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Cloned DNA Probes Specific for Detection of a Mycoplasmalike Organism Associated with Ash Yellows

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DNA was isolated from periwinkle (*Catharanthus roseus*) infected with a mycoplasmalike organism (MLO) that originated in white ash (*Fraxinus americana*) affected by ash yellows. The DNA was digested with *Eco*RI and *Hin*dIII, ligated to plasmid vector pIBI30, and cloned in *Escherichia coli* DH5α. Cloned DNA inserts were excised from four ash yellows-specific recombinant plasmids, labeled with biotin, and employed as probes in dot and Southern hybridizations. None of the probes hybridized with nucleic acid from healthy plants; all hybridized with nucleic acid from periwinkle infected by the ash yellows MLO. Southern hybridization analyses showed the probes to contain MLO chromosomal DNA. In dot hybridizations performed at 42° C, probe AA13I hybridized only with nucleic acid from plants infected by the ash yellows MLO, whereas the other three probes, designated AA82I, AA157I, and AA176I, hybridized with nucleic acid from plants infected by any of several MLOs, including the ash yellows MLO. Under conditions of high stringency (52° C), all four probes hybridized only with nucleic acid from plants infected by the ash yellows MLO. In diagnostic tests on naturally diseased plants, probes AA13I and AA176I hybridized with nucleic acid extracted from leaves, twigs, trunk phloem, and roots of white ash with symptoms of ash yellows but not with nucleic acid from healthy ash grown from seed. The findings provide means for specific detection and identification of ash yellows MLO and support the concept that this MLO represents a distinct strain cluster.

Ash yellows disease (AshY) (Matteoni and Sinclair 1985) is a common factor in the decline of ash in the central and eastern United States (Ferris *et al.* 1989; Han *et al.* 1991; Sinclair *et al.* 1990). This malady, which has been attributed to mycoplasmalike organisms (MLOs) (Hibben and Wolanski 1971; Matteoni and Sinclair 1985), is damaging to both forest and shade trees in North America. The known geographical distribution of AshY has expanded markedly in recent years (French *et al.* 1989; Matteoni and Sinclair 1988; Sinclair *et al.* 1987, 1990), underscoring the need to develop means to manage the disease, but many aspects of AshY and its pathogen are not yet understood. For example, the pathogen is presumed to be transmitted by insects, but the vector role suggested for two species (Matteoni and Sinclair 1988) has not been confirmed. Also, the possibility that alternative host plants may serve as reservoirs of AshY MLOs has not been evaluated.

A major problem in understanding and management of AshY also arises from difficulties in achieving accurate diagnosis. Although loss of vigor and progressive decline are typical of the AshY syndrome, these symptoms are not diagnostic (Matteoni and Sinclair 1985; Sinclair *et al.* 1989); factors other than mycoplasmal infection also cause ash to decline (Sinclair *et al.* 1990). Development of witches'-broom is diagnostic of the disease in ash, but only a small percentage of affected trees display this symptom at a given time (Matteoni and Sinclair 1985; Sinclair *et al.* 1989, 1990). The DAPI (4′,6-diamidino-2-phenylindole dihydrochloride) fluorescence test has become standard for diagnosis of AshY (Hibben and Franzen 1987; Sinclair *et al.* 1989, 1990; Smallidge 1989, 1990) and has contributed to current understanding of the occurrence of the disease, but this test is not specific for AshY MLOs, is of limited usefulness in efforts to detect other plant hosts, and is not applicable in the search for insect vectors. Without the means to distinguish AshY from other MLOs, much needed study of the disease has remained infeasible.

In recent years, cloned MLO DNA probes or riboprobes and amplification of specific MLO DNA sequences by the polymerase chain reaction (PCR) have afforded broad detection of MLOs associated with a wide variety of diseases and detection of some specific MLO strains in their plant and/or insect hosts (Bertaccini *et al.* 1990a; Davis *et al.* 1988a,b, 1990a,b; Deng and Hiruki 1990; Kirkpatrick *et al.* 1987; Lee *et al.* 1988a,b; Lee and Davis 1988, 1990; Kollar *et al.* 1990; Kuske and Kirkpatrick 1990; Schaff *et al.* 1990; Sears *et al.* 1989). In addition, such probes have permitted the recognition of distinct clusters of genetically related strains of MLOs and distinction among strains within a cluster (Davis *et al.* 1988b; Lee and Davis 1988; Lee *et al.* 1988a, 1990b). This progress encouraged the work reported herein on development of cloned DNA probes specific for AshY MLOs. Use of these specific probes makes it possible to identify AshY MLOs and to study the ecology of these mollicutes.

MATERIALS AND METHODS

MLO and molecular cloning of DNA. A strain of AshY MLO, designated AshY1 and collected in New York State (Matteoni and Sinclair 1983), was maintained in greenhouse-grown plants of Madagascar periwinkle (*Catharanthus roseus* (L.) G. Don) and used as the source of DNA for molecular cloning. Sieve cells were isolated by enzymatic

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digestion of veinal tissue from diseased plants, and a concentrated, MLO-enriched fraction was prepared from which DNA was purified (Lee and Davis 1988). For cloning, this DNA, which contained plant DNA as well as DNA of the AshY MLO, was double-digested with restriction endonucleases *Eco*RI and *Hin*dIII and ligated to plasmid pIBI30 (International Biotechnologies, Inc., New Haven, CT). Competent cells of *Escherichia coli* strain DH5α (Life Technologies, Inc., Gaithersburg, MD) were then transformed with the ligation mixture according to the supplier's protocol. Transformants containing recombinant plasmids were identified by screening on Luria-Bertani (Maniatis *et al.* 1982) agar medium containing X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) obtained from Life Technologies, Inc. Recombinant plasmids from transformant colonies picked at random were labeled by nick translation with biotin-7-dATP obtained from Life Technologies, Inc. and used as probes in dot hybridizations. Cloned DNA inserts were excised from recombinant plasmids, which hybridized with nucleic acid extracted from periwinkle infected with the AshY MLO but not with nucleic acid from healthy periwinkle. The inserts were excised from recombinant plasmids by double-digestion with *Eco*RI and *Hin*dIII and purified by electroelution after electrophoresis in 0.7% agarose gel. The inserts were labeled with biotin-7-dATP as above and used as probes in subsequent experiments.

Restriction sites within cloned fragments of MLO DNA were examined by use of the following restriction endonucleases, employed according to manufacturer's specifications: *Pst*I, *Sal*I, *Xba*I, *Eco*RI, *Hin*dIII, *Bam*HI, *Sma*I, *Xho*I, *Kpn*I, and *Sst*I (Life Technologies, Inc.).

Plant samples and MLO strains. Samples consisting of young leaves and/or succulent stems from MLO-infected or healthy periwinkle plants or of nucleic acid extracted from such plants were kindly supplied by the following researchers: dwarf western aster yellows (strain DAY) by Alexander Purcell, University of California, Berkeley; potato witches'-broom and clover proliferation by Chuji Hiruki, University of Alberta, Edmonton; western X by Bruce Kirkpatrick, University of California, Berkeley; Canada X by Lloyd Chiykowski, Agriculture Canada, Ottawa; tomato big bud by James Dale, University of Arkansas, Fayetteville; periwinkle little leaf (strain O-1) and eastern X disease (strain BX3) by Sharon Douglas, Connecticut Agricultural Experiment Station, New Haven; an MLO (strain GY) recovered in trap plants of periwinkle placed in a vineyard in Italy and supplied by Rino Credi, University of Bologna, Italy; and beet leafhopper-transmitted virescence MLO and *Spiroplasma citri* supplied by George N. Oldfield, University of California, Riverside. With the exception of strain GY, the designation noted for a strain of MLO is that provided by the supplier of the sample. Elm yellows MLO was obtained in a previous study (Braun and Sinclair 1979) from a diseased elm tree in New York State, was transferred to periwinkle through use of dodder (*Cuscuta* sp.), and was maintained by grafting in greenhouse-grown plants of periwinkle. Aster yellows MLO was field-collected in Maryland (Lee and Davis 1988).

Dot and Southern hybridizations. Plant samples consisting of 0.3 g of fresh or frozen (−20° C) tissues were submerged in liquid nitrogen and pulverized with a mortar and pestle. Nucleic acid was extracted and denatured as described elsewhere (Lee and Davis 1988; Davis *et al.* 1990a). After denaturation, a twofold dilution series of the nucleic acid was prepared (6× SSC [1× SSC = 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0] was the diluent), and a 3-μl vol of each dilution of each sample was applied to nitrocellulose membranes as described (Bertaccini *et al.* 1990a; Lee and Davis 1988; Davis *et al.* 1990a). The presence of a sufficient quantity of detectable MLO-specific DNA in each sample spot was verified separately by hybridization of control membranes to homologous DNA probes (data not shown). The temperature of the hybridization reaction was varied to achieve hybridizations at two levels of stringency. After a prehybridization treatment of 4-hr duration (Lee and Davis 1988), dot hybridizations were performed for 16 hr, either at 42° C (moderate stringency) or at 52° C (high stringency), in the presence of 5× SSC, 1× Denhardt's solution, 20 mM 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 DNA probe per milliliter. Membranes were washed twice for 3 min at room temperature with 2× SSC containing 0.1% sodium dodecyl sulfate (SDS), twice for 3 min at room temperature with 0.2× SSC containing 0.1% SDS, and twice for 15 min at 50 C with 0.16× SSC containing 0.1% SDS, and then were rinsed at room temperature with 2× SSC before signal detection was performed. Membrane blocking and visualization of the hybridized biotinylated probe, using Streptavidin -Alkaline phosphatase conjugate (BluGENE Nonradioactive Nucleic Acid Detection System, Life Technologies, Inc.) and nitroblue tetrazolium plus 5-bromo-4-chloro-3-indolyl phosphate, were as previously described (Lee and Davis 1988).

For Southern hybridization analyses, nucleic acid was extracted from plants according to the procedure of Dellaporta *et al.* (1983). Electrophoresis, staining, transfer of nucleic acid to nitrocellulose membranes, and hybridizations with biotin-labeled probes were performed as described (Bertaccini *et al.* 1990; Davis *et al.* 1990a; Lee and Davis 1988; Lee *et al.* 1990a). As controls for detection of extrachromosomal DNA, we employed samples containing DNA of both AshY MLO and AY MLO. Control samples consisted of DNA extracted from plant tissue infected by AshY MLO to which an equal fresh weight of tissue infected by AY MLO had been added. Electrophoresis and transfer to nitrocellulose membranes were carried out as for test samples. Samples on control membranes were hybridized against probe pAY24, which detects extrachromosomal DNA in AY MLO (I. M. Lee, unpublished).

Plant samples for diagnostic tests. Samples of periwinkle consisted of succulent stems and leaves, which displayed degrees of dwarfing and chlorosis, from diseased plants containing AshY1 MLO. Samples of white ash (*F. americana*) consisted of leaves, twigs, living bark from the base of the trunk, roots 2–6 mm in diameter (coarse roots), and feeder roots. The ash samples were collected in August 1989 from each of six trees, over 50 ft in height and estimated to be at least 75 yr old growing near Ithaca, NY,

that had symptoms of AshY disease, and in the roots of which MLOs had previously been detected by means of the DAPI fluorescence test (Sinclair *et al.* 1989). In the present work, this test was again performed on each sample, except on the fine roots, from each tree. Material sufficient for the DAPI test was preserved in 2.5% aqueous glutaraldehyde in 0.1 M phosphate buffer, pH 7.0, until sections were cut and examined as described (Sinclair *et al.* 1989). For examination of phloem of the trunk, radial sections were cut from a block of tissue 2 × 2 × 6 mm with the cambial surface on a long face. The remainder of each sample was frozen at −20° C until a portion was weighed out for nucleic acid extraction. Nucleic acid was extracted from rachises of leaves and midribs of leaflets; secondary phloem of twigs; the innermost millimeter, approximately, of phloem from the base of the trunk; secondary phloem of coarse roots; and clusters of feeder roots.

RESULTS

Probe selection. Four recombinant plasmids, which hybridized with nucleic acid from periwinkle containing AshY1 but not with nucleic acid from healthy periwinkle plants (data not shown), were selected for this study. Insert DNA fragments excised from these four recombinant plasmids are AA13I, AA82I, AA157I, and AA176I. DNA fragment AA13I is a sequence of approximately 950 bp that contains no apparent internal sites for digestion by any of the endonucleases tested. Fragment AA82I is approximately 2,200 bp in size and contains sites for *Sst*I, *Bam*HI, and *Pst*I. Fragment AA157I is a sequence of approximately 1,000 bp and contains a site for *Xba*I. Fragment AA176I is approximately 1,050 bp long and contains a site for *Sst*I. These four cloned fragments were labeled with biotin and employed as probes in dot hybridizations with total nucleic acid extracted from periwinkle, both healthy and infected with AshY1. Hybridizations at 42° and 52° C gave the same results. All four probes hybridized with nucleic acid from infected plants; no hybridization signals were seen with nucleic acid from healthy plants (Table 1).

Probe specificity in relation to hybridization stringency. The four probes derived from AshY1 were next tested for specificity by dot hybridizations against nucleic acid extracted from periwinkle plants infected by one or another of 13 different MLOs or *S. citri*. The influence of stringency of hybridization conditions on probe specificity for MLO detection was examined by performing the hybridizations at 42° C and at 52° C. At both temperatures, probe AA13I hybridized only with nucleic acid from plants infected by AshY1. At 42° C, the remaining three probes hybridized not only with nucleic acid from plants infected by AshY1 but also with nucleic acid from plants infected by other MLOs (Table 1). Under the more stringent hybridization conditions at 52° C, however, all four probes hybridized only with nucleic acid from plants containing AshY1. The range of hybridizations with probe AA176I at 42° C and its hybridization at 52° C only with nucleic acid from ash yellows MLO-infected plants are illustrated in Figure 1.

Southern hybridization analyses. Results from electrophoresis of undigested nucleic acid samples extracted from plants of periwinkle, either healthy or infected with AshY1, and Southern hybridizations with probe AA13I are illustrated in Figure 2. Probe AA13I hybridized with chromosomal DNA of AshY1 MLO; no evidence was observed for hybridization with extrachromosomal DNA. Probes AA82I, AA157I, and AA176I also hybridized only with chromosomal DNA of AshY1 MLO; ability to detect extrachromosomal DNA when present in nucleic acid samples was indicated by positive detection of extrachromosomal DNA of AY MLO by Southern hybridizations of control samples containing both DNA of AshY1 MLO and DNA of AY MLO (data not shown). Consistent with results from dot hybridizations summarized in Table 1, probe AA13I did not hybridize with DNA from MLOs associated with elm yellows, clover proliferation, potato witches'-broom, or aster yellows (data not shown).

Detection of AshY MLOs in ash. Two probes, AA13I and AA176I, were employed in attempts to detect AshY

Table 1. Influence of temperature on specificity of dot hybridizations

Probe[b]	Temperature[c]	Hybridization with nucleic acid of MLO strain indicated[a]												
		H	AA	AY	BB	CP	CX	DAY	EY	0-1	PWB	VR	WX	SC
AA13I	42	−	+	−	−	−	−	−	−	−	−	−	−	−
	52	−	+	−	−	−	−	−	−	−	−	−	−	−
AA82I	42	−	+	−	−	W	−	−	−	−	W	−	+	−
	52	−	+	−	−	−	−	−	−	−	−	−	−	−
AA157I	42	−	+	−	−	W	−	−	−	−	W	−	−	−
	52	−	+	−	−	−	−	−	−	−	−	−	−	−
AA176I	42	−	+	−	−	+	−	−	+	−	+	−	−	−
	52	−	+	−	−	−	−	−	−	−	−	−	−	−

[a] Nucleic acid samples were extracted from healthy (H) plants of *Catharanthus roseus* or from *C. roseus* singly infected by mycoplasmalike organisms (MLOs) of ash yellows (AA), aster yellows (AY), Canada X disease (CX), clover proliferation (CP), dwarf aster yellows (DAY), elm yellows (EY), periwinkle little leaf (0-1), potato witches'-broom (PWB), tomato big bud (BB), vinca virescence (VR) = beet leafhopper transmitted virescence western X disease (WX), or by *Spiroplasma citri* (SC). +, Positive hybridization signal. W, weak-positive hybridization signal. −, No hybridization signal.

[b] Each probe consisted of a cloned fragment of DNA from MLO strain AshY1, excised from a recombinant plasmid and labeled with biotin.

[c] Hybridizations were performed at 42° or 52° C in the presence of 45% formamide, 5× SSC (1× SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 1× Denhardt's solution (0.08% Ficoll, 0.08% PVP, 0.08% bovine serum albumin), 5% dextran sulfate, 20 mM sodium phosphate (pH 6.5), and salmon sperm DNA (0.2 mg/ml).

MLOs in naturally diseased white ash trees. Both probes detected AshY MLOs in all six trees examined (Table 2). Representative results obtained with probe AA176I are illustrated in Figure 3. Samples from trunk phloem consistently yielded positive hybridization signals with both probes, whereas samples from other plant parts yielded fewer and/or weaker hybridization signals. A similar pattern of results was obtained when the DAPI test was used on the same plant samples, except that with DAPI, positive results were obtained as often from coarse roots as from trunk phloem (Table 2). No hybridization was obtained with nucleic acid extracted from similar tissues from healthy ash.

DISCUSSION

In this and other work, the ability to clone MLO DNA has made possible the development of sensitive probes capable of detecting the presence of various MLOs in infected plant and insect tissues (Bertaccini *et al.* 1990a,b; Davis *et al.* 1988a,b,c, 1990a,b,c; Deng and Hiruki 1990; Kirkpatrick *et al.* 1987; Kollar *et al.* 1990; Kuske and Kirkpatrick 1990; Lee and Davis 1988; Lee *et al.* 1988a,b, 1990a,b,c; Schaff *et al.* 1990; Sears *et al.* 1989). The present study provides probes for apparently specific detection of an ash yellows MLO and provides data supporting the contention that ash yellows MLO is distinct from the other MLOs examined. Thus, AshY1 was distinguished from other MLOs by failure of one of the probes at 42° C and of all four probes at 52° C to hybridize with nucleic acid from plants with MLOs other than an ash yellows MLO.

Because probes AA13I, AA82I, AA157I, and AA176I hybridized at 52° C only with nucleic acid of AshY1 and not with nucleic acid of 12 other MLOs tested, all of these probes, when used at high stringency, are apparently spe-

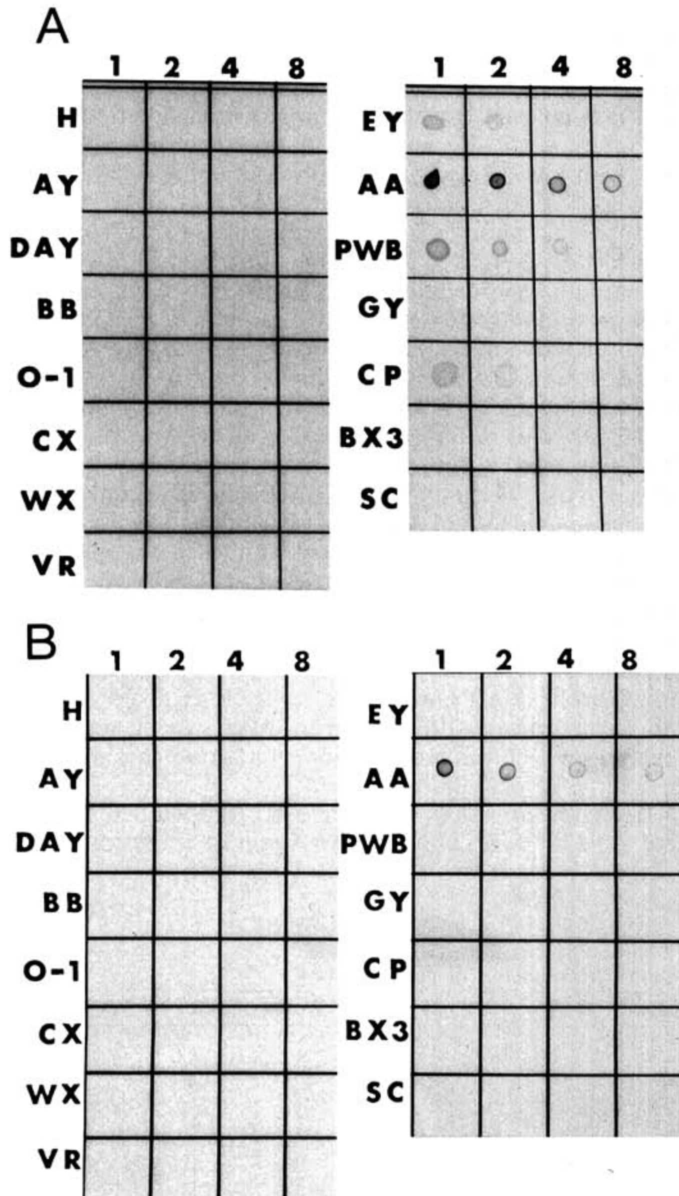

Fig. 1. Dot hybridizations of biotinylated DNA probe AA176I to nucleic acid from mycoplasmalike organism (MLO)-infected or healthy plants of periwinkle (*Catharanthus roseus*). **A,** hybridization performed at 42° C. **B,** hybridization performed at 52° C. H, healthy; AA, ash yellows MLO strain AshY1; AY, aster yellows; BB, tomato big bud; BX3, eastern X disease; CP, clover proliferation; CX, Canada X; DAY, western dwarf aster yellows; EY, elm yellows; GY, vineyard yellows; O-1, periwinkle little leaf; PWB, potato witches'-broom; SC, *Spiroplasma citri*; VR, beet leafhopper-transmitted virescence; WX, western X. 1, undiluted nucleic acid extract. 2, 4, 8, reciprocals of dilutions.

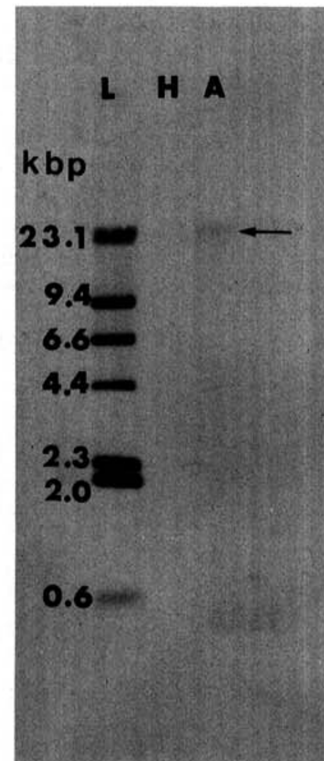

Fig. 2. Southern hybridization analyses of undigested DNA extracted from healthy and ash yellows (AshY1) mycoplasmalike organism (MLO)-infected plants of periwinkle (*Catharanthus roseus*). After electrophoresis in 0.7% agarose gel and transfer to nitrocellulose membrane, DNA was hybridized to biotinylated probe AA13I. H, nucleic acid sample extracted from healthy plants. A, nucleic acid sample extracted from AshY1 MLO-infected plants. L, biotinylated lambda DNA/*Hin*dIII fragments. Arrow denotes position of chromosomal DNA.

cific for AshY MLOs. Probe AA13I was specific for AshY MLOs even under conditions of moderate stringency. The possibility exists, however, that one or more of these probes may hybridize with nucleic acid of an as yet untested MLO, different from AshY1 but having a region of DNA homologous with that of the four AshY1 DNA probes. Nevertheless, because probes AA13I and AA176I hybridized under conditions of high stringency with nucleic acid from various organs of white ash trees naturally affected by AshY, we conclude that these trees contained MLOs identical with or very closely related to AshY1.

DAPI staining of sections from leaves, twigs, trunk phloem, and coarse roots yielded an MLO detection pattern similar to that obtained with the probes, except that DAPI more often revealed infection of coarse roots. We attribute this disparity in results to the relatively small proportion of conductive phloem in the root tissues that comprised the samples from which nucleic acid was extracted for dot hybridizations. These samples included all tissues between the vascular cambium and the periderm, whereas the phloem containing MLOs consisted, according to results of the DAPI test, only of a narrow band adjacent to the vascular cambium. For further field applications of the probes, increased sensitivity of AshY MLO detection conceivably could be attained by radiolabeling the probes.

The correspondence between results from DAPI staining and probe hybridizations should encourage further applications of these procedures in concert. For example, the more rapid DAPI staining might be employed in a general screening of plants for the presence of a mollicute, and those plants found to be infected could be tested for presence of a specific MLO, in this case an ash yellows MLO, employing plant tissues most appropriate for each test. It would be of interest to apply these methods to investigate the association of ash yellows MLO with decline of ash in regions other than New York State.

The four cloned DNA fragments from AshY1 employed in this work probably represent only a small percentage of the MLO genome (perhaps 2–3%, if the genome size of AshY MLO were about 800 kbp), and it is not known what significance these sequences may have in determining phenotype of AshY MLOs. Nevertheless, our results have revealed nucleotide sequence homologies between ash yellows and some other MLOs. For example, in dot hybridizations at moderate stringency, probe AA82I hybridized with nucleic acid extracted from periwinkle infected by MLOs associated with potato witches'-broom, clover proliferation, or western X disease, and probe AA176I hybridized with nucleic acid from plants infected by MLOs associated with elm yellows, potato witches'-broom, or clover proliferation. Southern hybridization analyses revealed that the probes contain chromosomal DNA of the ash yellows MLO, indicating that the homologies detected are at the level of chromosomal DNA.

Even at moderate stringency, hybridizations using the four AshY1 DNA probes did not reveal any genetic relatedness between ash yellows and certain other MLOs, including those associated with aster yellows, beet leafhopper-transmitted virescence, Canada X disease, periwinkle little leaf, tomato big bud, and western dwarf aster yellows. However, previous moderate stringency dot hybridization studies, with probes derived from MLOs other than AshY1, have shown ash yellows MLO to share some genomic DNA

Table 2. Detection of mycoplasmalike organisms (MLOs) in naturally diseased white ash by means of DNA probes specific for an ash yellows MLO and by means of the DAPI test

		Hybridization with nucleic acid and DAPI test result[a]				
Probe[b]	**Tree**	**Leaves**	**Twigs**	**Trunk phloem**	**Coarse roots**	**Fine roots**
AA13I	A	W (+)[c]	+ (−)	+ (+)	+ (+)	+(ND)
	B	W (+)	+ (−)	+ (+)	− (+)	+(ND)
	C	+ (−)	+ (−)	+ (+)	W (+)	+(ND)
	D	W (−)	W (−)	W (+)	W (+)	+(ND)
	E	W (−)	W (−)	+ (+)	+ (+)	+(ND)
	F	W (−)	W (−)	+ (+)	W (+)	+(ND)
AA176I	A	W (+)	− (−)	+ (+)	− (+)	+(ND)
	B	W (+)	W (−)	+ (+)	− (+)	W(ND)
	C	− (−)	− (−)	+ (+)	− (+)	−(ND)
	D	+ (−)	− (−)	W (+)	+ (+)	W(ND)
	E	− (−)	− (−)	+ (+)	+ (+)	W(ND)
	F	− (−)	− (−)	+ (+)	+ (+)	+(ND)

[a] Hybridizations were performed at 52° C; other conditions were as those given in Table 1. +, Positive hybridization signal. W, weak-positive hybridization signal. −, No hybridization signal.

[b] Each probe consisted of a biotin-labeled cloned fragment of DNA from MLO strain AshY1.

[c] Results from DAPI staining of plant tissues are in parentheses. +, Fluorescence in phloem sieve tubes. −, No DAPI fluorescence in sieve tubes. ND, not determined because feeder roots are not convenient for sectioning for the DAPI test.

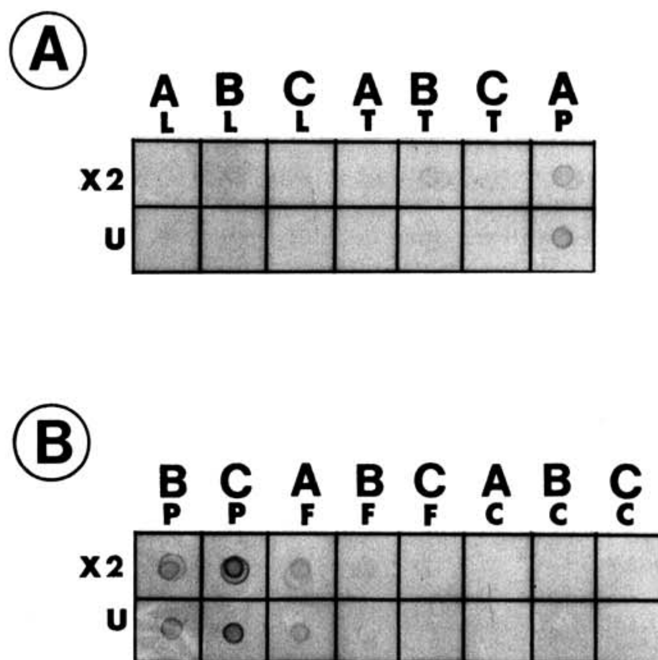

Fig. 3. Dot hybridization of biotinylated cloned DNA probe AA176I to nucleic acid extracted from tissue samples of white ash (*Fraxinus americana*) showing symptoms of ash yellows disease. **A** and **B,** field-collected samples from three separate, naturally infected trees of ash, designated A, B, and C, respectively. Samples from ash consisted of tissues from leaves (L), twigs (T), fine roots (F), coarse roots (C), or trunk phloem (P). Membranes were spotted with 6 μl (X2) or with 3 μl (U) of undiluted nucleic acid extract. Hybridizations were performed at 52° C in the presence of 45% formamide.

homology with all of these MLOs with the exception of the beet leafhopper-transmitted virescence MLO (Lee and Davis 1988; Davis *et al.* 1988b, 1990a). Both these studies and the present results distinguished ash yellows from all other MLOs studied and indicated that some apparently distant nucleotide sequence homologies exist between the ash yellows and several other MLOs.

In previous work, we have employed cloned MLO DNA probes to examine MLO interrelatedness. Patterns of hybridization of the probes with nucleic acid from MLOs associated with various diseases were interpreted to indicate the existence of MLO strain clusters, each containing strains that are more closely related to one another than to other MLOs (Bertaccini *et al.* 1990a,b; Davis *et al.* 1988b, 1990a,b; Lee and Davis 1988; Lee *et al.* 1990a,b). Results from PCR (Schaff *et al.* 1990) and from work with monoclonal antibodies (Lee *et al.* 1990c) are consistent with this interpretation. Lee and Davis (1988) proposed the existence of an MLO strain cluster that includes MLOs associated with aster yellows, periwinkle little leaf, and tomato big bud, but does not include ash yellows MLO. Our present results, as well as other data (Davis *et al.* 1988b, 1990a; Lee *et al.* 1990a), indicate that AshY1 is not as closely related genetically to aster yellows MLOs as are MLOs in that cluster. AshY1 appears to represent an MLO strain cluster that is distinct from the aster yellows strain cluster.

In areas where ash yellows occurs in North America, there also occur other MLO-associated diseases of trees and shrubs, including bunch disease of walnut, eastern X disease of stone fruits, elm yellows, lilac witches'-broom, and witches'-broom disease(s) of *Cornus* spp. Others have queried the possible relationship of these diseases to ash yellows (Hibben 1989; Hibben and Franzen 1987, 1989; Matteoni and Sinclair 1988). Data presented here and in previous reports from our laboratories show that the AshY and elm yellows MLOs are distinct from one another (Davis *et al.* 1988b, 1990a,b; Lee *et al.* 1990a). As yet, no information is available about relatedness of AshY MLOs to MLOs associated with the other diseases mentioned, but probes such as those developed in the present work make it feasible to explore this possibility. The present findings provide a basis for the possible detection of AshY MLOs in any plant, including alternate hosts of the MLOs, and for the investigation of symptomless MLO infections in ash (Carr and Tattar 1989; Matteoni and Sinclair 1985; Sinclair *et al.* 1989). Moreover, because MLO DNA in insects can be detected using cloned DNA probes (Davis *et al.* 1990a; Kirkpatrick *et al.* 1987), the DNA of probes specific for AshY1 should be useful in the search for vectors of AshY MLOs.

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