *Hin*dIII or *Eco*RI restriction enzymes. The fragments were ligated into plasmids pBR322 or pBR329 and amplified in *Escherichia coli*. Nineteen recombinant plasmids, each containing a different AP-MLO DNA fragment, were obtained. Four of these fragments were radioactively labeled and evaluated as probes for the detection of the AP-MLO in plant material. All four probes hybridized with DNA extracted from apple trees or periwinkle plants infected with the AP-MLO but not with DNA from correspondingly healthy plant material. Also, no hybridization was observed with DNA from plants infected by MLOs associated with 18 different plant diseases, with DNAs of two other mollicutes, including *Spiroplasma citri*, and with the extract from apple trees infected by rubbery wood disease. The probes detected the DNA of the AP-MLO in 7–15 ng of DNA from periwinkle infected with AP and in 15–30 ng of DNA from tissue of apple infected with AP. All five symptomatic apple trees examined and one of three asymptomatic apple trees gave a positive hybridization reaction with the probes. Southern hybridization of one of the probes with *Hin*dIII-restricted DNA from two different geographic and plant host isolates of the AP-MLO (grown in either periwinkle plants or apple trees) revealed the presence of a restriction fragment length polymorphism that indicates the occurrence of genetically different strains.

Additional keywords: apple proliferation mycoplasmalike organism, cloning mycoplasmalike organism DNA, detection of mycoplasmalike organisms.

Apple proliferation (AP), a serious disease of apple trees in southern regions of Europe, is associated with a mycoplasmalike organism (MLO). All attempts to culture the AP-MLO in cellfree media, as with all other plant MLOs, have failed. For that reason, the detection of MLOs in plants has been performed by electron and fluorescence microscopy. These techniques are nonspecific and do not differentiate the organisms associated with the various MLO diseases of plants. Differentiation and characterization have been based on symptomatology, host range, and specificity of pathogen transmission by insect vectors. Light microscopic methods usually are not sufficiently sensitive for routine diagnosis, especially for MLOs associated with woody plants.

The development of specific and more sensitive detection procedures, such as serological techniques and nucleic acid hybridization, have been delayed by difficulties in obtaining the antigens and DNA of the nonculturable MLO pathogens. Several approaches have recently been used for isolating MLO-enriched DNA from infected plants and leafhopper vectors. Lee and Davis (1988) and Sears *et al.* (1989) enriched the MLOs prior to DNA extraction; Kollar *et al.* (1990) isolated total DNA from infected plants and separated the MLO DNA from host plant DNA by bisbenzimide-CsCl buoyant density gradient centrifugation. Restriction fragments of MLO-enriched DNA have been cloned; MLO-specific sequences have been selected for use in detecting and differentiating MLO DNA in plant extracts and/or RNA probes have been developed from chromosomal DNA of the western X MLO (Kirkpatrick *et al.* 1987; Lee *et al.* 1988), tomato big bud MLO (Davis *et al.* 1988), and elm yellows MLO (Davis *et al.* 1988). Probes have also been made from chromosomal and/or extrachromosomal DNA of the aster yellows MLO (Lee and Davis 1988; Kuske and Kirkpatrick 1990), maize bushy stunt MLO (Davis *et al.* 1987), periwinkle little leaf MLO (Bertaccini *et al.* 1990), and the MLO causing phyllody and virescence in *Oenothera hookeri* T. & G. (Sears *et al.* 1989).

In this study, we report the cloning of AP-MLO DNA obtained by bisbenzimide-CsCl buoyant density gradient centrifugation. The bisbenzimide fluorescence stain, Hoechst 32258, binds preferentially to the adenine-thymine-rich DNA, characteristic of MLOs, thereby lowering the DNA buoyant density. After centrifugation, the MLO DNA appears as a well-separated band in the gradient (Kollar *et al.* 1990). Cloned DNA fragments of AP-MLO DNA were used to detect and differentiate various isolates of AP-MLOs.

MATERIALS AND METHODS

Isolates of Mollicutes. The AP-MLOs in this study were obtained from different sources: one was transmitted (Marwitz *et al.* 1974) by dodder from an apple tree in Heidelberg, Germany, to periwinkle (*Catharanthus roseus*

L.) and perpetuated in greenhouse-grown periwinkles by grafting. The others were from naturally infected or graft-inoculated apple trees grown on rootstock MM 106 in experimental orchards at Bordeaux.

Periwinkle-maintained MLOs associated with the following diseases were included in this study: tomato stolbur, clover phyllody, and cabbage chloranty, all collected by J. Gianotti (INRA, St. Christol les Alès, France) in southern France; gladiolus phyllody, obtained from G. T. N. De Leeuw (Phytopathologisch Laboratorium, Baarn, The Netherlands); the New Jersey strain of eastern aster yellows, provided by R. F. Whitcomb (USDA, Beltsville, MD); witches'-broom disease of lime trees from Oman (Bové *et al.* 1988); periwinkle virescence, collected by W. Heintz (Biologische Bundesanstalt, Dossenheim) at Dossenheim; periwinkle virescence, collected by C. E. Friboury in Lima, Peru; stolbur of red pepper, collected by D. Sutic (University of Zagreb, Yugoslavia) in Yugoslavia; safflower phyllody from Israel, originally provided by M. Klein (Volcani Center, Bet Dagan, Israel); yellowing of *Plantago coronopus* L. and virescence of primrose, collected by M. Marwitz (Biologische Bundesanstalt, Berlin) in Berlin; European aster yellows from Germany (Marwitz and Petzold 1972); virescence of *Diplotaxis erucoides* (L.) DC, collected in Spain (Moreno *et al.* 1985); and sandal spike from India (Dijkstra and Lee 1972).

Other nonculturable pathogens used in hybridization studies were the *Cuscuta odorata* latent MLO maintained in periwinkle (Heintz 1989) and the apple rubbery wood agent maintained in the apple cultivar Lord Lambourne. The latter disease has been reported to be induced by an MLO (Beakbane *et al.* 1971). DNAs from maize plants infected with the maize bushy stunt MLO, provided by M. J. Davis (University of Florida, Fort Lauderdale), and from periwinkle infected with the dwarf strain of western aster yellows, provided by B. C. Kirkpatrick (University of California, Davis), were also tested in these hybridization studies.

In addition, *Spiroplasma citri* (strain R8A2, ATCC 27556) and two strains of Mollicutes (F7 and F28) isolated from plant surfaces (Bonnet 1989; Saillard *et al.* 1987) were included for comparison. They were cultivated in BSR medium (Bové and Saillard 1979).

Purification of AP-MLO DNA. Extraction and purification of AP-MLO DNA were conducted as described by Kollar *et al.* (1990). Briefly, the DNA was extracted from diseased periwinkles by using the cetyltrimethylammonium bromide (CTAB) procedure (Murray and Thompson 1980). The MLO DNA was separated from host plant DNA by repeated bisbenzimide-CsCl density gradient centrifugation as described previously (Kollar *et al.* 1990).

Construction of DNA probes. Four micrograms of AP-MLO DNA was digested with 80 units of *Hin*dIII or *Eco*RI restriction enzymes (Bethesda Research Laboratories, Gaithersburg, MD) at 37° C for 5 hr. The solution was treated twice with an equal volume of phenol-chloroform-isoamyl alcohol (50:48:2) saturated with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.8) and once with an equal volume of chloroform-isoamyl alcohol (24:1). The DNA fragments were precipitated from the aqueous phase by adding one-tenth volume of 3 M sodium acetate and 2 volumes of ethanol (96%) at −70° C for 2 hr. They were recovered by centrifugation at 100,000 × *g* for 2 hr at 4° C, dried under vacuum, and dissolved in 60 μl of sterile water. One hundred nanograms of *Hin*dIII-digested DNA was ligated with 200 ng of *Hin*dIII-linearized plasmid pBR322. The same quantities were used in ligation of *Eco*RI DNA fragments to *Eco*RI-digested pBR329. Ligation (0.5 units of T4 DNA ligase, Appligene, Strasbourg, France) was conducted at room temperature for 5 hr in a final volume of 100 μl. One third of each ligation mixture was used to transform competent *Escherichia coli* cells (strain HB101) prepared by the procedure described by Hannahan (1983).

Ampicillin-resistant and tetracycline-sensitive colonies that were obtained after cloning at the *Hin*dIII site of plasmid pBR322 and ampicillin-resistant and chloramphenicol-sensitive colonies resulting from cloning at the *Eco*RI site of plasmid pBR329 were identified. The recombinant plasmids were purified according to Birnboim and Doly (1979). The *Hin*dIII- or *Eco*RI-digested plasmids were analyzed by 0.7% agarose gel electrophoresis, and plasmids containing a DNA insert of 2 kilobase pairs (kbp) or longer were selected. The bands corresponding to the insert DNA were excised, and the DNA was eluted from the agarose gel on a GeneClean column (Bio-101, La Jolla, CA) following the procedure of Vogelstein and Gillespie (1979). The eluted DNA was labeled by random priming with [α-^{32}P]dCTP (Amersham Corporation, Arlington Heights, IL), providing a specific activity of 2×10^9 cpm per microgram of insert DNA. Inserts hybridizing only with DNA from periwinkle plants infected with the AP-MLO and not with DNA from healthy plants were saved for subsequent studies.

Extraction of DNA for hybridization. Midribs, shoots, and roots of healthy and infected periwinkle plants and the bark of healthy and diseased apple shoots were used to extract large amounts of DNA following the procedure of Murray and Thompson (1980), with some modification. The plant material was frozen in liquid nitrogen, lyophilized, and stored at room temperature. Five grams of lyophilized plant tissue was ground to a fine powder in a Waring blender. The powder was then dispersed in 40 ml of extraction buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 700 mM NaCl, 1.8% CTAB, 0.1% dithiothreitol) and incubated for 30 min at room temperature or at 55° C. For incubations at room temperature, sodium dodecyl sulfate (SDS) was added to a final concentration of 1.0%, and the lysate was incubated at 55° C for an additional 30 min. The DNA was extracted three times with an equal volume of chloroform-isoamyl alcohol (24:1). After centrifugation (5,000 × *g*, 15 min), one-tenth volume of extraction buffer containing 10% CTAB was added to the aqueous phase. The DNA was precipitated at room temperature with 1 volume of precipitation buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% CTAB), and the CTAB-DNA complex was recovered by centrifugation at 4,000 × *g* for 10 min. The pellet was washed four times with 96% ethanol to remove CTAB and dried under vacuum. The dried pellet was then dissolved in 10 ml of TE buffer. In some cases, the dissolved

pellet was treated with RNase (50 μg/ml), followed by proteinase K (50 μg/ml) and SDS (0.5%) at 37° C for 1 hr. The DNA was recovered by centrifugation after three treatments with chloroform-isoamyl alcohol and precipitation with 96% ethanol at −20° C. The resulting pellet was suspended in 5 ml of sterile water and kept at −20° C. The DNA concentration was estimated spectrophotometrically at 260 nm. From 5 g of lyophilized tissue, approximately 2 mg of DNA was extracted from periwinkle plants and 1 mg of DNA from apple shoots.

For detection of the AP-MLO by dot hybridization, the extraction procedure was simplified. Two hundred fifty milligrams of fresh periwinkle or apple plant material was ground in liquid nitrogen, and the nucleic acids were extracted as described above, except for the omission of the RNase, proteinase K, and SDS treatments. The final pellet, which usually contained 3–5 μg of DNA, was dissolved in 50 μl of sterile water.

DNA from the culturable mollicutes was obtained as described by Carle *et al.* (1983).

Dot hybridization. DNA was denaturated with 50 mM methyl mercury at room temperature for 10 min. Twofold serial dilutions of DNA in sterile water were prepared. Both the undiluted and diluted denatured DNAs were dot blotted (20 μl per spot) on a nitrocellulose sheet presoaked in 5× SSC (1× SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0). The filters were air-dried and then baked at 80° C for 2 hr under vacuum. The filters were prehybridized for 2 hr at 37° C in a solution containing 50% formamide, 5× SSC, 5× Denhardt's solution (1× = 0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin), 1% SDS, and 200 μg/ml of denatured salmon sperm DNA. For hybridization, the solution was replaced by a buffer obtained by adding 10% sodium dextran sulfate and heat-denaturated ^{32}P-labeled DNA (2×10^6 cpm per milliliter) to the prehybridization buffer. After overnight hybridization at 37° C, the filters were washed three times at room temperature for 15 min with 2× SSC containing 1% SDS and twice at 60° C for 30 min with 0.1× SSC containing 1% SDS. The filters were then air-dried and exposed to X-ray film (Fuji RX) for 48 hr with an intensifying screen (Du Pont, Wilmington, DE, Chronex).

Southern blot hybridization. Two micrograms of DNA from healthy or infected plants was digested with *Hin*dIII or *Eco*RI restriction endonucleases, electrophoresed in 0.7% agarose gels, alkali-denaturated (0.2 N NaOH in 0.5 M NaCl for 45 min), and transferred to nitrocellulose filter paper according to Southern (1975). The filters were then baked at 80° C for 2 hr, prehybridized at 37° C, and hybridized with ^{32}P-labeled probes as described in the previous section. After washing, the filters were dried and exposed to X-ray film for 48 hr with an intensifying screen.

Restriction mapping of the cloned inserts. Four selected cloned inserts were excised from the pBR322 or pBR329 recombinant plasmid and digested with *Hin*dIII, *Eco*RI, *Eco*RV, *Hinc*II, *Cla*I, *Acc*I, *Pst*I, *Rsa*I, *Sph*I, *Hpa*I, *Bam*HI, *Pvu*II, or *Dde*I restriction endonucleases, used singly or in combination, according to the supplier's recommendations. The resulting fragments were analyzed by electrophoresis in 0.7% agarose gels.

4′-6 Diamidino-2-phenylindole (DAPI) test. Samples from young apple shoots collected in September were fixed in glutaraldehyde. Frozen microtome sections (20 μm) were stained with DAPI and examined for the presence of MLOs by fluorescence microscopy as described by Seemüller (1976).

RESULTS

Cloning and screening of MLO-specific fragments. The transformation of *E. coli* cells with the ligation mixtures resulted in a total of 325 ampicillin-resistant colonies. Recombinant plasmids were detected in 247 colonies, 19 with inserts of 2 kbp or longer. None of these inserts cross-hybridized with each other.

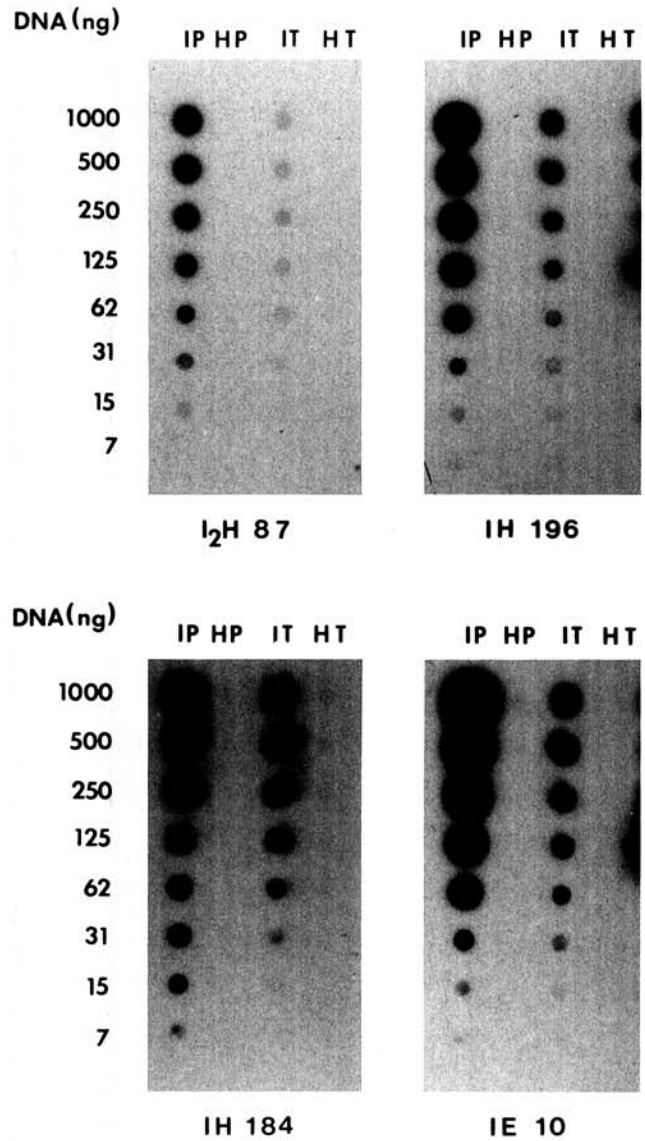

Fig. 1. Dot hybridization of the four ^{32}P-labeled DNA probes I_2H87, IH196, IH184, and IE10 with DNA from periwinkle plants infected with apple proliferation disease (IP) or apple trees infected with apple proliferation disease (IT) and with DNA from healthy periwinkle (HP) or apple (HT).

In dot hybridization, the inserts of all 19 recombinant plasmids reacted with DNA from periwinkle plants infected with the AP-MLO but not with DNA from healthy plants. The inserts of recombinant plasmids pH87 (3.9 kbp), pH184 (3.1 kbp), and pH196 (3.7 kbp), resulting from ligation of *Hin*dIII fragments in pBR322, and of recombinant plasmid pE10 (5.5 kbp), resulting from ligation of an *Eco*RI fragment in pBR329, were selected for further work. The corresponding inserts were designated I_2H87, IH184, IH196, and IE10, respectively.

Dot hybridizations between the four inserts used as probes and total DNA from healthy periwinkle plants and apple trees and periwinkle and apple infected with the AP-MLO are shown in Figure 1. None of the probes hybridized with DNA from healthy plants but all reacted with DNA from periwinkle or apple infected with the AP-MLO. The strongest signal was obtained with probe IE10 and the weakest with I_2H87. The probes detected AP-MLO DNA in as little as 7 to 15 ng of total DNA from infected periwinkle plants and in 15 to 31 ng of total DNA from infected apple trees.

The four inserts were also used in Southern blot hybridization experiments. Total DNA from healthy periwinkle plants and apple trees and periwinkle and apple infected with the AP-MLO was restricted with *Hin*dIII (Fig. 2A, B, and D) or *Eco*RI (Fig. 2C) and hybridized with one of the four probes, namely I_2H87 (Fig. 2A), IH196 (Fig. 2B), IH184 (Fig. 2D), or IE10 (Fig. 2C). Hybridization occurred only with DNA from periwinkle plants and apple trees infected with the AP-MLO. No hybridization was obtained with DNA from healthy plants. With probes I_2H87, IH196, and IE10, the length of the AP-MLO DNA fragment from both diseased periwinkle and apple that hybridized with the respective probes was identical to the length of the cloned DNA insert used as a probe (Fig. 2A, B, and C). However, with probe IH184 (Fig. 2D), the hybridization patterns obtained with DNA isolated from periwinkles and apple infected with the AP-MLO were different. With DNA from infected periwinkle, the restriction fragment detected by hybridization had a length identical to that of the probe (3.1 kbp), whereas the length of the DNA fragment from infected apple that hybridized with the probe was considerably shorter (2.2 kbp).

Homology to other mollicutes. Total DNA from periwinkle plants infected with the MLOs associated with tomato stolbur, cabbage chloranty, clover phyllody, gladiolus phyllody, and witches'-broom disease of lime trees and from an apple tree affected by rubbery wood was hybridized in dot blot experiments with the four probes. No hybridization was observed (data not shown). Also, no hybridization occurred with DNA of *S. citri* and two other strains of Mollicutes, F7 and F28 (data not shown). The DNAs from periwinkle or maize infected with the 12 remaining MLOs listed in Materials and Methods were

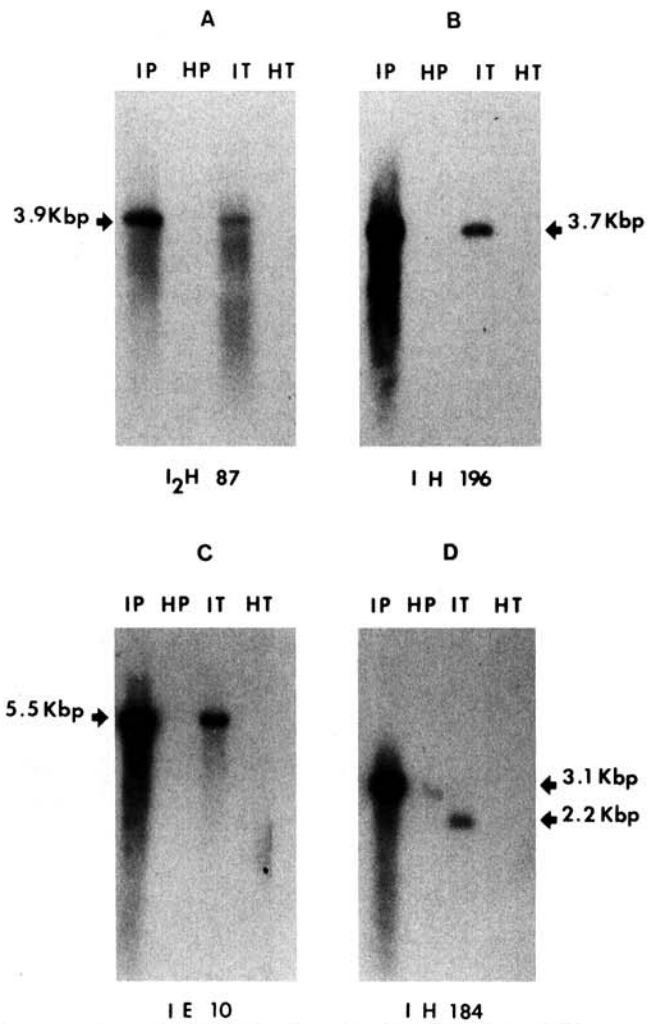

Fig. 2. Southern blot hybridization of the four ^{32}P-labeled DNA probes I_2H87, IH196, IH184, and IE10 with DNA from periwinkle plants infected with apple proliferation disease (IP) or apple trees infected with apple proliferation disease (IT) and with DNA from healthy periwinkle (HP) or apple (HT). The DNA in panels **A**, **B**, and **D** was digested with *Hin*dIII, and in **C** it was digested with *Eco*RI.

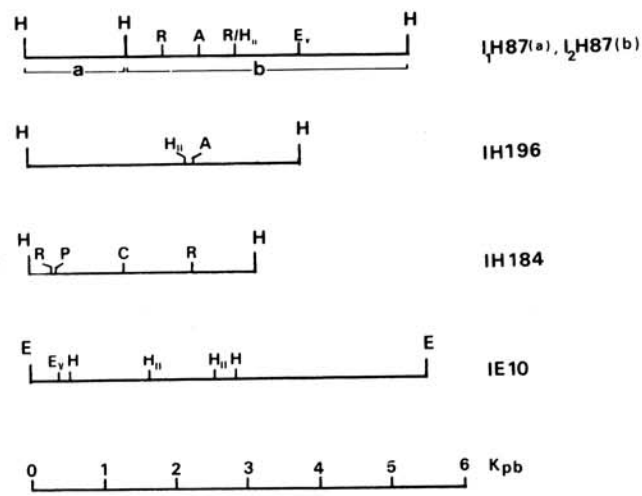

Fig. 3. Partial restriction map of the four inserts obtained by cloning *Hin*dIII restriction fragments in pBR322 (IH87, IH196, and IH184) or an *Eco*RI fragment in pBR329 (IE10). The recombinant plasmid pH87 produced two *Hin*dIII fragments, a and b: fragment a, I_1H87 (1.4 kilobase pairs [kbp]), has no restriction site for the endonucleases tested and was not used in hybridization, and fragment b, I_2H87 (3.8 kbp), contains *Acc*I, *Eco*RV, *Rsa*I, and *Hin*cII sites. A denotes *Acc*I; C, *Cla*I; E, *Eco*RI; E_v, *Eco*RV; H, *Hin*dIII; H_{II}, *Hin*cII; P, *Pst*I; and R, *Rsa*I.

hybridized in Southern blots with probe IH196. No hybridization was detected (data not shown).

Partial restriction maps of the cloned MLO DNA. The partial restriction maps of the four inserts are shown in Figure 3. Plasmid pH87 produced a 1.4-kbp *Hin*dIII fragment (I_1H87), which contained no restriction site for any of the endonucleases tested, and a 3.9-kbp *Hin*dIII fragment (I_2H87) that possessed two *Rsa*I sites and three unique sites (*Acc*I, *Hinc*II, and *Eco*RV). In insert IH196, two unique sites for *Hinc*II and *Acc*I were identified. Insert IH184 contained two *Rsa*I sites and unique sites for *Pst*I and *Cla*I. The *Eco*RI fragment IE10 had two sites for *Hin*dIII and *Hinc*II and a unique site for *Eco*RV. No cuts occurred with the other enzymes tested.

Detection of the AP-MLO in proliferation-affected trees. Probes IE10 (Fig. 4A) and IH196 (Fig. 4B) were used in dot hybridization experiments to detect DNA of AP-MLO in total DNA of naturally infected or experimentally inoculated apple trees. Strong hybridization signals were obtained with DNA from the diseased tree used as a positive control (Fig. 4, lane a). No hybridization was observed with DNA from healthy plant material (Fig. 4, lane b).

The DNA samples of the eight trees selected for testing were spotted on lane c. Trees 1, 2, 4, 5, 6, and 7 were experimentally infected with the AP-MLO by graft inoculations with infected shoots from various naturally infected apple trees. DNA samples from trees 1, 2, 4, and 7 each gave a strong hybridization signal with the two probes. Tree 5 gave a weak signal, while tree 6 failed to react. Trees 3 and 8 were both exposed to natural contamination in the field. Only tree 3 showed symptoms of AP; it was the only one of the two where hybridization with both probes, IE10 and IH196, was observed.

Table 1 presents the results of the study of the correlation between symptom expression and tests performed to confirm the presence of AP-MLOs.

Each of the trees that developed AP symptoms gave a positive hybridization signal and had a positive DAPI test (trees 1, 2, 3, 4, and 7). Symptomless tree 5 was weakly positive by hybridization but negative in the DAPI test. Symptomless trees 6 and 8 were found to be negative in both hybridization and DAPI tests.

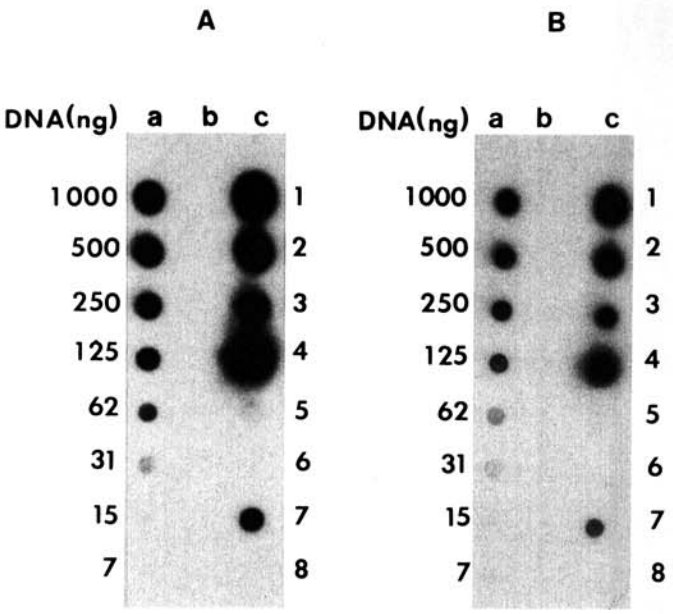

Fig. 4. Detection of the DNA from the mycoplasmalike organism associated with apple proliferation disease in total DNA of apple trees by dot hybridization with the probes IE10 (**A**) and IH196 (**B**). In lane a is DNA from a diseased apple tree spotted in a dilution series. In lane b is DNA from a healthy apple tree spotted in a dilution series. In lane c, 1, 2, 4, 5, 6, and 7 indicate DNA samples from graft-inoculated trees; 3 and 8 represent DNA samples from naturally infected apple trees.

DISCUSSION

The first attempts to detect MLOs in plants were made when heterologous rRNA gene probes from animal mycoplasmas and plant spiroplasmas became available. They proved to be unsuitable for that purpose because they hybridize with the rRNA genes of chloroplasts (Nur *et al.* 1986). Subsequently, fragments of chromosomal and extrachromosomal DNA of several MLOs were found that did not share homology with the DNA of healthy plants or spiroplasmas. The specificity of the cloned fragments for the detection of MLOs varies considerably. Probes such as those cloned from the DNA of the aster yellows MLO can recognize different MLOs (Lee and Davis 1988). Other probes, including fragments of the DNA of western X MLO and the elm yellows MLO, have a narrower detection range (Kirkpatrick *et al.* 1987; Davis *et al.* 1988).

The results we have described here indicate that probes made with the cloned DNA fragments of the AP-MLO have a narrow detection range and little affinity for the DNA of MLOs of herbaceous hosts. Probes of the AP-MLO detected several sources where the AP-MLO was the presumed pathogen, but did not hybridize with the DNA of MLOs associated with 18 different MLO diseases, most of which occur in herbaceous hosts. The 19 AP-MLO DNA probes obtained in this study did not cross-hybridize and showed no hybridization with the pMC5 probe corresponding to the rRNA gene of *Mycoplasma capricolum* (data not shown). However, an earlier study showed that the probes do share homology with the DNA of the MLO associated with apricot chlorotic leaf roll (Bonnet *et al.* 1990). This is not surprising, since Llacer *et al.* (1980)

Table 1. Correlation between symptom expression and the 4′-6 diamidino-2-phenylindole (DAPI) test and dot blot hybridizations

Tree no.	Infection[a]	Symptoms[b]	DAPI test[c]	Hybridization with probe[d]	
				IE10	IH196
1	E	++	+	+++	+++
2	E	+	+	+++	+++
3	N	+	+	++	++
4	E	+	++	+++	+++
5	E	−	−	+	+
6	E	−	−	−	−
7	E	+	+	++	++
8	N	−	−	−	−

[a] E, experimental; N, natural.
[b] No (−), weak (+), and moderate (++) symptoms.
[c] No (−), weak (+), and moderate (++) DAPI reactions.
[d] No (−), weak (+), intermediate (++), and strong (+++) dot blot hybridization signals.

provided evidence that AP and apricot chlorotic leaf roll are induced by closely related organisms.

One probe (IH184), when used in Southern hybridization, detected DNA fragments of different sizes in an isolate originally collected from an apple tree in Heidelberg and in an isolate obtained from an apple tree grown in Bordeaux. Such a restriction fragment length polymorphism could result from mutations that occurred during maintenance of the AP-MLO in the experimental host, *C. roseus*. On the other hand, the two isolates may actually be genetically different strains. Naturally occurring AP-MLO strains that differ significantly in virulence have been reported by Kunze (1976).

The probes we have developed have been shown to be suitable for sensitive detection of the AP-MLO. They detected the organisms in all five of the symptomatic trees tested and in one among the three inoculated but asymptomatic trees. This result is slightly better than that obtained with the DAPI procedure, which is considered to be a sensitive detection procedure. The failure to detect the MLOs in asymptomatic trees may be due to the MLO colonization behavior, which is closely related to symptom expression. The AP-MLO overwinters in the roots and recolonizes the stem in spring when new phloem is being formed. Recolonization is usually constant in the first few years after the disease becomes evident, and symptom expression is observed during that period. Later, the organisms often fail to reinvade the stem, or the population is reduced, or they are unevenly distributed. Under these conditions, trees do not develop symptoms (Seemüller *et al.*1984). In our study, the two symptomless trees in which MLOs were not detected may not have been colonized in the stem.

ACKNOWLEDGMENTS

We thank F. Dosba for providing proliferation-diseased apple trees, M. Lansac for assistance with the DAPI tests, and R. Marwitz for supplying most of the MLO isolates maintained in periwinkle.

LITERATURE CITED

Beakbane, A. B., Mishra, M. D., Posnette, A. F., and Slater, C. H. 1971. Mycoplasma-like organisms associated with chat fruit and rubbery wood disease of apple, *Malus domestica* Borkh., compared with those in strawberry green petal. J. Gen. Microbiol. 66:55-62.

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. Plant Dis. 74:40-43.

Birnboim, H. C., and Doly, J. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. 7:1513-1523.

Bonnet, F. 1989. Position taxonomique de divers mollicutes et étude d'organismes de ce type (MLO) par hybridation ADN-ADN. Thèse de l'Université de Bordeaux II, France, n. 71 (Science de la vie, option biologie et santé).

Bonnet, F., Saillard, C., Kollar, A., Seemüller, E., Dosba, F., and Bové, J. M. 1990. Molecular probes for the apple proliferation MLO. Pages 908-909 in: Recent Advances in Mycoplasmology. Zbl. Bakt. Suppl. 20. G. Stanek, G. M. Cassell, J. G. Tully, and R. F. Whitcomb, eds. Gustav Fischer Verlag, Stuttgart.

Bové, J. M., and Saillard, C. 1979. Cell biology of spiroplasmas. Pages 83-153 in: The Mycoplasmas: Plant and Insect Mycoplasmas. R. F. Whitcomb and J. G. Tully, eds. Academic Press, Inc., New York.

Bové, J. M., Garnier, M., Mjeni, A. M., and Khayrallah, A. 1988. Witches' broom disease of small fruited acid lime trees in Oman: First MLO disease of citrus. Pages 307-309 in: Proc. Conf. Int. Organ. Citrus Virol., 10th. L. W. Timmer, S. M. Garnsey, and L. Navarro, eds. Univ. California, Plant Pathol., Riverside.

Carle, P., Saillard, C., and Bové, J. M. 1983. DNA extraction and purification. Pages 295-300 in: Methods in Mycoplasmology, Vol. 1. S. Razin and J. G. Tully, eds. Academic Press, New York.

Davis, M. J., Tsai, J. H., Cox, R. L., McDaniel, L. L., and Harrison, N. A. 1987. DNA probes for detecting the maize-bushy-stunt mycoplasma-like organism (MBS-MLO). (Abstr.) Phytopathology 77:1769.

Davis, R. E., Lee, I.-M., Dally, E. L, Dewitt, N., and Douglas, S. M. 1988. Cloned nucleic acid hybridization probes in detection and classification of mycoplasmalike organisms (MLOs). Acta Hortic. 234:115-121.

Dijkstra, J., and Lee, P. E. 1972. Transmission by dodder of sandal spike disease and the accompanying mycoplasma-like organisms via *Vinca rosea*. Neth. J. Plant Pathol. 78:218-228.

Hannahan, D. 1983. Studies on transformations of *Escherichia coli* with plasmids. J. Mol. Biol. 166:357-580.

Heintz, W. 1989. Transmission of a new mycoplasma-like organism (MLO) from *Cuscuta odorata* (Ruiz et Pav.) to herbaceous plants and attempts to its elimination in the vector. J. Phytopathol. (Berlin) 125:171-186.

Kirkpatrick, B. C., Stenger, D. C., Morris, T. J., and Purcell, A. H. 1987. Cloning and detection of DNA from a nonculturable plant pathogenic mycoplasma-like organism. Science 238:197-200.

Kollar, A., Seemüller, E., Bonnet, F., Saillard, C., and Bové, J. M. 1990. Isolation of the DNA of various plant pathogenic mycoplasmalike organisms from infected plants. Phytopathology 80:233-237.

Kunze, L. 1976. The effect of different strains of apple proliferation on growth and crop of infected trees. Mitt. Biol. Bundesanst. Land-Forstwirtsch. Berlin-Dahlem 170:107-115.

Kuske, C. R., and Kirkpatrick, B. C. 1990. Identification and characterization of plasmids from the western aster yellows mycoplasmalike organism. J. Bacteriol. 172:1628-1633.

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.

Lee, I.-M., Davis, R. E., Hammond, R., and Kirkpatrick, B. 1988. Cloned riboprobe for detection of a mycoplasma-like organism. Biochem. Biophys. Res. Commun. 155:443-448.

Llacer, G., Sanchez-Capuchino, J. A., Casanova, R., and Forner, J. B. 1980. Graft transmission of apricot chlorotic leaf roll from an apple tree affected by proliferation. Acta Phytopathol. Acad. Sci. Hung. 15:239-240.

Marwitz, R., and Petzold, H. 1972. Nachweis von mycoplasmaahnlichen organismen in sommerastern. Jahresber. Biol. Bundesanst. (Braunschweig) 1972:41-42.

Marwitz, R., Petzold, H., and Ozel, M. 1974. Untersuchungen zur ubertragbarkeit des möglichen erregers der triebsucht des apfels auf einen krautigen wirt. Phytopathol. Z. 81:85-91.

Moreno, P., Llacer, G., and Medina, V. 1985. Descripcion y comparacion de varias micoplasmosis en *Vinca rosea* L. An. INIA Ser. Agric. N. Extr. 28:287-309.

Murray, M. G., and Thompson, W. F. 1980. Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res. 8:4321-4325.

Nur, I., Bové, J. M., Saillard, C., Rottem, S., Whitcomb, R. M., and Razin, S. 1986. DNA probes in detection of spiroplasmas and mycoplasma-like organisms in plants and insects. FEMS Microbiol. Lett. 35:157-162.

Saillard, C., Vignault, J. C., Bonnet, F., Rose, D. L., Tully, J. G., Carle, P., and Bové, J. M. 1987. Further characterization of an usual plant Mollicutes species of uncertain taxonomic status. Isr. J. Med. Sci. 23:696-697.

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.

Seemüller, E. 1976. Investigation to demonstrate mycoplasma-like organisms in diseased plants by fluorescence microscopy. Fruit tree virus diseases. Acta Hortic. (Heidelberg) 67:109-111.

Seemüller, E., Kunze, L., and Schaper, U. 1984. Colonization behavior of MLOs, and symptom expression of proliferation-diseased apple trees and decline-diseased pear trees over a period of several years. Z. Pflanzenkrankh. Pflanzenschutz 91:525-532.

Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98:503-517.

Vogelstein, B., and Gillespie, D. 1979. Preparative and analytical purification of DNA from agarose. Proc. Natl. Acad. Sci. USA 76:615-619.