Molecular Cloning and Detection of Chromosomal and Extrachromosomal DNA of the Mycoplasmalike Organism Associated with Little Leaf Disease in Periwinkle (*Catharanthus roseus*)

R. E. Davis, I.-M. Lee, S. M. Douglas, and E. L. Dally

Microbiology and Plant Pathology Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, MD 20705; and Connecticut Agricultural Experiment Station, New Haven, CT 06504.

We gratefully acknowledge the excellent technical assistance of Natalie Dewitt and thank Ernest Bantarri, Lloyd Chiykowski, James Dale, Jacqueline Fletcher, Chuji Hiruki, George N. Oldfield, Alexander Purcell, and Wayne Sinclair for kindly supplying samples of mycoplasmalike organisms used in this work.

Mention of a trade name, proprietary product, or vendor does not constitute a guarantee of the product by the USDA and does not imply its approval to the exclusion of other vendors that may also be suitable.

Accepted for publication 25 January 1990 (submitted for electronic processing).

ABSTRACT

Davis, R. E., Lee, I.-M., Douglas, S. M., and Dally, E. L. 1990. Molecular cloning and detection of chromosomal and extrachromosomal DNA of the mycoplasmalike organism associated with little leaf disease in periwinkle (*Catharanthus roseus*). Phytopathology 80:789-793.

Preparations enriched for periwinkle little leaf mycoplasmalike organism (MLO) were obtained by a method involving isolation of sieve cells from diseased plants of periwinkle (*Catharanthus roseus*). DNA was extracted from the preparations, ligated into plasmids pSP64 and pSP65, and cloned in *Escherichia coli* JM83. Selected recombinant plasmids and cloned DNA inserts were labeled with biotin and employed as probes in dot hybridizations. The probes hybridized with nucleic acid extracted from periwinkle plants infected by periwinkle little leaf, aster yellows, or tomato big bud MLOs, but not with extracts from healthy plants. Some hybridized also with nucleic acid from plants infected by other MLOs. Southern hybridization analyses revealed the occurrence of extrachromosomal DNA in periwinkle little leaf, tomato big bud, and aster yellows MLOs. These and other results indicate genetic relatedness at the level of chromosomal DNA among these MLOs, and nucleotide sequence homology among their extrachromosomal DNAs. Biotinylated cloned DNA probes were successfully applied in dot hybridizations for detection of an Oklahoma strain of aster yellows MLO in the insect vector *Macrosteles fascifrons* and for detection of this and other MLOs in plant species which serve as their hosts in nature.

Additional keywords: disease diagnosis, indexing, Mollicutes.

Mycoplasmalike organisms (MLOs) are believed to be responsible for numerous plant diseases worldwide, but inability thus far to isolate and culture these unique prokaryotes in vitro has long hindered the development of efficient and accurate means for their detection and identification. In recent years, both polyclonal antisera and monoclonal antibodies have shown promise in MLO detection and identification (2,3,11,19,23,24). Another approach for these applications involves molecular cloning of MLO DNA and use of the cloned DNA fragments as hybridization probes. Infected insect hosts have been used as sources of DNA in the development of probes for detection of western X MLO (12) and maize bushy stunt MLO (4,5). The western X DNA probes hybridized with nucleic acid extracted from plants and insects carrying a strain of the western X-disease pathogen but not with nucleic acid from healthy hosts or from plants containing MLOs associated with aster yellows, elm yellows, or vinca virescence (beet leafhopper-transmitted virescence) diseases. An RNA probe prepared from one of the cloned DNA fragments was similarly specific for the western X MLO (18). Probes containing DNA of maize bushy stunt MLO hybridized with DNA extracted from maize bushy stunt MLO-infected plants and insects and not with DNA extracted from healthy hosts; one probe hybridized with DNA from *Spiroplasma kunkelii*, the causal agent of corn stunt disease (5). In other work, MLO-infected plants have been used as starting material for development of cloned probes containing DNA of the aster yellows, elm yellows, or tomato big bud MLOs (7,8,16,17). In that work, several different MLOs, in addition to those from which cloned nucleotide sequences were derived, could be detected by use of the sequences as ^{32}P-labeled or biotinylated DNA and RNA probes. Hybridization patterns indicated existence of a cluster of MLOs, including the aster yellows MLO, that share greater nucleotide sequence homology with one another than with other MLOs.

In a separate study in 1986, one of us (S. M. D.) discovered an MLO in trap plants of annual periwinkle (*Catharanthus roseus* (L.) C. Don) placed in Connecticut peach orchards seriously affected by eastern X-disease. This MLO, elsewhere termed ORCH1 MLO (7), is associated with a severe "yellows disease" syndrome in *C. roseus* that includes little leaf symptoms and has therefore been designated periwinkle little leaf disease (strain 0-1) MLO. In this paper, we report the molecular cloning and characterization of 12 MLO-specific DNA fragments derived from plants containing periwinkle little leaf MLO, and we describe results from investigations on their use as biotinylated DNA hybridization probes (6).

MATERIALS AND METHODS

Sources of mycoplasmalike organisms (MLOs). MLO strains were maintained by grafting in greenhouse-grown plants of a white flowered clone of periwinkle or were obtained in samples of infected hosts provided by others. Aster yellows MLO was field collected in Beltsville, MD (16). MLO 0-13, considered a strain of periwinkle little leaf MLO, was recovered by one of us (S. M. D.) in trap plants of *C. roseus* placed in peach orchards during the same period of time when strain 0-1 was recovered. Symptoms associated with the presence of 0-13 MLO in periwinkle are similar to those associated with 0-1 MLO in this host. Other MLO strains and *S. citri* were kindly provided by the following researchers, who provided each strain separately in tissue of periwinkle, unless noted otherwise: aster yellows in aster (*Callistephus chinensis* (L.) Nees) (Minnesota strain from Ernest Bantarri, University of Minnesota, St. Paul, and strain OKAY from Jacqueline Fletcher, Oklahoma State University, Stillwater) and in celery (*Apium graveolens* L.) and *Macrosteles fascifrons* Stål) (J. Fletcher);

blueberry stunt and tomato big bud (James Dale, University of Arkansas, Fayetteville); alfalfa witches' broom in alfalfa (*Medicago sativa* L.), clover proliferation, and potato witches' broom (Chuji Hiruki, University of Alberta, Edmonton, Canada); western X (strain GX) (B. C. Kirkpatrick, University of California, Davis); western dwarf aster yellows (strain DAY) (Alexander Purcell, University of California, Berkeley); clover phyllody in white clover (*Trifolium repens* L.) and aster, X-disease (strain CX) in periwinkle and (strain PX) in clover and celery, clover yellow edge in clover, CAY strain of aster yellows in aster, and NAY strain of aster yellows in clover and aster (Lloyd Chiykowski, Agriculture Canada, Ottawa, Ontario); ash yellows in periwinkle and ash (*Fraxinus americana* L.) and elm yellows in periwinkle (Wayne Sinclair, Cornell University, Ithaca, NY); vinca (*C. roseus*) virescence (beet leafhopper-transmitted virescence agent) and *S. citri* (George N. Oldfield, University of California, Riverside). The designation noted for a strain of MLO is that provided by the supplier of the strain.

Molecular cloning and labeling of DNA probes. Sieve cells were isolated from periwinkle little leaf strain 0-1 MLO-infected plants of periwinkle by means of an enzyme digestion procedure (15). A concentrated MLO-enriched fraction was prepared by gently rupturing the sieve cells with a glass homogenizer to release the MLOs into suspending medium (0.5 M mannitol, 30 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid] buffer, 0.1% polyvinylpyrrolidone [PVP] 40, pH 7.0). The suspension was clarified by centrifugation at 482 *g* for 10 min; the supernatant liquid was centrifuged at 17,300 *g* for 40 min; and the resulting pellet was resuspended in 2 ml of PBS-sucrose solution (0.145 M NaCl, 8 mM Na_2HPO_4 1.4 mM NaH_2PO_4, 0.146 M sucrose, pH 7.2) and stored at −70 C.

DNA for cloning was prepared from sieve cells of infected plants of *C. roseus* as described elsewhere (16). For cloning, the periwinkle little leaf strain 0-1 MLO DNA plus plant DNA was digested with restriction endonucleases *Eco*RI and *Hin*dIII, ligated into plasmid pSP64 or pSP65 (Promega Biotec, Madison, WI), and used to transform competent cells of *Escherichia coli* JM83 according to a standard procedure (20).

In an attempt to isolate cloned DNA fragments that might have nucleotide sequence homology with MLOs other than strain 0-1 of periwinkle little leaf MLO, 50 transformant colonies were screened for the presence of fragments capable of hybridizing with DNA from aster yellows MLO-infected (but not healthy) plants. DNA was prepared from transformants by the alkaline lysis method (20), denatured, spotted on nitrocellulose membranes, and the membranes baked, prehybridized, and hybridized as described elsewhere (16). DNA extracted from healthy plants of periwinkle or from plants infected by the aster yellows disease agent was radiolabeled by nick translation and employed in hybridizations as described (16). Hybridization with labeled DNA from aster yellows MLO-diseased (but not healthy) plants identified recombinant plasmids as candidates for probe development. Ten additional recombinant plasmids were chosen for further work on the basis of sizes of cloned DNA inserts determined by restriction endonuclease digestion and electrophoresis in 0.7% agarose gels.

Recombinant plasmids (p), or cloned insert DNA (I) excised from recombinant plasmids, were labeled by nick translation with biotin-7-dATP (Bethesda Research Laboratories, Inc. Gaithersburg, MD) and used as probes in tests for detection of homologous nucleotide sequences in MLO-infected hosts.

MLO detection in host tissues. MLO detection was performed by dot hybridizations employing biotinylated DNA probes and nucleic acid extracted from healthy or infected hosts. Each sample consisted of 0.3 g of plant tissue (whole, or fragments of, young leaves, shoots, and/or pieces of whole stem) or of a pool of 25 individual insects. The plant tissue or insects (I) were pulverized in liquid nitrogen, transferred to a microfuge tube, and triturated in 400 μl of DNA extraction buffer (16) plus 2 μl of 2-mercaptoethanol and 20 μl of 20% sodium dodecyl sulfate. Samples were then centrifuged in a microfuge at 325 *g* for 10 minutes, and the supernatant liquid conserved. The samples were further centrifuged at 5,220 *g* for 10 minutes, and the second supernatant combined with the first. The combined supernatants were then heated at 65 C for 5 min, centrifuged at 16,000 *g* for 10 min, the supernatant subjected to two extractions with TE saturated phenol and chloroform-isoamyl alcohol, and the nucleic acid precipitated from the aqueous phase by addition of two volumes of cold (−20 C) absolute ethanol and collected by centrifugation for 10 min at 16,000 *g*. Each nucleic acid pellet was dried with a stream of nitrogen gas, resuspended in 100 μl of 6× SSC, pH 7.0, and stored frozen at −20 C until use.

For dot hybridizations, nucleic acid samples were thawed, denatured by addition of 6 μl of 2 N NaOH per 100-μl sample, held in a boiling water bath for 10 minutes, cooled in an ice bath, and neutralized by addition of 6 μl of 2 M Tris, pH 7.0, and 2 μl of 1.5 M NaOAc, pH 5.0, per sample. Samples were diluted in twofold dilution series in 6× SSC (undiluted, about 7 μg of nucleic acid per spot), and aliquots (3 μl each) were applied to clean nitrocellulose membranes. Membranes were air-dried, baked under vacuum at 80 C for 2 hr, and stored in a desiccator until use. Prehybridizations, hybridizations, washing, blocking, and probe detection procedures were performed using the BRL BluGENE Nonradioactive Nucleic Acid Detection System (Cat. No. 82795A). Following a prehybridization, hybridization of membranes was performed for 16 hr at 42 C in the presence of 5× SSC, 1× Denhardt's solution, 20 mmol sodium phosphate, pH 6.5, 5% dextran sulfate, 45% formamide, 0. 2 mg of denatured salmon sperm DNA per milliliter, and 0.2 μg of biotin-labeled probe DNA per milliliter. Membranes were then washed at room temperature for 3 min twice in 2× SSC containing 0.1% SDS and twice in 0.2× SSC containing 0.1% SDS, and then twice at 50 C for 15 min in 0.16× SSC containing 0.1% SDS. Membrane blocking was performed for 1 hr at 64 C in a buffer (0.1 M Tris, pH 7.5) containing 3% bovine serum albumin (Pentex Fraction V, Miles Scientific, Naperville, IL) and 0.15 M NaCl. Visualization of hybridized biotinylated probe was performed using Strepavidin-Alkaline phosphatase conjugate and nitroblue tetrazolium plus 5-bromo-4-chloro-3-indolyl phosphate (BRL BluGENE System).

For Southern hybridization analyses (25), nucleic acid was extracted from plants according to the procedure of Dellaporta et al (10), electrophoresed in 0.7% agarose gel, stained with ethidium bromide for photography, destained with deionized water, denatured by addition of 1.5 M NaCl plus 0.5 M NaOH for 30 min, and neutralized for 30 min at room temperature in 1.5 M NaCl plus 0.5 M Tris, pH 7.5. Nucleic acid was then transferred from gel to nitrocellulose membranes (20). Membranes were baked, prehybridized, and hybridized with biotin-labeled probes as described above.

RESULTS

A total of 367 transformant colonies of *E. coli* were obtained in the cloning of DNA from plant tissues infected with the periwinkle little leaf MLO. No recombinant plasmids from 50 transformants hybridized with DNA from healthy plants, but seven hybridized with DNA from aster yellows diseased plants. Two of the latter (designated pCN42 and pCN10, respectively) showed strong hybridization signals and were selected for further work.

Subsequently, transformants not previously tested were examined for cloned periwinkle little leaf MLO DNA fragments capable of hybridizing with DNA from periwinkle little leaf MLO-infected (but not healthy) plants. Ten transformants were selected to include a range of cloned DNA insert sizes, and the plasmids or cloned inserts were labeled by biotinylation. The labeled recombinant plasmids were then used as probes in dot hybridizations with nucleic acid extracted from healthy plants of periwinkle or from periwinkle plants infected with the periwinkle little leaf MLO. All 10 probes hybridized with nucleic acid from plants infected by periwinkle little leaf MLO; none hybridized with nucleic acid from healthy plants.

Detection of MLOs in plants of *C. roseus*. Twelve recombinant

TABLE 1. Summary of results from dot hybridizations of biotinylated cloned periwinkle little leaf strain 0-1 mycoplasmalike organism (MLO) DNA probes to nucleic acid preparations extracted from MLO-infected plants of periwinkle (*Catharanthus roseus*)

Probe DNA (insert size, Kbp)	Hybridization with nucleic acid from plant indicated[a]											
	0-1	AY	DAY	BB	DB	WX	CX	AA	CP	PWB	EY	VR
CN25I(0.4), CN35I(0.4), CN50I(0.4), CN41I(1.1), CN5I(1.2), CN10I(1.2), CN43I(1.4), CN120I(2.1), CN55I(3.1), and CN42I(3.2)	+	+	+	+	+	−	−	−	−	−	−	−
CN44I(1.6)	+	+	+	+	+	+	+	−	−	−	−	−
CN40I(1.7)	+	+	+	+	+	+	+	+	+	+	−	−

[a] +, Positive hybridization. −, no hybridization signal. For each data point, labeled probe was hybridized to nucleic acid extracted from plants affected by a given MLO-associated disease. 0-1, periwinkle little leaf; AY, aster yellows; DAY, dwarf western aster yellows; BB, tomato big bud; DB, blueberry stunt; WX, western X-disease; CX, Canada X-disease; AA, ash yellows; CP, clover proliferation; PWB, potato witches' broom; EY, elm yellows; and VR, beet leafhopper-transmitted virescence agent. Results with periwinkle little leaf strain 13 (0-13) were the same as those obtained with strain 0-1. All probes consisted of labeled, cloned MLO DNA inserts. None of the probes hybridized with nucleic acid from plants with *Spiroplasma citri*.

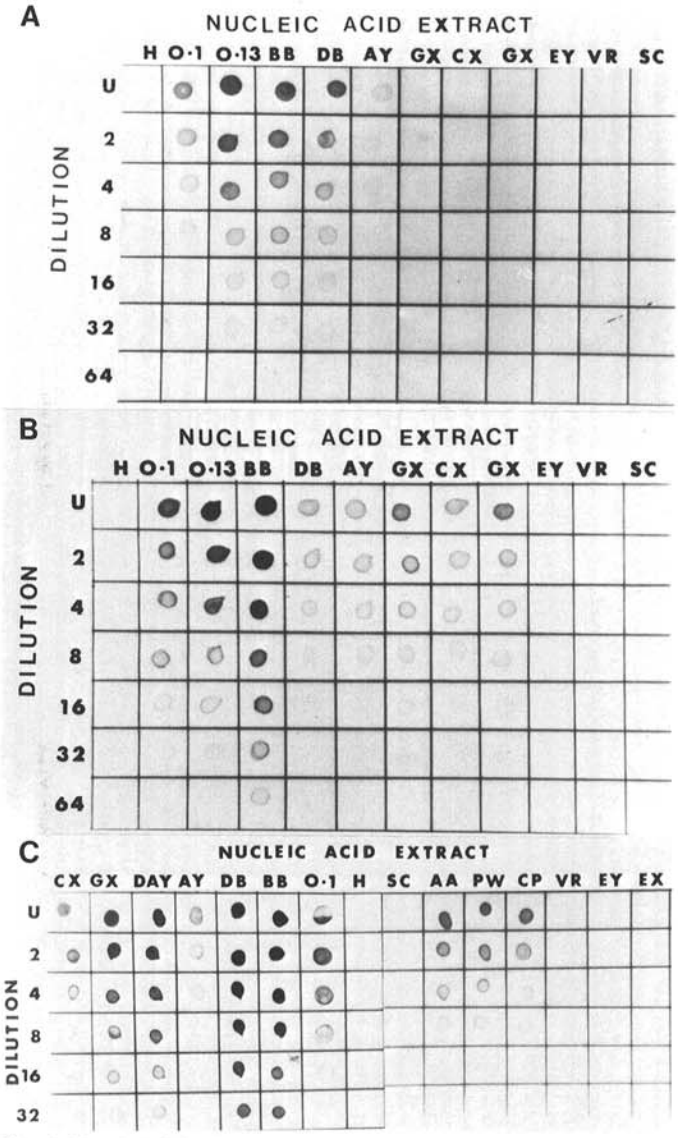

Fig. 1. Dot hybridizations of biotinylated cloned periwinkle little leaf (0-1) mycopolasmalike organism (MLO) DNA probes to nucleic acid preparations extracted from MLO-infected or healthy plants of periwinkle (*Catharanthus roseus*). **A,** probe CN44I. **B,** probe CN5I. **C,** probe CN40I. AY, aster yellows; DAY, western dwarf aster yellows; BB, tomato big bud; DB, blueberry stunt; GX, western X-disease; CX, Canada X-disease; EY, elm yellows; VR, beet leafhopper-transmitted virescence; AA, ash yellows; CP, clover proliferation; PWB, potato witches' broom; SC, *Spiroplasma citri*; and H, healthy. Samples of nucleic acid extracted from plants were applied to nitrocellulose membranes in a twofold dilution series, 3 μl per spot. U, undiluted. 2, 4, 8, 16, 32, and 64, reciprocals of dilutions.

plasmids containing periwinkle little leaf MLO DNA, and their respective MLO DNA inserts, were labeled with biotin and hybridized against nucleic acid extracted from plants affected by a variety of MLO-associated plant diseases. Sizes of the 12 cloned DNA inserts are given in Table 1. Representative results from dot hybridizations are illustrated in Figure 1. There was no evidence of hybridization of any probe with nucleic acid extracted from healthy periwinkle plants or from plants infected by *Spiroplasma citri*. Strong hybridization signals were obtained with extracts from various MLO-infected plants. Labeled recombinant plasmids and labeled DNA inserts gave equivalent results. As seen from Figure 1, the pattern of hybridizations between probe and nucleic acid extract varied with probe and with infecting MLO. The results from several separate experiments of this type, involving 12 cloned DNA inserts employed as probes, are summarized in Table 1. Each combination of probe and nucleic acid extract was tested at least twice; all tests of a given combination gave the same results. All 12 probes hybridized with nucleic acid extracted from plants of periwinkle infected by strains 0-1 or 0-13 of periwinkle little leaf MLO, or by aster yellows, western dwarf aster yellows, tomato big bud, or blueberry stunt MLOs. None gave hybridization signals with extracts from healthy plants or from plants infected by *S. citri*. Of the 12 probes, only CN40I and CN44I hybridized with nucleic acid from plants infected by any of the additional MLOs examined. Probe CN44I hybridized with nucleic acid from plants infected with the MLOs of western X and Canada X diseases; and probe CN40I hybridized with nucleic acid from plants with the MLOs of western X, Canada X, clover phyllody, potato witches' broom, and ash yellows diseases.

Detection of MLOs in natural host species. Use of biotinylated probes permitted detection of several MLOs in plant and insect species known to harbor the MLOs in nature. Figure 2 illustrates hybridization signals obtained in dot hybridizations of probe CN40I with nucleic acid extracted from periwinkle, aster, celery, and clover. Samples from healthy plants generally gave no discernible hybridization signal. In a few cases (for example with aster), presence of plant components in nucleic acid samples sometimes resulted in a slightly darkened dot on the nitrocellulose, but this was readily distinguished from colored hybridization signals. The results from several experiments of this type are summarized in Table 2. Infection by MLOs was detected by dot hybridizations with probe CN40I in the case of several diseases in different hosts, including an insect vector. Positive hybridizations were observed in the case of aster yellows in clover, China aster, celery, and in the insect vector *M. fascifrons*; in the case of clover phyllody in clover and China aster; of alfalfa witches' broom in alfalfa; of ash yellows in ash; of clover yellow edge in clover, and of X-disease pathogen in clover.

Southern hybridization analyses. Electrophoresis of undigested nucleic acid extracted from MLO-infected or healthy plants is illustrated in Figure 3A. A visible band corresponding to chromosomal DNA occurs in the case of each extract. Figures 3B and 3C illustrate results from Southern hybridizations with

probe CN5I (Fig. 3B) and CN42I (Fig. 3C). Probe CN5I hybridized with chromosomal DNA of periwinkle little leaf MLO, and also hybridized with chromosomal DNA of aster yellows and tomato big bud MLOs. Probe CN42I gave weak or no hybridization signals with MLO chromosomal DNA, but did hybridize with bands of extrachromosomal DNA from plants infected with periwinkle little leaf, aster yellows, and tomato big bud MLOs.

DISCUSSION

The feasibility of molecular cloning of DNA fragments from uncultured mycoplasmalike plant pathogens has been illustrated in the present work and in other recent studies (4,5,7,12–14,16–18). Although this technology has been applied in relatively few laboratories thus far, it promises to become increasingly pursued as an important approach for the detection and identification of MLOs and for investigations of their genetic relatedness. In contrast to some other studies (5,12), MLO DNA suitable for molecular cloning was obtained from infected plants in the present work. Our approach exploited the fact that MLOs are confined to phloem sieve cells in infected plants and that these cells not only comprise a small proportion of the entire plant mass, but they also contain no nuclei when mature. In this way, we attempted to obtain preparations in which the concentration of MLO-bearing cells was maximized, while we minimized the concentration of host cell DNA that was harvested. Infected insect vectors have been used as starting material for molecular cloning of MLO DNA in other work (5,12). Use of infected plants as starting material presents some advantages over use of infected insect vectors. These arise in part because rearing of insect vectors may be obviated by quarantine restrictions, lack of knowledge on vector biology, or absence of appropriate facilities, and because insect vectors of many MLOs have not yet been identified. The ability to clone MLO DNA from periwinkle is advantageous because it is an easily propagated, congenial host for numerous different MLOs.

DNA probes currently used in plant disease diagnosis are most often radioactively labeled (21). Of MLO DNA probes, most reported have been labeled with ^{32}P (4,5,12–14,16). Our results indicate that biotinylation can yield labeled probes capable of detecting infection by MLOs in plant and insect hosts. Sensitivity of MLO detection using the biotin-labeled probes could be estimated on the basis of minimum amount of plant tissue needed for MLO detection, as has been done in other work (5). In our hands, this amount varied with the probe used and MLO detected. We attribute this result in part to copy numbers of homologous nucleotide sequences in an MLO and to differences in MLO concentrations in infected hosts. Nevertheless, we were able to detect MLOs in small quantities of plant tissue. For example, MLOs of periwinkle little leaf, tomato big bud, blueberry stunt, dwarf aster yellows, western X, and Canada X were detected by hybridizing probe CN40I to dots containing nucleic acid from less than 300 μg of plant tissue (see Figure 1).

The aster yellows and tomato big bud MLOs differ markedly from one another in the symptoms they induce in infected plants. Whereas the aster yellows MLO induces a virescence and phyllody in flowers of periwinkle, the tomato big bud MLO induces formation of small but normally colored flowers, and there is no phyllody or virescence associated with the presence of the tomato big bud MLO in periwinkle. Yet, it is possible to perceive a relationship among these distinctly different MLOs. Dot hybridizations indi-

TABLE 2. Host species in which infection by a mycoplasmalike organism (MLO) was detected by dot hybridization with biotinylated DNA probe CN40I

Host	MLO strain (disease) detected
Alfalfa (*Medicago sativa*)	Alfalfa witches' broom
Ash (*Fraxinus americana*)	Ash yellows
Celery (*Apium graveolens*)	Aster yellows (strain CAY)
Clover (*Trifolium repens*)	Clover yellow edge
	Clover phyllody
	X-disease of peach
	Aster yellows (strain NAY)
China aster (*Callistephus chinensis*)	Aster yellows (strains OKAY, NAY, CAY, and Minnesota strain)
	Clover phyllody
Aster leafhopper (*Macrosteles fascifrons*)	Aster yellows (strain OKAY)

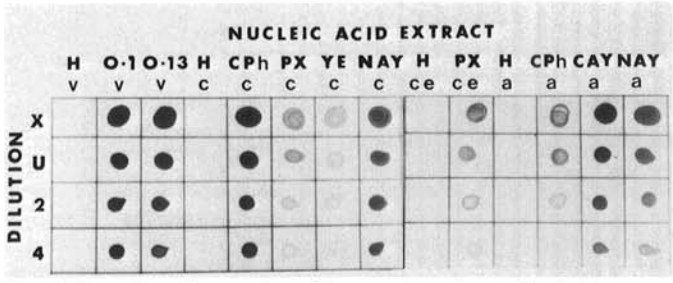

Fig. 2. Detection of mycoplasmalike organisms in species that serve as their hosts in nature. v, *Catharanthus roseus*; c, clover (*Trifolium repens*); ce, celery (*Apium graveolens*); a, aster (*Callistephus chinensis*); 0-1, periwinkle little leaf; AY, aster yellows; CPh, clover phyllody; YE, clover yellow edge; NAY, strain of aster yellows; and PX, X-disease. Samples of nucleic acid extracted from plants were applied to a nitrocellulose membrane in a twofold dilution series. X, 6 μl of undiluted sample per spot. U, 2, and 4, 3 μl of undiluted, 1:2, and 1:4 dilution of sample, respectively, per spot.

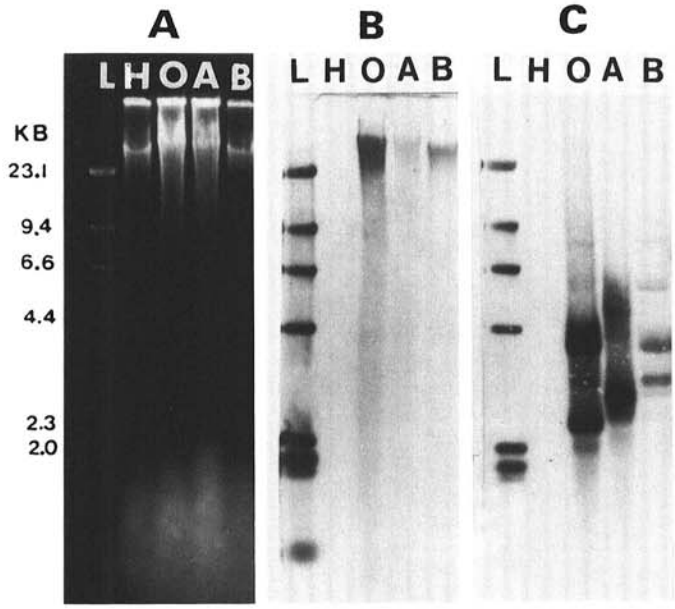

Fig. 3. Southern hybridization analyses of undigested DNA extracted from healthy and mycoplasmalike organism (MLO)-infected plants of periwinkle (*Catharanthus roseus*). **3A,** Electrophoresis of nucleic acid samples extracted from healthy (lane H) and MLO-infected (lanes O, A, and B) plants in 0.7% agarose gel followed by staining with ethidium bromide. Lane O, periwinkle little leaf MLO; lane A, aster yellows; lane B, tomato big bud; and lane L, biotinylated λ DNA/*Hin*dIII fragments. **3B and C,** Southern hybridizations of DNA following electrophoresis in 0.7% agarose gel and transfer to nitrocellulose membranes. Membranes were hybridized to biotinylated DNA probes CN5I (Fig. 3B) and CN42I (Fig. 3C). Lane designations same as for Fig. 3A.

cated that periwinkle little leaf, aster yellows, and tomato big bud MLOs share nucleotide sequence homologies. Southern hybridizations revealed that homologies exist among both chromosomal and extrachromosomal DNAs. The results from Southern hybridizations indicated presence of extrachromosomal DNA, possibly plasmids, in a strain of aster yellows MLO, confirming other work (13,14,16). The data further revealed the presence of extrachromosomal DNA in periwinkle little leaf strain 0-1 MLO and tomato big bud MLO and provided evidence of nucleotide sequence homologies between chromosomal DNAs and between extrachromosomal DNAs. The results with periwinkle little leaf and tomato big bud MLOs are the first to indicate presence of extrachromosomal DNA in these prokaryotes. The finding of nucleotide sequence homology among chromosomal DNAs of these MLOs indicates that they are genetically related. Since the relationship among these MLOs involves both genetic relatedness at the level of chromosomal DNA and the sharing of nucleotide sequence homology between extrachromosomal DNAs, probes containing either chromosomal or extrachromosomal DNA of periwinkle little leaf MLO yielded positive MLO detection.

Previous work has also indicated that the periwinkle little leaf, aster yellows, and tomato big bud MLOs are interrelated (16), although they can be differentiated from one another. Thus, hybridizations involving RNA probes transcribed from cloned aster yellows MLO chromosomal DNA distinguished between periwinkle little leaf and aster yellows MLOs (16). Recently, 12 plant diseases, considered to be caused by closely related MLO strains from Japan, were classified by Shiomi and Sugiura (22) into three groups on the basis of plant host range. In the future it should be possible to classify and differentiate MLOs such as these on the basis of nucleotide sequence homologies.

It is evident that some of the cloned periwinkle little leaf MLO DNA fragments obtained in the present work contain nucleotide sequences having homology with sequences that are present in a relatively broad array of MLO strains. This array includes MLOs associated with aster yellows, tomato big bud, western X of stone fruits, X-disease in Canada, ash yellows, alfalfa witches' broom, clover yellow edge, clover phyllody, clover proliferation, potato witches' broom, and other diseases. All are associated with diseases of economically important forest or agricultural crops. Infection by several of these MLOs was detected in agronomically important plant hosts and in an insect vector by the use of a biotinylated DNA probe. The results encourage consideration of nonradioactive DNA probes in diagnosis of possible MLO-induced plant disease.

LITERATURE CITED

1. Boulton, M. I., Markham, P. G., and Davies, J. W. 1984. Nucleic acid hybridization techniques for the detection of plant pathogens in insect vectors. Pages 181-186 in: Proc. British Crop Prot. Conf–Pest Dis. Vol. I.
2. Clark, M. F., Davies, D. L., and Barbara, D. J. 1983. Production and characteristics of antisera to *Spiroplasma citri* and clover phyllody-associated antigens derived from plants. Ann. Appl. Biol. 103:251-259.
3. Clark, M. F., Morton, A., and Buss, S. L. 1989. Preparation of mycoplasma immunogens from plants and a comparison of polyclonal and monoclonal antibodies made against primula yellows MLO-associated antigens. Ann. Appl. Biol. 114:111-124.
4. 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 mycoplasmalike organism (MBA-MLO). (Abstr.) Phytopathology 77:1769.
5. 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.
6. Davis, R. E., Lee, I.-M., Douglas, S. M., and Dally, E. L. 1988. Biotinylated cloned DNA fragments from an unknown mycoplasma-like organism (MLO) detect infection in several plant diseases. (Abstr.) Phytopathology 78:1541.
7. Davis, R. E., Lee, I.-M., Douglas, S. M., Dally, E. L., and Dewitt, N. 1988. Cloned nucleic acid hybridization probes in detection and classification of mycoplasmalike organisms (MLOs). Acta Hortic. 234:115-122.
8. Davis, R. E., Lee, I.-M., Douglas, S. M., Dally, E. L., and Dewitt, N. 1990. Development and use of cloned nucleic acid hybridization probes for disease diagnosis and detection of sequence homologies among uncultured mycoplasmalike organisms (MLOs). Zbl. Bakt. Hyg.A (Int. J. Microbiol. Hyg. Ser.A) (in press).
9. Davis, R. E., Lee, I.-M., Sinclair, W., and Douglas, S. M. 1988. Biotinylated cloned DNA probe detects ash yellows mycoplasmalike organism in ash. (Abstr.) Phytopathology 78:1555.
10. Dellaporta, S. L., Wood, J., and Hicks, J. B. 1983. A plant DNA minipreparation: Version II. Plant Mol. Biol. Rep. 1:19-21.
11. Kirkpatrick, B. C., and Carrot, D. C. 1984. Detection of X-disease in plant hosts by enzyme-linked immunosorbent assay. (Abstr.) Phytopathology 74:825.
12. 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.
13. Kuske, C. R., Davis, M. J., and Kirkpatrick, B. C. 1988. Evidence for homology between plasmids from western aster yellows MLO and plasmids from maize bushy stunt MLO. (Abstr.) Phytopathology 78:1603.
14. Kuske, C. R., and Kirkpatrick, B. C. 1988. Isolation and cloning of plasmids from the western aster yellows MLO. (Abstr.) Phytopathology 78:1569.
15. Lee, I.-M., and Davis, R. E. 1983. Phloem-limited prokaryotes in sieve elements isolated by enzyme treatment of diseased plant tissue. Phytopathology 73:1540-1543.
16. 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.
17. Lee, I.-M., Davis, R. E., and Dewitt, N. D. 1988. Molecular cloning of and screening by a new method for DNA fragments from elm yellows (EY) and tomato big bud (BB) mycoplasmalike organisms (MLOs). (Abstr.) Phytopathology 78:1602.
18. Lee, I.-M., Davis, R. E., Hammond, R., and Kirkpatrick, B. 1988. Cloned riboprobe for detection of a mycoplasmalike organism (MLO). Biochem. Biophys. Res. Commun. 155:443-448.
19. Lin, C. P., and Chen, T. A. 1985. Monoclonal antibodies against the aster yellows agent. Science 227:1233-1235.
20. Maniatis, T. I., Fritsch, E. F., and Sambrook, J. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 545 pages.
21. Miller, S. A. 1988. Biotechnology-based disease diagnostics. Plant Dis. 72:188.
22. Shiomi, T., and Sugiura, M. 1984. Grouping of mycoplasma-like organisms transmitted by the leafhopper vector, *Macrosteles orientalis* Virvaste, based on host range. Ann. Phytopathol. Soc. Jpn. 50:149-157.
23. Sinha, R. C. 1979. Purification and serology of mycoplasmalike organisms from aster yellows-infected plants. Can. J. Plant Pathol. 1:65-70.
24. Sinha, R. C., and Chiykowski, L. N. 1984. Purification and serological detection of mycoplasmalike organisms from plants affected by peach eastern X-disease. Can. J. Plant Pathol. 6:200-205.
25. Southern, E. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98:503-517.