Identification and Analysis of a Genomic Strain Cluster of Mycoplasmalike Organisms Associated with Canadian Peach (Eastern) X Disease, Western X Disease, and Clover Yellow Edge

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Genetic interrelatedness among 13 strains of mycoplasmalike organisms (MLOs) from various sources was evaluated by dot hybridization and restriction fragment length polymorphism analyses using cloned DNA probes derived from Canadian peach X (CX) and western X (WX) MLOs. Dot hybridization analysis indicated that CX, WX, and clover yellow edge MLOs are closely related and form a distinct strain cluster that is only distantly related to the 10 other MLOs. Similarity coefficients derived from restriction fragment length polymorphism analysis revealed that CX, WX, and clover yellow edge MLOs represent three distinct genomic types.

X disease, an economically important disease of stone fruit trees which is particularly devastating to peach and cherry trees, is believed to be caused by a mycoplasmalike organism (MLO) (9, 10, 26, 28). Two strains of X-disease MLO, eastern and western, are found in distinct geographic regions of North America. The eastern strain is transmitted predominantly by *Paraphlepsius irroratus* (Say) insect vector and is seen in the eastern United States and eastern Canada, while the western strain is transmitted predominantly by *Colladonus montanus* (Van Duzee) and is prevalent in the western United States (3, 9, 15, 31). Because of the inability to isolate MLOs in pure culture in vitro, the relatedness of these two strains of X-disease MLO was postulated on the basis of similarity of host range and symptoms produced in common hosts (15, 32). Another MLO-caused disease, associated with red clover (*Trifolium pretense*) and Ladino clover (*Trifolium repens*), clover yellow edge (CYE), was reported to be transmitted by the leafhopper *Aphrodes bicinctus* (Schrank) (1). Recently, CYE has been described as being quite similar to peach X disease in terms of symptoms induced in common plant hosts and similar plant host range (2, 3). These biological characteristics, although helpful for grouping these diseases, cannot accurately identify the MLOs and define the genetic interrelationships among this group of MLOs. The lack of sensitive and specific means for detection and identification of the X-disease MLO(s) has hampered disease diagnosis and epidemiological study.

Progress in MLO detection and identification has been made through the use of recombinant DNA technology (4-8, 12, 14, 16, 17, 19, 21, 23, 30) and the ability to clone MLO-specific DNA fragments directly from infected hosts. Cloned MLO-specific DNAs, when used as probes, allow rapid and accurate detection of MLOs and improved diagnosis of MLO-induced diseases (4-8, 11, 12, 16, 19, 21, 23, 30). Further, these methods provide researchers a means to investigate the genetic interrelationships among MLOs and

to differentiate or classify related MLOs on the basis of genotype (18, 20, 23).

Generally, nucleic acid dot hybridization using random DNA probes derived from various MLO strains is initially employed to investigate the overall genetic interrelationships among numerous strains of MLOs (5-8, 19, 21, 23). Dot hybridization assay is particularly useful for preliminary categorization of MLOs on the basis of genetic interrelatedness. MLO strains having extensive sequence homology can be separated from unrelated or less related strains. Each group that consists of closely related MLO strains thereby represents a distinct strain cluster. This approach has already led to the recognition of several strain clusters (5, 7, 20, 22-24). More-detailed genotypic information can be obtained by restriction fragment length polymorphism (RFLP) analysis, which provides the means for closer investigation of the genetic interrelationships and for differentiation among closely related MLO strains, such as members of a given strain cluster.

Recently, using cloned DNA probes derived from a western strain of X-disease MLO for dot hybridization analysis, Kirkpatrick et al. have suggested that an eastern (Canadian peach X [CX]) and a western strain of X-disease MLO are closely related (15). Serological data obtained by using monoclonal antibodies also indicated that these two MLOs are antigenically related (13). However, the relationship of the X-disease MLOs to CYE MLO and to other MLOs remained undetermined.

In this work, we have cloned MLO-specific DNA fragments from an eastern (CX) as well as a western (WX) (Green Valley X) strain of X-disease MLO. CX MLO- and WX MLO-specific DNA probes were used in dot hybridization and RFLP analyses to determine the genetic relationships among representative MLO strains associated with CX, WX, and CYE diseases and to investigate the interrelatedness among 10 other MLOs associated with a wide variety of plant diseases.

(A brief abstract has been published elsewhere [24].)

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Sources of healthy and diseased plants. Diseased plants of periwinkle (*Catharanthus roseus* [L.] G. Don) were maintained by being grafted in a white-flowered clone of periwinkle maintained in our greenhouse in Beltsville, Md. Clover phyllody MLO-infected periwinkle and MLO strains representative of CX in periwinkle and CYE in Ladino clover were provided by L. N. Chiykowski. An MLO strain representative of WX in periwinkle was provided by B. C. Kirkpatrick, University of California, Davis. An aster yellows (AY) MLO was originally field collected in Beltsville, Md., as described elsewhere (19). Other MLOs (causing the indicated diseases) were kindly provided by various researchers as follows (each strain was provided separately in periwinkle tissue unless noted otherwise): severe AY, A. H. Purcell, University of California, Berkeley; tomato big bud, J. Dale, University of Arkansas, Fayetteville; New Jersey AY, T. A. Chen, Rutgers—The State University, New Brunswick, N.J.; potato witches' broom and clover proliferation (CP), C. Hiruki, University of Alberta, Edmonton, Canada; ash yellows (AshY) and elm yellows, W. A. Sinclair, Cornell University, Ithaca, N.Y.; and beet leafhopper-transmitted virescence agent (VR), G. N. Oldfield, University of California, Riverside.

Molecular cloning and preparation of DNA probes. Molecular cloning and preparation of DNA probes for CX and WX MLO-infected plants were performed separately in a like manner. Isolation of sieve elements from MLO-infected periwinkle plants, preparation of an MLO-enriched fraction, DNA extraction, and molecular cloning were performed as previously described (19, 21). The MLO DNAs were partially digested with both *EcoRI* and *HindIII* restriction endonucleases; the DNA fragments were then ligated to plasmid SP64 (Promega Biotec, Madison, Wis., and then *Escherichia coli* JM 83 was transformed. To select MLO-specific recombinant plasmids, ampicillin-resistant colonies were screened by dot hybridization with biotin-labeled DNA preparations from MLO-diseased plants as well as from healthy plants by a procedure previously reported (21). Colonies from which DNA hybridized with biotin-labeled DNA from MLO-infected plants but not with labeled DNA from healthy plant tissues were considered candidates for development of MLO-specific DNA probes. MLO-specific probes were prepared by nick translation of cloned recombinant plasmids with biotin-7-dATP according to the instructions of the manufacturer (Bethesda Research Laboratories, Gaithersburg, Md.).

Dot hybridization. DNA samples from healthy plants and plants infected with CX MLO, WX MLO, or other MLO strains were prepared as previously described (23). Eighteen cloned CX MLO chromosomal DNA probes (pCX4, pCX8, pCX10, pCX12, pCX14, pCX15, pCX17, pCX30, pCX32, pCX35, pCX36, pCX49, pCX55, pCX56, pCX61, pCX78, pCX87, and pCX95) and three cloned WX MLO chromosomal DNA probes (pWX54, pWX56, and pWX58) were used in dot hybridization to study the genetic interrelatedness among CX MLO, WX MLO, and 10 other MLOs. The insert sizes of the 21 probes ranged from 0.3 to 4.9 kbp. Dot hybridization and signal detection were performed as described elsewhere (19, 21).

Approximately equal amounts of denatured nucleic acid prepared from CX MLO-, WX MLO-, and other MLO-infected plants were blotted onto nitrocellulose membranes (undiluted nucleic acid, 4 µg per spot). The membranes were then baked and hybridized with each of the cloned CX and

TABLE 1. Summary of results from dot hybridizations of biotin-labeled cloned CX and WX MLO DNA probes to nucleic acid preparations extracted from healthy or MLO-infected plants^a

Probe ^b	Hybridization with nucleic acid from plants infected with ^c :			
	AshY	WX	CX	CYE
CX (18)	1	15	18	16
WX (3)	1	3	1	3

^a All hybridizations were performed under stringent conditions (50 to 52°C) in the presence of 45% formamide as described in the text. No probes were positive for healthy *C. roseus* (periwinkle) plants or for plants infected with AY, severe AY, tomato Big Bud, New Jersey AY, clover phyllody, potato witches' broom, CP, VR, or elm yellows MLOs. See text for details.

^b Total number of probes used is given in parentheses.

^c Values are numbers of probes giving a positive signal.

WX MLO probes. The presence of a sufficient quantity of detectable MLO-specific DNA in each sample spot was verified separately by hybridization to a homologous DNA probe (data not shown). Hybridizations were performed at 50 to 52°C in the presence of 45% formamide as described elsewhere (6, 21). None of the 18 CX or 3 WX MLO probes hybridized with nucleic acid from healthy plants or with nucleic acids from AY, severe AY, tomato big bud, New Jersey AY, clover phyllody, potato witches' broom, CP, VR, or elm yellows MLO-infected plants. Of the 21 probes used, only 2 (pCX95 and pWX58) weakly hybridized with nucleic acid from AshY MLO-infected plants (Table 1). All 18 CX MLO probes hybridized strongly with nucleic acid from CX MLO-infected plants. A total of 15 of the 18 CX MLO probes and all 3 WX MLO probes hybridized with nucleic acid from WX MLO-infected plants. A total of 16 of the 18 CX MLO probes and all 3 WX MLO probes hybridized with nucleic acid from CYE MLO-infected plants. It is clear that the CX, WX, and CYE MLOs form a distinct strain cluster, peach X MLO strain cluster, being closely related to one another and only distantly related to the other MLOs examined in this study.

Southern hybridization and RFLP analysis. The genetic interrelatedness among CX, WX, and CYE MLOs in the peach X MLO strain cluster was further analyzed by RFLP analyses. The same 18 CX and 3 WX MLO probes were used to compare CX and WX MLOs and 12 CX MLO probes (pCX4, pCX8, pCX12, pCX14, pCX32, pCX36, pCX49, pCX56, pCX61, pCX78, pCX87, and pCX95) were used to compare CYE, CX, and WX MLOs. Total nucleic acid samples (about 4 µg total nucleic acid per sample) from healthy and diseased plants were doubly digested with *EcoRI* and *HindIII*, electrophoresed through a 0.7% agarose gel, alkali denatured, and transferred to nitrocellulose membranes by the method of Southern as described by Maniatis et al. (27). The membranes were air dried, baked, prehybridized, and hybridized with biotin-labeled probes. In addition, a cocktail probe containing 0.5 µg of biotin-labeled DNA from each of the 18 CX and 3 WX MLO clones was used for RFLP analysis.

The RFLP patterns (Fig. 1 [representative data] and 2) of CX, WX, and CYE MLOs indicated that the three MLOs are related but distinct from one another. Common bands were seen among CX, WX, and CYE MLOs (Fig. 1A through C, E, and G, and 2) and between CX and WX MLOs (Fig. 1 and 2). For example, in Fig. 1A, there were two bands (3.2 and 3.4 kbp) which were common to CX, WX, and CYE MLOs. CX and WX MLOs are more closely related to each other

FIG. 1. Southern hybridization analyses of genomic DNAs prepared from healthy and MLO-infected periwinkle plants (*C. roseus*). DNA samples were digested with the restriction endonucleases *Eco*RI and *Hind*III and hybridized with the indicated cloned CX MLO DNA probes (lower right of each panel). Lanes S, biotinylated lambda phage DNA *Hind*III digest with molecular sizes (from top to bottom) of 23, 9.4, 6.6, 4.4, 2.3, 2.0, and 0.6 kbp; lanes H, healthy plant DNA. The other lanes contain the indicated MLO DNAs.

than to CYE MLO. This is indicated by the finding of more bands in common between CX and WX MLOs than between CX and CYE MLOs or between WX and CYE MLOs when 21 probes (for CX and WX MLOs) or 12 probes (for CX, WX, and CYE MLOs) were employed individually or when a cocktail probe of 21 probes (for CX, WX, and CYE MLOs) was employed for RFLP analysis (representative data shown in Fig. 1 and 2). When the cocktail probe was employed for analysis of the other MLOs (clover phyllody, AY, VR, and AshY, etc.) which appeared unrelated to CX, WX, and CYE MLOs on the basis of dot hybridization, none of the other 10 MLOs, with the exceptions of AshY (with a single band) and CP (with very faint bands), showed any homology to or reactivity with CX or WX MLO DNA probes, as no bands were seen (Fig. 2). The weak hybridization signal of the AshY and CP MLO DNAs was not due to an insufficient amount of MLO-specific DNA. This was verified separately by hybridization to homologous DNA probes cloned from AshY and CP MLOs (data not shown). Probes pWX54 and pWX56 did not hybridize to Southern-blotted DNA from CX MLO, while probes pCX10, pCX15, pCX30, pCX35, and pCX55 either did not hybridize or only slightly hybridized to Southern-blotted DNA from WX MLO (data not shown). Probes pCX87 and pCX95 did not hybridize to DNA from CYE MLO (data not shown). Dual or multiple infection by CX, WX, and CYE MLOs might occur in nature. However, the lack of hybridization of CX MLO DNA samples with pWX54 and pWX56, of WX MLO DNA samples with pCX10, pCX15, pCX30, pCX35, and pCX55, and of CYE MLO DNA samples with pCX87 and pCX95 indicated the absence of mixed infection. The similar RFLP patterns

FIG. 2. Southern hybridization analyses of genomic DNAs prepared from healthy and MLO-infected periwinkle plants (*C. roseus*). DNA samples were digested with the restriction endonucleases *Eco*RI and *Hind*III (unless otherwise noted) and hybridized with a cocktail probe consisting of cloned CX and WX MLO DNAs as described in the text. Lane S, biotinylated lambda phage DNA *Hind*III digest; lane H, healthy plant DNA; lanes CX-s and WX-s, CX and WX MLO DNAs digested with *Eco*RI only, respectively. The other lanes contain the indicated MLO DNAs. AY1, aster yellows; BB, tomato big bud; CN1 and CN13, two strains of field-collected periwinkle little-leaf MLO; EY, elm yellows; PWB, potato witches' broom; MPY, Malaysian periwinkle yellows. Sizes (in kilobase pairs) are shown on the left.

observed for these three MLOs with certain DNA probes are not due to multiple infection.

The similarity coefficient, F , values based on the average of 18 RFLP analyses of CX and WX MLO strains and 12 RFLP analyses of CX and CYE MLO strains and of WX and CYE MLO strains were 0.53 for CX and WX MLOs, 0.39 for WX and CYE MLOs, and 0.31 for CX and CYE MLOs (Table 2). The similarity coefficient (F) for strains x and y was calculated as $F = 2N_{xy}/(N_x + N_y)$, where N_x and N_y are the total numbers of hybridized DNA fragments in strains x and y , respectively, and N_{xy} is the number of hybridized-fragment band positions shared by strains x and y (25, 29).

RFLP analysis comparing CX MLO with WX MLO (Fig. 1 shows representative data) indicated that while WX MLO is closely related to CX MLO, exhibiting a similarity coefficient of 0.53, these two MLOs are in fact more distinct than previously thought (15). It is known that CX and WX MLOs are transmitted predominantly by different species of insect vectors (3, 15). Each insect vector has been known to transmit more than one type of MLO and has its own preferred plant hosts (3, 15). The differences in genome

TABLE 2. Similarity coefficients derived from RFLP analyses^a

MLO	F with ^b :		
	CX	WX	CYE
CX	1.00	0.53	0.31
WX	0.53	1.00	0.39
CYE	0.31	0.39	1.00

^a Eighteen DNA probes derived from CX MLO and restriction endonucleases *Eco*RI and *Hind*III were used.

^b Values for RFLP analyses involving CYE are averages of 12 analyses; all others are averages of 18 analyses.

composition between CX and WX MLOs might be explained by divergent evolution upon geographical separation and concomitant differences in plant host range of MLO and/or insect vector species. A difference between their biological niches may result in a difference in exchange or rearrangement of genetic information in these two MLOs.

RFLP analysis comparing CYE MLO with eastern and western strains of X-disease MLOs indicates that CYE MLO has significant genome similarity to both eastern and Western strains of X-disease MLOs, though perhaps somewhat less similarity with the eastern strain. On the basis of these similarities, CYE MLO is classified as a member of the peach X MLO strain cluster.

Use of a cocktail probe consisting of biotin-labeled recombinant plasmid DNA from each of the 18 CX and 3 WX MLO DNA probes for RFLP analysis further supports the establishment of the peach X MLO strain cluster. The restriction fragment pattern seen in Fig. 2 clearly indicates the similarity among CX, WX, and CYE MLOs and their dissimilarity as a group from the other various MLOs investigated in this study, providing an overall picture of the peach X MLO strain cluster. Likewise, the restriction fragment pattern clearly shows that CX, WX, and CYE MLOs, while similar, represent three distinct genomic types. With a combination of MLO DNA probes, for example pCX8, pCX12, and pCX14 (Fig. 1A, F, and H, respectively), it is possible to identify and differentiate members of the peach X strain cluster on the basis of RFLP patterns. This approach may be used in the future to include more related MLOs in the peach X strain cluster.

It is interesting to examine the significance of CYE MLO as a peach X strain cluster member in terms of evolutionary and epidemiological implications. As a whole, the X-disease organisms have been previously associated with disease of stone fruit trees. CYE MLO provides evidence of a similar and most likely evolutionarily linked disease-causing organism that infects predominantly the clover, a host that is only distantly related to stone fruits. A study of diverse MLOs and an attempt to cluster them on the basis of genotype may help to identify a possible common evolutionary origin of the MLOs and aid in the study of the phylogeny of otherwise varied MLOs. The close phylogenetic relationship of CYE MLO with CX and WX MLOs implies that CYE MLO may in fact have evolved from the same organism as CX and WX MLOs.

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