Detection and Investigation of Genetic Relatedness among Aster Yellows and Other Mycoplasmalike Organisms by Using Cloned DNA and RNA Probes

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A method was developed for enriching the concentration of mycoplasmalike organism (MLO) DNA in diseased plant extracts. With this method, fragments of DNA of the aster yellows (AY) MLO were cloned in pSP6 plasmid vectors and amplified in *Escherichia coli* strain JM83. Labeled double-stranded DNA probes were prepared by nick translation of recombinant plasmids by using either ^{32}P nucleotides or biotinylated nucleotides. Single-stranded RNA probes (riboprobes) were synthesized *in vitro* by using SP6 polymerase and linearized recombinant plasmids and were labeled with ^{32}P. The probes all hybridized with nucleic acid from AY MLO-infected, but not healthy, plants. Some hybridized also with nucleic acid from plants infected by other MLOs. Hybridization patterns indicated existence of a cluster of MLOs, including AY MLO, that share greater nucleotide sequence homology with one another than with other MLOs. Riboprobes were used to differentiate AY MLO from others in the AY MLO cluster.

Aster yellows (AY) disease is believed to be caused by wall-less mycoplasmalike organisms (MLOs). Since Kunkel (1926) reported the first incidence of AY disease (then called AY viral disease) in China aster plants, numerous diseases in various crops have been claimed to be caused by this same pathogen or related strains (Murtomaa 1966; Ploaie 1969, 1981). Due to the inability to culture AY MLOs *in vitro*, nearly all identification and classification done in the past depended entirely on biological properties such as symptoms in diseased plants, plant host range (Chiykowski 1967; Westdal and Richardson 1969), and specificity of pathogen transmission by insect vectors (Freitag 1967; Granados 1965). Some progress in development of polyclonal antisera for detection of MLOs has been reported (Clark *et al.* 1983; Sinha 1979; Sinha and Benhamou 1983). However, the general lack of sensitive, rapid, and accurate means for detection and identification of the pathogen has hindered progress in etiological, epidemiological, and phylogenetic studies, and has impeded the elimination of the pathogen from important crop germplasm. Hybridoma and recombinant DNA technologies may overcome these hindrances.

Lin and Chen (1985) produced two AY-specific monoclonal antibodies that reacted with extracts from AY-infected plants, but not with extracts from healthy plants. However, application of these highly specific probes may not be adequate for broad phylogenetic or taxonomic

studies. By using recombinant DNA technology, Kirkpatrick *et al.* (1987) cloned fragments of DNA purified from extracts of insects infected with the western X (WX) disease MLO. WX disease-specific cloned DNA, when labeled and used as probes, specifically hybridized with DNA from WX-diseased plants and insects. Subsequently, single-stranded riboprobes specific to this disease were constructed elsewhere and proved to be powerful and efficient tools with high sensitivity and specificity for detection and identification of the WX MLO in plants (Lee *et al.* 1987). In other work, cloning of DNA fragments from a strain of the maize bushy stunt (MBS) disease MLO was reported (Davis *et al.* 1987). These cloned fragments, when used as ^{32}P-labeled DNA probes, were able to detect MBS MLO but not other MLOs. This progress has encouraged our application of recombinant DNA methods to the AY disease problem.

In this paper we describe the preparation and cloning of MLO DNA from infected plants and report the use of cloned DNA and RNA probes in detection of AY MLO and in investigation of genetic relatedness between AY and other MLOs.

MATERIALS AND METHODS

Sources of healthy and diseased plants. Diseased plants of periwinkle (*Catharanthus roseus*) were maintained by grafting in a white flowered clone of periwinkle grown in a greenhouse. Aster yellows (AY)-infected periwinkle used for DNA cloning in this experiment was originally field collected and maintained in our greenhouse at Beltsville. *Spiroplasma citri* and MLOs were kindly provided separately in infected periwinkle plants or other hosts by the following researchers: *S. citri* in periwinkle (George N. Oldfield, USDA, University of California, Riverside); beet

leafhopper-transmitted virescence (VR) in periwinkle (Golino *et al.* 1987) (George N. Oldfield); aster yellows in aster (OKAY-2) (Jacqueline Fletcher, Oklahoma State University, Stillwater); aster yellows in periwinkle (OKAY-1) (Jacqueline Fletcher); aster yellows in aster (CAY-aster) (Lloyd N. Chiykowski, Agriculture Canada, Ottawa); aster yellows in periwinkle (NYAY) (Chuji Hiruki, University of Alberta, Edmonton, Canada); aster yellows in clover (NAY) (Lloyd N. Chiykowski); western aster yellows in periwinkle (WAY) (Chuji Hiruki); blueberry stunt (DB) and tomato big bud (BB) in periwinkle (James Dale, University of Arkansas, Fayetteville); clover proliferation (CP) and potato witches' broom (PWB) in periwinkle (Chuji Hiruki); western X disease (WX) and western dwarf aster yellows (DY) in periwinkle (B.C. Kirkpatrick, University of California, Davis); Canada X disease (CX) in periwinkle (Lloyd N. Chiykowski); elm yellows (EY) and ash yellows (AA) in periwinkle (Wayne Sinclair, Cornell University, Ithaca, New York); and unknown MLO (CN and CN13 = ORCH1 and ORCH13, respectively) in periwinkle (Sharon M. Douglas, Connecticut Agricultural Experiment Station, New Haven).

Preparation of AY MLO-enriched fraction from infected periwinkle plants. Fifty grams of young leaves showing early stage symptoms were surface-sterilized with 1% sodium hypochlorite for 5 min and then rinsed twice in sterile distilled water. Midribs of leaves were cut longitudinally with sharp forceps in such a way that only central portions containing vascular tissues were removed. The vascular tissues were transferred aseptically into petri plates containing macerating enzymes in solution (Lee and Davis 1983) and were incubated overnight in the dark at 4° C. At this stage the vascular tissues became partially digested; phloem tissue, consisting mainly of layers of sieve elements and some incompletely digested phloem parenchyma cells, could be easily separated from xylem tissue with forceps. The phloem tissue was transferred into a petri plate containing 25 ml of sterile suspending medium (0.5M mannitol, 30 mM HEPES buffer, 0.1% polyvinylpyrrolidone [PVP] 40, pH 7.0). The sieve elements were gently ruptured with glass tissue homogenizers to release AY MLOs into the suspending medium.

To remove the host organelles and cell wall debris, the suspension was clarified at least once by centrifugation at 2,000 rpm (482 × g; in a Sorvall centrifuge SS-34 rotor) for 10 min. The supernatant liquids, which contained AY MLOs, were centrifuged at 12,000 rpm (17,300 × g) for 40 min. The resulting pellets were resuspended in 2 ml of PBS-sucrose solution (0.145 M NaCl, 0.008 M Na_2HPO_4, 0.0014 M NaH_2PO_4, 0.146 M sucrose, pH 7.2) and stored at −70° C.

Isolation of nucleic acid from AY MLO-enriched preparation. The AY MLOs in PBS-sucrose were concentrated by centrifugation at 12,000 rpm for 40 min. The pellet was then resuspended in 2 ml of DNA extraction buffer (0.1 M Tris, 0.05 M EDTA, 0.5M NaCl, pH 8.0). One hundred μl of 20% sodium dodecyl sulfate (SDS) and 1.5 μl of 2-mercaptoethanol were added. The components were mixed thoroughly by vortexing. The mixture was incubated at 65° C in a water bath for 10 min and then cooled to room temperature. The resulting clear solution was extracted twice with 1 ml of TE (10 mM Tris HCl, 1 mM EDTA, pH 8.0) saturated phenol and 1 ml of chloroform-isoamyl alcohol (24:1) and once with 2 ml of chloroform-isoamyl

alcohol. The aqueous phase was transferred to a clean silicon-treated Corex glass tube, and 2 volumes of cold 95% ethanol was added. The crude AY MLO nucleic acids were precipitated by incubating the solution at −70° C for one-half hr or at −20° C overnight. After centrifugation at 12,000 rpm for 40 min, the pellet (nucleic acid) was air-dried or dried by passing a slow stream of nitrogen over it. The pellet was resuspended in 200 μl of TE buffer and transferred to an Eppendorf microfuge tube. Twenty μl of 3 M sodium acetate (NaOAc) (pH 4.6) was added, and the nucleic acids were reprecipitated with ethanol and centrifuged as described above. The resulting pellet was resuspended in 25 μl of sterile deionized water or TE buffer. This AY MLO plus plant host nucleic acids solution was used as source of DNA for molecular cloning.

Construction of DNA and RNA (riboprobe) probes. The AY MLO DNA plus plant DNA (in a solution of 10–20 μg of total nucleic acids) was digested with both *Eco*RI and *Hin*dIII restriction endonucleases at 37° C for 1 hr. The reaction was stopped by incubating at 65° C for 5 min, and the solution was then extracted once with a half volume of TE-saturated phenol and a half volume of chloroform-isoamyl alcohol and once with an equal volume of ether. The DNA fragments in the aqueous phase were precipitated by adding 1/10 volume of 3 M NaOAC and 2 volumes of ethanol at −70° C for one-half hr. The DNA fragments were ligated with *Eco*RI- and *Hin*dIII-digested plasmid pSP64 or pSP65 (Promega Biotec, Madison, Wisconsin) at 16° C for 12–16 hr and then used to transform competent *E. coli* strain JM83 according to the procedure described by Maniatis *et al.* (1982).

Ampicillin-resistant colonies were screened by dot hybridization (Maniatis *et al.* 1982) with ^{32}P-labeled nick-translated nucleic acid from AY-diseased plants as well as healthy plant host nucleic acid prepared as noted above. DNA was prepared from ampicillin-resistant transformants by the alkaline lysis method (Maniatis *et al.* 1982). About 3.5 μl (containing 2–3 ng denatured plasmid DNA) of this preparation was spotted on nitrocellulose papers that were presoaked with 10× saline sodium citrate (SSC; 1× = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0). The filter papers were baked, prehybridized, and hybridized as described below. Colonies, from which nucleic acid hybridized with labeled nucleic acid from AY MLO-diseased plants but not with labeled nucleic acid from healthy plant tissues, were considered as candidates for development of AY MLO DNA probes. The presence of an insert in plasmid pSP64 or pSP65 was confirmed by restriction endonuclease digestion and agarose gel electrophoresis. AY MLO-specific DNA probes were made by nick-translation of cloned recombinant plasmids or of cloned inserts by using either α^{32}P ATP (Dupont New England Nuclear, Boston, MA; 800 Ci/m mol) labeled or biotinylated α-ATP (Bethesda Research Laboratories, Bethesda, MD) nucleotides. α^{32}P UTP (Dupont New England Nuclear; 800 Ci/m mol) labeled single-stranded (ss) RNA probes (Melton *et al.* 1984) were synthesized *in vitro* by using SP6 RNA polymerase and the linearized recombinant pSP64 plasmid DNA templates containing AY MLO-specific DNA fragments as described in manufacturer's literature (Promega Biotec).

Extraction of nucleic acids from plants. Nucleic acids from healthy plants and from plants infected with *Spiroplasma citri*, aster yellows, and other MLO diseases

were prepared. Approximately 0.3 g of plant tissues (midribs or young shoots) was pulverized in liquid nitrogen. The pulverized samples were triturated (in 400 μl of DNA extraction buffer containing 0.5% 2-mercaptoethanol and 0.5% SDS) in Eppendorf microfuge tubes with plastic mini-pestles, and centrifuged at 2,000 rpm for 10 min. The supernatants were transferred to clean centrifuge tubes, and the loose pellets were centrifuged again at 8,000 rpm for 10 min. The second supernatants were combined with the first, heated at 65° C for 5 min, and then centrifuged at 14,000 rpm for 5 min to remove the coagulated debris. The resulting supernatants were transferred to clean Eppendorf microfuge tubes and extracted with 200 μl of TE-saturated phenol and 200 μl of chloroform-isoamyl alcohol. After centifugation at 14,000 rpm for 5 min, the aqueous phase containing nucleic acid was collected and used for dot hybridization. When biotinylated probes were used in dot hybridization, nucleic acid samples were further purified by additional extraction with phenol and chloroform-isoamyl alcohol and finally by ethanol precipitation, centrifugation, and resuspension of pellets in 6× SSC (50-100 μl per pellet).

Dot hybridization. Nucleic acid samples prepared from healthy and MLO-infected periwinkle plants were denatured (by boiling in the presence of 3 μl of 2 N NaOH per 50 μl of sample, and neutralizing by addition of 3 μl of 2 M Tris, pH 7.0) and dot blotted (3 μl per spot) in twofold serial dilutions in 6× SSC (undiluted, 7 μg of nucleic acid per spot) on clean nitrocellulose paper. For hybridizations with ^{32}P-labeled probes, the filter papers were air-dried and then baked at 80° C for 2 hr under vacuum. The filter papers were prehybridized for 1 hr at 65° C in a solution containing 6× SSC, 4× Denhardt's (1× = 0.08% Ficoll, 0.08% PVP, 0.08% bovine serum albumin), 0.5% SDS, and 150 μg/ml denatured calf thymus or salmon sperm DNA.

For hybridization, the solution was replaced with a similar solution (12 ml per filter paper) containing denatured ^{32}P-labeled DNA (120 ng; specific activity 1.7 × 10^8 cpm/μg) or ^{32}P ssRNA probes (at concentrations between 3 × 10^5 cpm and 10^6 cpm per ml). The ^{32}P-labeled DNA probes were alkali-denatured (1 μl of 2 N NaOH in 40 μl of DNA or nucleic acid sample) and incubated in a boiling water bath for 2 min, followed by immediate cooling in ice water. After overnight incubation at 65° C, the filters were subjected to either high- or low-stringency washes.

Under high stringency, filters were washed for 15 min once at room temperature with excess of 3× SSC containing 0.5% SDS, twice at 65° C with 3× SSC containing 0.5% SDS, twice at 65° C with 2× SSC containing 0.5% SDS, and twice at 65° C with 0.1× SSC containing 0.1% SDS. Under low stringency, the washes with 0.1× SSC containing 0.1% SDS were omitted. In separate experiments, using riboprobes for hybridizations, an RNase treatment was performed following washes under low-stringency conditions. For RNase treatment, the filters were rinsed three times in 2× SSC for 5 min. After final incubation at room temperature for 15 min in 2× SSC containing 1 μg/ml RNase A, filters were washed with 0.1× SSC plus 0.1% SDS at 50° C for 30 min. After washing, the filters were dried and exposed to X-ray film (Kodak XAR) for 24 hr with an intensifying screen (MCI Optonix, Inc., Cedar Knolls, NJ).

Hybridizations with biotinylated probes were carried out according to the procedure of "Nonradioactive Nucleic Acid Detection System," published by Bethesda Research Laboratories (Gaithersburg, Maryland). Hybridizations with biotinylated double-stranded (ds) DNA probes (1-2 μg per filter paper) were performed at 42° C in 5× SSC, 1× Denhardt's solution, 5% dextran sulfate, 45% formamide, 20 mM sodium phosphate (pH 6.5), and 0.2 mg/ml of salmon sperm DNA. After overnight incubation, filters were washed for 3 min twice at room temperature with 2× SSC containing 0.1% SDS; 3 min twice at room temperature with 0.2× SSC containing 0.1% SDS; 15 min twice at 50° C with 0.16× SSC containing 0.1% SDS, and then were rinsed at room temperature with 2× SSC before proceeding to signal detection (color reaction).

Southern blot hybridization. DNAs from healthy AY-, BB-, and WX-infected periwinkle plants were digested with *Eco*RI and *Hin*dIII, electrophoresed in 1% agarose gels, alkali-denatured (0.2 N NaOH in 0.5 M NaCl for 30 min), and transferred by using a Bio-Rad trans blot cell (Bio-Rad Laboratories, Richmond, CA) to Zeta-probe membranes (0.45 μm; Bio-Rad Laboratories) according to manufacturer's operating instructions. The membranes were then baked at 80° C for 2 hr, prehybridized at 65° C, and then hybridized with ^{32}P-labeled riboprobes AY18-r and AY27-r, separately, as described in the previous section. After washing, the filters were dried and exposed to X-ray film for 24 hr with an intensifying screen.

In tests to determine whether cloned MLO DNA might have derived from MLO chromosomal DNA, undigested DNA isolated from healthy AY-, BB-, CN-, or WX-infected periwinkle plants was electrophoresed in 0.7% agarose gels and transferred onto Zeta-probe membranes as described above. The membranes then were hybridized with biotinylated DNA probes, pAY4, pAY7, pAY9, pAY19, pAY18, pAY22, pAY27, and pAY30, separately. Another probe, probe X, which was known to hybridize extensively with chromosomal DNA from healthy periwinkle plants, was used as a control in hybridizations with a replicate set of transblotted Zeta-probe membrane.

RESULTS

Partial purification of AY MLO from infected periwinkles by the sieve elements separation technique (Lee and Davis 1983) enriched the titer of AY MLOs in the preparation to more than 30 times that of extracts made from undigested leaf midribs. This conclusion is based on estimation of AY MLO-specific nucleic acids in the preparations by dot hybridization of serial dilutions of each DNA sample with a known AY-specific DNA probe (data not shown). About 40 μg of total nucleic acids was recovered from 6 g of midribs that was dissected from 50 g of whole leaves from AY MLO-infected periwinkle plants. Agarose gel electrophoretic analysis of crude DNA preparations from AY MLO- and BB MLO-infected, and from healthy periwinkle plants, indicated the presence, in the profiles of DNA preparations from the MLO-infected plants, of bands not present in profiles from healthy plants, suggesting occurrence of extrachromosomal DNA in AY and BB MLOs (Fig. 1).

Forty-three recombinant plasmids out of 175 were identified by dot hybridization to react with nucleic acid from AY-infected, but not from healthy, periwinkle plants. Among these 43 recombinants, at least 30 were unique, based on endonuclease restriction analysis and sizes of their

cloned insert DNA fragments. The sizes of inserts ranged from 400 to more than 4,000 bp nucleotides. Figure 2 illustrates representative results from this type of experiment.

Several AY MLO-specific recombinant plasmids were chosen for constructing DNA and RNA probes and for studying the specificity of these probes for detection of MLOs in periwinkle plants. Probes were labeled with either ^{32}P nucleotide or biotinylated nucleotide. ^{32}P-labeled recombinant plasmid DNAs, pAY18 and pAY27, hybridized broadly under low- (Fig. 3, A and B) and high- (Fig. 3, C and D) stringency wash conditions, with nucleic acid from AY-infected and several other MLO-infected, but not healthy, plants. The biotinylated pAY18 and pAY27 DNA probes exhibited similar sensitivity and cross hybridization (Fig. 4). When these two biotinylated DNA probes were used for dot blot hybridizations, it was possible to detect several strains of eastern and western AY MLO in infected plants, including periwinkle, China aster, and clover collected from various geographic locations (Table 1).

The generally broad reactivity of dsDNA probes was evident in the case of all DNA probes tested (Table 2). All AY MLO DNA probes hybridized with nucleic acid from plants infected by AY, BB, DB, or CN (= ORCH) MLOs. Eight out of nine probes also hybridized with WX and CX MLO nucleic acid, but only five and six probes hybridized with EY and VR MLO nucleic acid, respectively. None

Fig. 2. Endonuclease restriction analyses of some clones of AY-specific recombinant plasmids. Plasmids were digested with HindIII and PvuII, and electrophoresed in 1% agarose gel. Lane A, pAY1; lane B, pAY2; lane C, pAY3; lane D, pAY4; lane E, pAY5; lane F, pAY6; lane G, pAY7; lane H, pAY8; lane I, pAY9; lane J, pAY14; lane K, molecular weight (kilobase pairs) markers (lambda DNA/HindIII fragments and φX174, RF DNA/HaeIII fragments). From top band to bottom band (kb): 23.1, 9.4, 6.6, 4.4, 2.3, 2.0, 1.4, 1.1, 0.87, 0.60, 0.31; lane L, pAY17; lane M, pAY19; lane N, pAY20; lane O, pAY21; lane P, pAY24; lane Q, pAY25. Recombinant plasmids containing the plasmid pSP64 cloning vector are shown in lanes A, C, D, G, H, I, L, N, O, and P. Arrows indicate position of excised plasmid pSP64 fragment (plasmid pSP64 minus a 231-bp fragment). Additional band(s) represents insert(s) plus the 231-bp fragment. Recombinant plasmids containing the plasmid pSP65 are shown in lanes B, E, F, J, M, and P. The upper bands represent fragments of insert DNA and pSP65 plasmid minus a 238-bp fragment. The lower bands are the 238-bp fragments.

Fig. 1. Electrophoretic analyses of total nucleic acid extracted from MLO-infected and healthy periwinkle plants (Catharanthus roseus). Lane a, AY-infected; lane b, BB-infected; and lane c, healthy periwinkle. Note extra bands present in nucleic acid profiles from MLO-infected periwinkle.
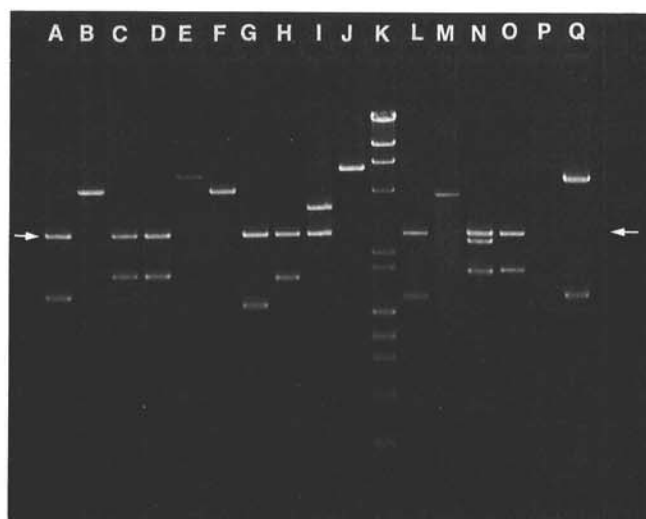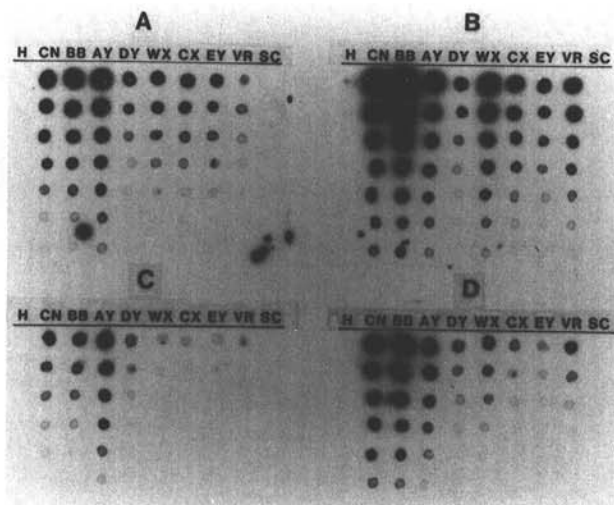

Fig. 3. Dot hybridizations of cloned AY MLO DNA to nucleic acid preparations from several MLO-infected and healthy periwinkle plants. Recombinant plasmids pAY18 and pAY27 were ^{32}P-labeled by nick-translation and hybridized separately with replicate filters followed by low- (A and B) or high- (C and D) stringency wash conditions. A and B, DNA probes pAY18 and pAY27, respectively; C and D, DNA probes pAY18 and pAY27, respectively. AY, aster yellows; DY, Western dwarf aster yellows; CN, unknown MLO (= ORCH1); BB, tomato big bud; WX, western X disease; CX, Canada X disease; EY, elm yellows; VR, beet leafhopper-transmitted virescence; SC, Spiroplasma citri; H, healthy.

hybridized with nucleic acid from *S. citri*-infected or healthy plants.

[32]P-labeled, ss AY18-r, transcribed from pAY18, riboprobe was found to hybridize more narrowly than its corresponding DNA probe used under the same conditions for hybridization and washing (Figs. 3 and 5). For example, pAY18 DNA probe under low stringency hybridized strongly with heterologous (non-AY) nucleic acid preparations from CN-, BB-, WX-, CX-, and EY-infected periwinkle plants and weakly with VR (Fig. 3A). The AY18-r riboprobe under the same conditions hybridized strongly with BB and EY nucleic acid, but weakly with CN, WX, and
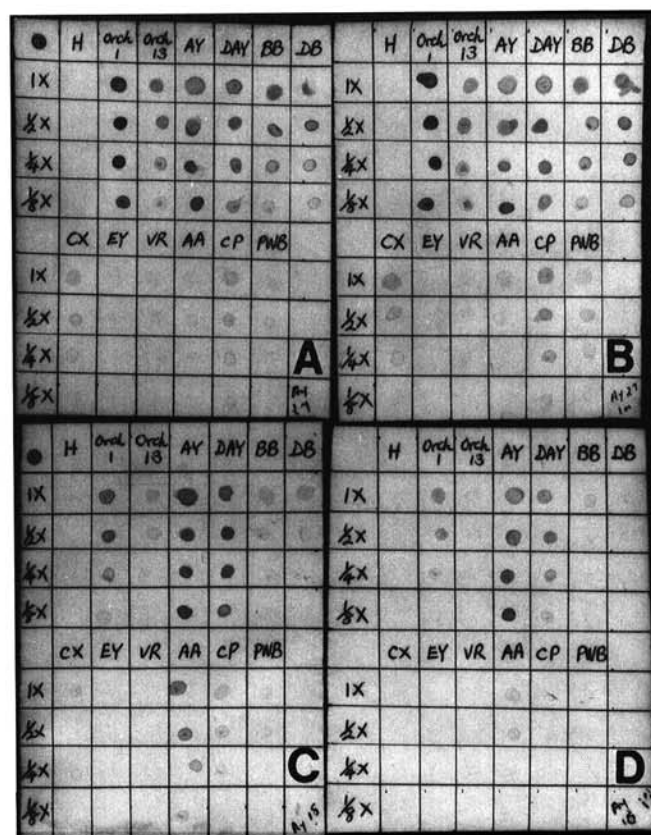

Fig. 4. Dot hybridizations of biotinylated cloned AY DNA probes to nucleic acid preparations extracted from several MLO-infected and healthy periwinkle plants. **A** and **C**, DNA probes pAY27 and pAY18, respectively, constructed by nick-translating the entire recombinant plasmids (pSP64 or pSP65 plasmid plus insert) with biotinylated nucleotides. **B** and **D**, DNA probes AY27 and AY18, respectively, constructed by labeling the excised inserts only. Note the similar patterns of hybridization when the entire plasmid and the excised insert were used as probes. Abbreviations are same as for Figure 3. In addition: AA, ash yellows; ORCH1 = CN; ORCH13 = CN13; DAY= DY; CP, clover proliferation; PWB, potato witches broom.

CX, and not at all with VR nucleic acid (Fig. 5A). Hybridization signals from the AY18 DNA probe and riboprobe with homologous (AY strain) nucleic acids were similar (Figs. 3A and 5A). The hybridization pattern obtained with riboprobe AY27-r was similar to that obtained with DNA probe pAY27. However, hybridization with AY27-r followed by RNase treatment resulted in strong signals with only two (CN and BB) heterologous (non-AY) MLOs, a weak signal with VR, and a very faint signal with WX (Fig. 6B). In the case of riboprobe AY18-r, following RNase treatment hybridization signal was seen only with homologous (AY) strains (Fig. 6A). Another two ^{32}P-labeled riboprobes used with RNase treatment, AY4-r and AY19-r, were found to hybridize with AY, DY, BB, and VR MLO DNA, and with AY, CN, and BB MLO DNA, respectively (Fig. 6, C and D). Four distinctly different hybridization patterns were obtained with these four riboprobes. These patterns, and those obtained with other riboprobes (see Table 2), confirmed that CN, BB, and AY disease MLOs were interrelated (Fig. 6B), but that these MLOs can be differentiated by using riboprobes.

Results from Southern blot analyses are shown in Figures 7 and 8. Figure 7 shows results from Southern blots of undigested DNA from healthy, AY-, BB-, CN-, (= ORCH1), and WX-infected plants. All four probes hybridized with chromosomal DNA; no evidence was seen of hybridization with extrachromosomal DNA. An additional five probes (pAY7, pAY18, pAY19, pAY27, and pAY30) were employed in the same type of experiment; all hybridized with chromosomal but not extrachromosomal DNA (data not shown). Figure 8 shows results from blots in which nucleic acid was isolated from healthy and AY-, WX-, and BB-infected plants, digested with *Eco*RI and *Hin*dIII endonucleases, and hybridized with riboprobes AY18-r and AY27-r. Riboprobe AY27-r hybridized strongly with several bands of AY and BB DNA, weakly with WX DNA, and not with healthy *C. roseus* DNA. Riboprobe AY18-r hybridized specifically with several bands of AY-DNA but not with BB, WX, and healthy plant DNA. Multiple bands may result from incomplete digestion of MLO DNA.

DISCUSSION

Because the MLOs associated with plant disease have not yet been obtained in pure culture *in vitro*, infected hosts must serve as sources of MLO DNA for molecular cloning. In their hosts, MLOs are present in relatively low titer and represent a minute amount of total components. Thus, for efficient cloning of MLO DNA, it is essential to concentrate MLO DNA while avoiding a similar concentration of host DNA. Infected insect vectors may be a useful source of

Table 1. Detection of different AY MLO strains by hybridization with cloned AY MLO DNA fragments as probes

Probe[b]	Hybridization with nucleic acid from indicated strain[a]										
	AY	DY	CAY	NYAY	OKAY-1	OKAY-2	WAY	NAY	H1	H2	H3
AY18	+	+	+	+	+	+	+	+	−	−	−
AY27	+	+	+	+	+	+	+	+	−	−	−

[a] AY, DY, NYAY, OKAY-1, and WAY in periwinkle (*Catharanthus roseus*) and healthy control H1; CAY, OKAY-2 in China aster (*Callistephus chinensis*) and healthy control H2; NAY in clover (*Trifolium repens*) and healthy control H3. Sources of infected and healthy hosts are described in the text. DY is Western dwarf aster yellows strain; WAY is western strain of AY. NAY is a noncelery-infecting strain of eastern AY. Nucleic acid was extracted from plants infected by the AY MLO strain indicated or from healthy plants.
[b] Biotinylated insert DNA fragments excised from cloned recombinant plasmids were used as probes for dot-blot hybridization analysis.

Table 2. Results from dot-blot hybridizations with labeled recombinant plasmids containing AY MLO DNA or with labeled RNA complementary to AY MLO DNA[a]

Probe	Insert size (bp)[c]		Type of probe[d]	Hybridization with nucleic acid from indicated source plants[b]										
				AY	DY	BB	DB	CN	WX	CX	EY	VR	SC	H
pAY4	1,660	(0)	DNA-B	+	NT[e]	+	NT	+	+	+	+	+	−	−
pAY7	1,180	(0)	DNA-B	+	NT	+	NT	+	+	+	+	+	−	−
pAY9	3,530	(0)	DNA-B	+	NT	+	NT	+	+	+	+	+	−	−
pAY16	1,640	(0)	DNA-B	+	+	+	+	+	+	+	−	−	−	−
pAY18	1,640	(0)	DNA-B	+	+	W[f]	W	+	+	+	−	−	−	−
pAY19	1,530	(0)	DNA-B	+	NT	+	NT	+	−	−	−	−	−	−
pAY27	2,330	(1)	DNA-B	+	+	+	+	+	+	+	−	+	−	−
pAY18	1,640	(0)	DNA-P	+	+	+	NT	+	+	+	+	W	−	−
pAY27	2,330	(1)	DNA-P	+	+	+	NT	+	+	+	W	+	−	−
AY4-r	1,660	(0)	RNA-P	+	+	W	NT	−	−	−	−	W	−	−
AY7-r	1,180	(0)	RNA-P	+	NT	+	NT	−	−	NT	−	−	−	−
AY-9	3,530	(0)	RNA-P	+	NT	+	NT	−	−	NT	−	−	−	−
AY18-r	1,640	(0)	RNA-P	+	+	−	NT	−	−	−	−	−	−	−
AY19-r	1,530	(0)	RNA-P	+	NT	+	NT	+	−	−	−	−	−	−
AY22-r	2,910	(0)	RNA-P	+	NT	+	NT	+	W	NT	−	W	−	−
AY27-r	2,330	(1)	RNA-P	+	+	+	NT	+	−	−	−	W	−	−
AY29-r	2,770	(0)	RNA-P	+	NT	+	NT	+	−	NT	−	−	−	−
AY30-r	4,164	(0)	RNA-P	+	NT	+	NT	+	−	NT	−	−	−	−

[a] Dot hybridizations were carried out as described in the text.
[b] Source plants of *C. roseus* (periwinkle) contained; AY, aster yellows; DY, western dwarf aster yellows; BB, tomato big bud; DB, blueberry stunt; CN, an unknown MLO from a Connecticut peach orchard; WX, Western X; CX, Canada X; EY, elm yellows; VR, beet leafhopper-transmitted virescence MLO; SC, *S. citri*; H, healthy.
[c] Sizes of double-stranded AY MLO DNA insert in recombinant plasmids are given. RNA probes were transcribed *in vitro* from cloned DNA inserts. The number of internal *Eco*RI and/or *Hin*dIII sites in cloned insert DNA is in parentheses.
[d] DNA-B, biotinylated DNA probe; DNA-P and RNA-P, ^{32}P-labeled double-stranded DNA probe and single-stranded riboprobe, respectively.
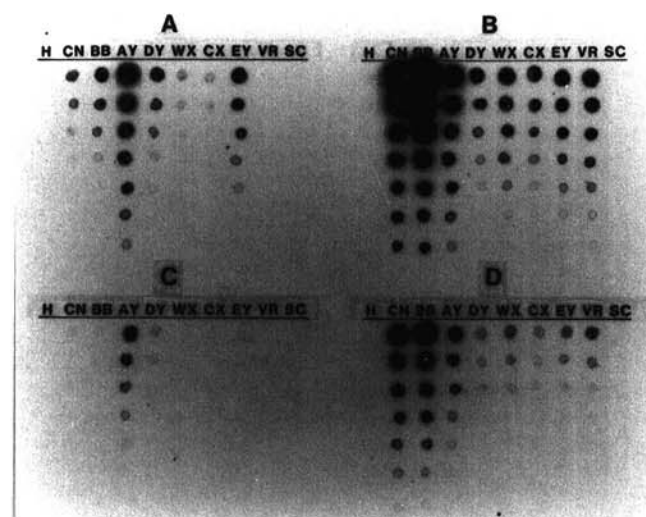[e] NT, not tested.
[f] W, weak hybridized signal.

Fig. 5. Dot hybridizations of ^{32}P-labeled riboprobes to nucleic acid preparations from several MLO-infected and healthy periwinkle plants. Riboprobes AY18-r (**A** and **C**) and AY27-r (**B** and **D**) were prepared by *in vitro* transcription from pAY18 and pAY27, respectively. Posthybridization washes were performed at low (**A** and **B**) or high (**C** and **D**) stringency. Filters received no posthybridization RNase treatment. Abbreviations are the same as in Figure 3.
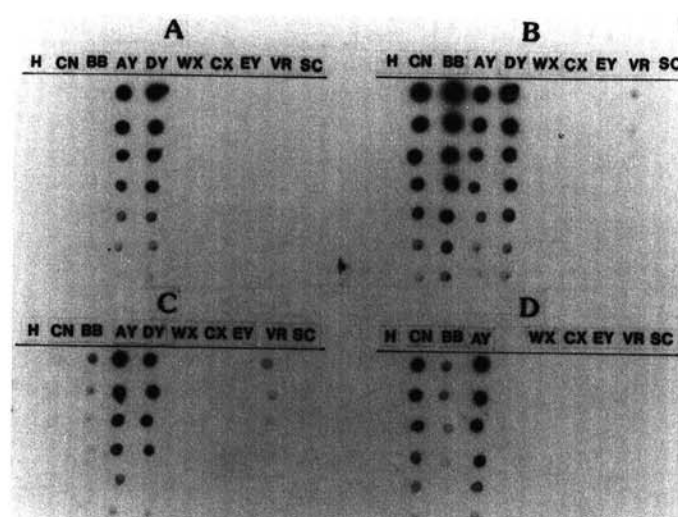

Fig. 6. Results from RNase treatment following dot hybridizations of ^{32}P-labeled riboprobes to nucleic acid preparations from several MLO-infected and healthy periwinkle plants. **A**, AY18-r; **B**, AY27-r; **C**, AY4-r; **D**, AY19-r. Note distinct hybridization patterns obtained with these four riboprobes. Abbreviations are the same as in Figure 3.

MLO DNA for cloning (Davis *et al.* 1987; Kirkpatrick *et al.* 1987), but some problems can be associated with this approach. For some MLO diseases, specific insect vectors are difficult to raise, and insect rearing can be time consuming. A laboratory may not be able to rear appropriate insect vectors because of facility or quarantine restrictions, and for many MLO diseases the identities of insect vectors are unknown. The use of infected plants as the source of MLO DNA obviates these problems. MLOs purified from infected plants would be a valuable source of DNA for molecular cloning. Attempts to purify uncultured MLOs directly from extracts of infected plants have yielded some encouraging reports (Clark *et al.* 1983; Jiang and Chen 1987; Sinha 1979). Our approach was to begin by exploiting the fact that MLOs are confined to sieve elements in infected plants and that these cells constitute only a small proportion of the entire plant mass.

By employing the sieve elements enzymatic separation technique developed earlier in this laboratory (Lee and Davis 1983), we have obtained preparations enriched for AY MLO. DNA extracted from such preparations proved to be highly suitable for cloning and screening of AY MLO-specific DNA. This strategy has also been successfully used for cloning MLO-specific DNA from plants infected by other MLO diseases, including tomato big bud, elm yellows, and an unknown MLO termed ORCH1 (Davis et al. 1988; Lee et al. 1988). The strategy provides a general procedure that should be applicable for the cloning of DNA from any phloem-inhabiting pathogen.

Under the hybridization conditions described above, both DNA probes and riboprobes hybridized with nucleic acid preparations from AY-infected periwinkle plants but not with preparations from healthy plants. However, single-stranded riboprobes hybridized more discriminately with nucleic acid from heterologous MLO diseases than did their corresponding dsDNA probes, which contained sequences of both plasmid pSP64 or pSP65 and MLO-specific insert or insert only (see Figs. 3, 4, and 5). An advantage of using dsDNA probes for detection is the possible amplification of signals of MLO-specific hybridization. This could conceivably result from hybridization among sequences in the probes and/or consequent network formation with excess dsDNA probe under prolonged hybridization (Meinkoth and Wahl 1984). However, this enhancement of hybridization signal with DNA probes may not necessarily be proportional to the degree of probe and target MLO nucleotide sequence similarity. Thus, a strong hybridization signal may be observed with a given DNA probe-heterologous target combination, whereas only weak or no signal may be observed with a corresponding single-

stranded riboprobe (see Figs. 3A and 5A). For these reasons, and because RNase treatment removes mismatched bound probe, the use of single-stranded riboprobes can be particularly useful where identification and differentiation of uncultured MLOs is important. Nevertheless, signal enhancement by network formation may confer an advantage to DNA probes, provided that an unsatisfactory signal-to-noise ratio is not obtained by use of excessive amounts of probe.

By employing several riboprobes for preliminary study of interrelatedness among MLOs, we were able to differentiate various MLO-associated diseases. For example, using riboprobes AY18-r, AY27-r, AY19-r, and AY4-r, three MLO-associated diseases (AY, BB, and CN) could be differentiated (see Fig. 6). The MLOs in these diseases were related, based on detection of all three by probe AY27, but distinct from one another based on differences in hybridization patterns of the diseases with RNA probes AY18-r, AY19-r, and AY4-r. In contrast to other MLOs tested, BB and CN hybridized with a high proportion of the riboprobes prepared from cloned DNA of AY MLO. On the basis of our data (see Table 2), we suggest that these three MLOs are distinctly different strains that probably belong to a cluster of strains that are more closely related genetically to one another than to other MLOs.

Genetic relatedness among several MLOs (including AY, BB, CN, DB, WX, EY, and others) is inferred from hybridizations between these MLOs and our probes, because results indicated that the cloned MLO-specific DNA was MLO chromosomal DNA. Although we cloned AY chromosomal DNA, other results from this work
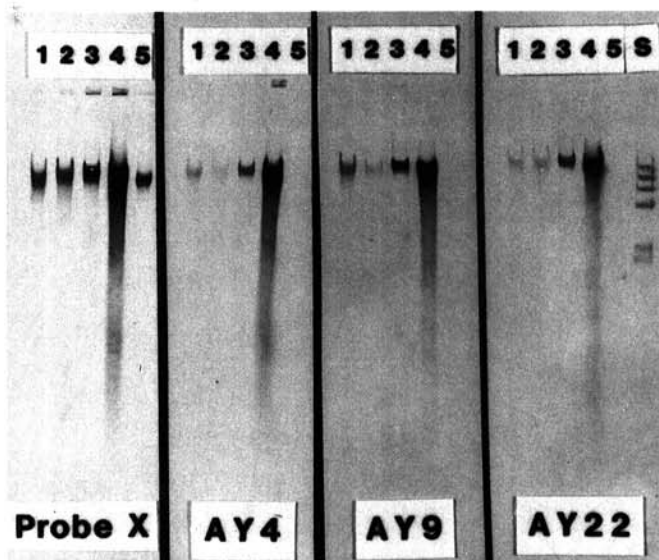

Fig. 7. Southern blot hybridization analysis of undigested DNA from healthy or MLO-diseased periwinkle plants. Nucleic acid extracted from plants was electrophoresed in 0.7% agarose gel, then transferred to Zeta-probe membrane, hybridized to biotinylated DNA probes. Probe X, a probe known to hybridize with chromosomal DNA of healthy periwinkle. AY4, pAY4; AY9, pAY9; AY22, pAY22. Nucleic acid preparations: lane 1, western X disease MLO; lane 2, MLO strain CN (= ORCH1); lane 3, tomato big bud; lane 4, Beltsville aster yellows; lane 5, healthy periwinkle. Lane S, molecular weight markers lambda DNA/HindIII fragments of the following sizes (kb), from top to bottom land: 23.1, 9.4, 6.6, 4.4, 2.3, 2.0.
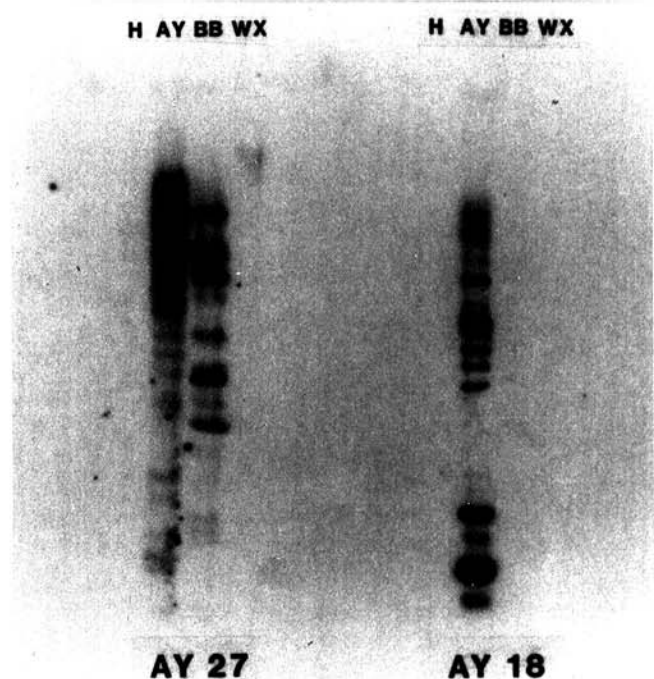

Fig. 8. Southern blot analyses. ^{32}P-labeled RNA probes, complementary to cloned AY MLO DNA inserts pAY18 and pAY27, were hybridized with electrophoresed (1% agarose gel) nucleic acid preparations that had been extracted from infected (AY, BB, and WX) and healthy periwinkle plants and digested with EcoR1 and HindIII restriction endonucleases. Abbreviations are the same as in Figure 3.

suggest that uncultured plant-inhabiting MLOs, including AY MLO and BB MLO, contain extrachromosomal DNA (see Fig. 1). It is possible that examination of extrachromosomal DNA may also reveal nucleotide sequence similarities among some MLOs.

It has become feasible to illustrate, by direct nucleic acid hybridizations, the occurrence of nucleotide sequence homologies between different MLOs and gain insight into their genetic interrelatedness. MLOs that are associated with numerous diseases have been claimed to belong to or be closely related to AY MLO entirely on the basis of biological properties. In this work, we have confirmed that an eastern AY strain is closely related to a western AY strain and have postulated the existence of a cluster of AY-related but distinct MLOs.

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