Genetic Interrelatedness among Clover Proliferation Mycoplasmalike Organisms (MLOs) and Other MLOs Investigated by Nucleic Acid Hybridization and Restriction Fragment Length Polymorphism Analyses

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DNA was isolated from clover proliferation (CP) mycoplasmalike organism (MLO)-diseased periwinkle plants (Catharanthus roseus (L.) G. Don.) and cloned into pSP6 plasmid vectors. CP MLO-specific recombinant DNA clones were biotin labeled and used as probes in dot hybridization and restriction fragment length polymorphism analyses to study the genetic interrelatedness among CP MLO and other MLOs, including potato witches'-broom (PWB) MLO. Results from dot hybridization analyses indicated that both a Maryland strain of aster yellows and a California strain of aster yellows are distantly related to CP MLO. Elm yellows, paulownia witches'-broom, peanut witches'-broom, loofah witches'-broom, and sweet potato witches'-broom may be very distantly related, if at all, to CP MLO. A new Jersey strain of aster yellows MLO, tomato big bud MLO, clover phyllody MLO, beet leafhopper-transmitted virescence MLO, and ash yellows MLO are related to CP MLO, but PWB MLO is the most closely related. Similarity coefficients derived from restriction fragment length polymorphism analyses revealed that PWB and CP MLOs are closely related strains and thus provided direct evidence of their relatedness in contrast to reliance solely on biological characterization.

Clove proliferation (CP), first found in Alberta, Canada, is a disease believed to be caused by a mycoplasmalike organism (MLO) (4). On the basis of its characteristic biological properties (e.g., symptomatology, host range, and vector transmissibility of the MLO), Chiykowski suggested in 1965 that CP is distinct from clover phyllody (CPh) and aster yellows (AY) but that it may be related to potato witches'-broom (PWB) (6, 7). This raises the question of whether CP and PWB MLOs are distinct MLOs or are two strains of the same MLO. The answer to this question is important for understanding the epidemiology of the two diseases. Because of the inability to isolate any MLO in pure culture in vitro, until recently it has not been possible to gain a definitive answer to this question. Recent development of hybridoma and recombinant DNA technologies has made it possible to study the genetic relatedness of the uncultured MLOs (5, 8–12, 16–20, 22). The development of recombinant DNA technology has made it feasible to clone MLO-specific DNA fragments directly from infected hosts (8–12, 16–20, 27). The cloned fragments, when used as 32P- or biotin-labeled DNA probes, enable detection and accurate diagnosis of MLOs in hosts and make it possible to investigate the genetic interrelatedness among diverse MLOs (2, 3, 8–11, 16–20). In the past few years, several distinct MLO strains and strain clusters (each cluster consisting of a number of closely related strains) were identified mainly on the basis of dot hybridization analyses with cloned DNA probes derived from various MLOs. Studies of the interrelatedness among the MLOs revealed that CP MLO is distinct from AY, tomato big bud (BB), CPh, and periwinkle little leaf (CN1 0-1) MLOs and that CP and PWB MLOs are genetically related (9–12, 19, 20). However, relationships among related MLO strains in a given strain cluster have been difficult to determine simply by comparisons of dot hybridization patterns. Thus, it remained to be determined whether CP and PWB MLOs are distinct MLOs or are two strains of the same MLO. In this study, we prepared homologous DNA probes that contained fragments of CP MLO DNA and used them in hybridization analyses. Combined analyses of dot hybridization and restriction fragment length polymorphism (RFLP) were used to investigate the genetic relatedness between CP and PWB MLOs and the interrelatedness between the CP MLO and 13 other MLOs with a wide variety of plant diseases. This analysis resulted in the identification of the CP MLO and PWB MLO as closely related strains, distinct from the other 13 MLOs investigated.

MATERIALS AND METHODS

Sources of healthy and diseased plants. Diseased plants of periwinkle (Catharanthus roseus) were maintained by grafting to a white flowered clone of periwinkle maintained in our greenhouse in Beltsville, Md. The Maryland strain of AY-infected periwinkle was originally collected in Beltsville, Md. CP MLO-infected plants were provided by one of us (C.H.). PWB MLO-infected periwinkle plants were originally obtained by one of us (C.H.) by dodder (Cuscuta subinclusa) transmission of the MLO from field-collected potatoes in Canada. The following additional MLO strains were kindly provided by the indicated researchers, who provided each strain separately in periwinkle tissue unless noted otherwise: severe AY (SAY), A. H. Purcell (University of California, Berkeley); BB, James Dale (University of Arkansas, Fayetteville); New Jersey AY (NJAY), T. A. Chen (Rutgers University, Piscataway, N.J.); CPh, Lloyd N. Chiykowski (Agriculture Canada, Ottawa, Ontario, Can-
Nucleic acid preparations from plants infected by paulownia witches'-broom (PaWB), peanut witches'-broom (PnWB), loofah witches'-broom (LFWB), and sweet potato witches'-broom (SPWB) MLOs were kindly provided by H. J. Su and M. C. Tsai (National Taiwan University, Taipei, Taiwan).

**Molecular cloning and preparation of DNA probes.** Isolation of sieve elements from CP-infected periwinkle plants, preparation of the CP MLO-enriched fraction, DNA extraction, and molecular cloning of DNA were done as previously described (19, 20). The CP MLO DNA plus plant DNA was partially digested with both EcoRI and HindIII restriction endonucleases, ligated in pSP64 (Promega Biotec, Madison, Wis.), and then used to transform Escherichia coli JM 83. To select CP MLO-specific recombinant clones, ampicillin-resistant colonies were screened by dot hybridization with biotin-labeled DNA preparations from CP-diseased plants as well as from healthy plants, according to the procedure previously reported (20). Colonies from which DNA hybridized with biotin-labeled DNA from CP MLO-infected plants but not with labeled DNA from healthy plant tissues were considered candidates for the development of CP MLO-specific DNA probes. CP 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.).

**Extraction of nucleic acids from plants.** DNA samples from healthy plants and plants infected with CP MLO or with other strains of MLO were prepared by a modified procedure for preparation of genomic DNA from plant tissue and from bacteria as described by Ausubel et al. (1). This procedure was designed to obtain DNA samples that are relatively free of contaminants which may interfere with digestion of MLO DNA by restriction endonucleases. In brief, for each sample about 1.0 g of leaf midribs stripped by a sharp forceps was pulverized in liquid nitrogen in a mortar. The pulverized sample was triturated in 2 to 4 ml of DNA extraction buffer (100 mM Tris-HCl [pH 8.0], 100 mM EDTA, 250 mM NaCl, 100 μg of proteinase K per ml). Sarkosyl (in 10% stock solution) was added to the samples after trituration to give a final concentration of 1%. The sample was then incubated for 1 h at 55°C and clarified by centrifugation at 6,000 rpm (4,300 × g) for 10 min. Crude DNA was precipitated from the supernatant liquid by the addition of 0.6 volume of isopropanol and pelleted by centrifugation at 8,000 rpm (7,700 × g) for 15 min. The pellet was resuspended in 1 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]) containing 100 μg of proteinase K per ml and 0.5% sodium dodecyl sulfate (SDS) and incubated for 1 h at 37°C. Then 175 μl of 5 M NaCl was added to and mixed thoroughly with the sample, and 140 μl of 10% cetyltrimethylammonium bromide in 0.7 M NaCl was added to the sample. After incubation for 10 min at 65°C, the sample was extracted at least once with an equal volume of chloroform-isooamyl alcohol (24:1) until no precipitate was visible at the interface. The aqueous fraction was extracted once with an equal volume of TE-saturated phenol-chloroform-isooamyl alcohol (25:24:1), and the DNA was precipitated from the aqueous phase by the addition of 0.6 volume of isopropanol and pelleted by centrifugation for 10 min at 8,000 rpm. The pellet was then washed with 70% ethanol to remove residual cetyltrimethylammonium bromide, briefly dried in air, and resuspended in TE buffer.

**Dot hybridization.** Nine CP MLO cloned DNA probes were selected and used in dot hybridization to study the genetic interrelatedness among the CP MLO and 14 other MLOs. In tests to determine whether cloned CP MLO DNA fragments might have derived from MLO chromosomal or extrachromosomal (plasmid) DNA, undigested DNA samples (4 μg of total nucleic acid each) from healthy periwinkle plants and from CP MLO-infected periwinkle plants were electrophoresed in a 0.7% agarose gel, transferred to nitrocellulose membrane, and hybridized with each of the cloned CP MLO DNA probes (19). Dot hybridization and signal detection were performed as described elsewhere (19, 20). Approximately equal amounts of denatured nucleic acid prepared from CP and each of the other MLO-infected plants were blotted onto nitrocellulose membranes (4 μg of undenatured nucleic acid per spot), baked, and hybridized with each of the cloned CP MLO DNA 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. Hybridizations were performed at 50 to 55°C in the presence of 45% formamide as described previously (10, 20). Posthybridization washes were performed twice at room temperature with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0–0.1% SDS, twice at room temperature with 0.2× SSC-0.1% SDS, and twice at 50°C with 0.16× SSC-0.1% SDS (20).

**Southern hybridization and RFLP analyses.** DNA samples (about 2 μg of total nucleic acid per sample) from healthy or MLO-infected periwinkle plants were digested with EcoRI and HindIII at 37°C for 4 h, electrophoresed in a 0.7% agarose gel, alkali denatured, and transferred to nitrocellulose membrane by the method of Southern as described by Maniatis et al. (23). The membranes were air dried, baked, prehybridized, and hybridized with biotin-labeled DNA probes. Six CP MLO cloned DNA probes were used for RFLP analyses. For each probe, the similarity coefficient (F) of strains x and y was calculated as $F = 2N_x/(N_x + N_y)$, where $N_x$ and $N_y$ are the numbers of restriction fragments in strains x and y, respectively, and $N_{xy}$ is the number of fragments shared between two strains (26). The F values are the means from the six hybridization probes.

**RESULTS**

More than 1,000 transformant colonies of E. coli JM 83 were obtained in the cloning of DNA from CP MLO-infected periwinkle plants. Of 100 transformants screened, 70 were CP disease specific. Nine clones were selected, and their recombinant plasmids (insert sizes ranged from 0.6 to 6.0 kbp [Table 1]) were extracted and biotinylated to make probes for dot hybridization and Southern hybridization analyses.

**Dot hybridization.** The results from dot hybridization of nine cloned CP MLO DNA probes to nucleic acid preparations from healthy plants and to nucleic acid from plants infected by one of 15 different MLOs are summarized in Table 1. None of the nine probes hybridized with nucleic acid from healthy plants, nor did they hybridize with nucleic acid from CY, PaWB, PnWB, LFWB, or SPWB MLO-infected plants. All nine probes hybridized strongly with nucleic acid prepared from both CP and PWB MLO-infected plants. Of the nine (eight when hybridized to NJAY or SAY MLO) probes, three (pCP61, pCP63, and pCP67) hybridized strongly and three (pCP2, pCP75, and pCP95) hybridized
TABLE 1. Summary of results from dot hybridizations of biotin-labeled cloned CP MLO DNA probes to nucleic acid preparations

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<th>Probe</th>
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<th>AY</th>
<th>SAY</th>
<th>BB</th>
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a All hybridizations were performed under stringent conditions (50 to 52°C) in the presence of 45% formamide as described in the text.

b H, healthy C. roseus (periwinkle); all other abbreviations are for MLOs (infecting C. roseus) as in the text. ND, not determined; --, no hybridization signal; +, weak to moderate hybridization signal; ++, strong hybridization signal.

Weakly with nucleic acid from plants containing AshY MLO, four (pCP63, pCP67, pCP75, and pCP95) hybridized weakly with nucleic acid from CPh MLO, three (pCP2, pCP75, and pCP95) hybridized weakly and one (pCP67) hybridized strongly with nucleic acid from WX MLO, four (pCP61, pCP63, pCP67, and pCP95) hybridized weakly with nucleic acid from NJAY MLO, three (pCP61, pCP63, and pCP95) hybridized with nucleic acid from BB MLO, two (pCP67 and pCP75) hybridized with nucleic acid from AY MLO, and one (pCP67) hybridized with nucleic acid from SAY MLO. Figure 1 shows the dot hybridization patterns from 15 strains of MLOs associated with a wide variety of plant diseases. Probe pCP54 hybridized strongly with nucleic acid from CP and PWB MLO-infected plants and weakly with nucleic acid from VR MLO-infected plants (Fig. 1A). Probe pCP67 hybridized broadly with nucleic acid from CP, PBW, AshY, WX, CPh, NJAY, SAY, and AY MLO-infected plants (Fig. 1B).

Southern hybridization and RFLP analyses. Southern hybridization analyses revealed that all nine probes hybridized with CP MLO chromosomal DNA (data not shown), indicating that these cloned DNA fragments originated from CP MLO chromosomal DNA. Six probes (pCP61, pCP67, pCP54, pCP63, pCP2, and pCP75) were employed for RFLP analyses. The RFLP patterns of CP and PWB MLOs appeared identical or nearly identical, as shown in Fig. 2A, C, E, and F, when probes pCP61, pCP54, pCP2, and pCP75, respectively, were used for Southern hybridization. The patterns of CP and PBW MLOs were also very similar when probes pCP67 and pCP63 were used (Fig. 2B and D). The RFLP patterns of other MLOs (i.e., CPh, VR, and AshY) which appear to be related to CP MLO on the basis of dot hybridization analyses were distinguished from CP MLO by RFLP analyses. For example, no DNA fragments of CPh MLO appeared to hybridize with probe pCP61, pCP54, or pCP2 (Fig. 2A, C, and E), whereas a different DNA fragment hybridized with probes pCP67, pCP63, and pCP75 (Fig. 2B, D, and F). Similarly, no DNA fragments from VR MLO hybridized with probes pCP61, pCP67, pCP63, and pCP75 (Fig. 2A, B, D, and F), while different DNA fragments hybridized weakly with probes pCP54 and pCP2 (Fig. 2C and E); no DNA fragments from AshY MLO hybridized with probe pCP54 (Fig. 2C), while different DNA fragments did hybridize with probes pCP61, pCP67, pCP63, pCP2, and pCP75 (Fig. 2A, B, and D through F). The similarity coefficients (F) derived from RFLP analyses with the six CP MLO cloned DNA probes were 0.94 between CP and PBW MLOs, 0.14 between CP and AshY MLOs, 0.06 between CP and CPh MLOs, and 0.08 between CP and VR MLOs.

DISCUSSION

Nucleic acid dot hybridization has been employed to study the genetic relatedness among various strains of MLOs (this study and references 9-12, 16-20). New information about the genetic relationships of the MLOs studied revealed that they can be classified into several distinct strains or strain clusters (9, 10, 19, 20). However, dot hybridization analysis generally fails to give a definitive strain identity within a given cluster. RFLP analysis has been employed widely in biological sciences (13, 14, 21, 24-26, 28) to study changes in a genome due to sequence rearrangements and to distinguish between strains which have considerable sequence homology. Recently, RFLP analyses have been applied to differentiate MLOs (18, 19). In the present study, we applied this analysis in the identification of strains whose genomic cluster affinity had been established by dot hybridization analysis. Our study illustrates that the identity of a strain can be determined by a combined analysis of the data obtained from dot hybridization and RFLP analysis with MLO chromosomal DNA probes. For example, dot hybridizations may first be conducted to separate clusters of related strains from distantly related ones on the basis of patterns of hybridiza-
RFLP analysis may then be employed to differentiate and identify a strain within a cluster. When this approach was used, the CP and PWB MLOs were found to be very closely related strains. A similar approach could be used to assess the identities of MLOs that are known to be associated with several hundred important diseases in nature and in agricultural systems.

Our and other earlier reports of dot hybridizations with probes prepared from DNAs of other MLOs have indicated that CP MLO is distantly related to AY, BB, and CN1 (= 0-1) MLOs (9-12, 19, 20). The results of dot hybridizations in the present study with probes containing cloned CP MLO DNA fragments confirm those findings. Furthermore, the results also revealed that CP MLO may be only very distantly related to EY, PaWB, PnWB, LfWB, and SPWB MLOs (as indicated by the lack of hybridization with the nine CP MLO DNA probes used) but that it has greater nucleotide sequence homology with other MLOs, i.e., those of AshY, CPh, VR, NJAY, and WX (as indicated by hybridization with three to six of the nine CP MLO DNA probes).

On the basis of dot hybridizations with all nine CP MLO DNA probes, PWB MLO is clearly shown to be the most closely related to CP MLO. This apparent close relatedness between CP and PWB MLOs was confirmed by RFLP analysis. Nearly identical RFLP patterns were exhibited by these two MLOs in Southern hybridizations with six CP MLO DNA probes (Fig. 2). The combined sizes of DNA fragments that hybridized against the six probes represent approximately a random one-fifth of the total genome, assuming the average genome size of MLOs to be about 750 kb (15). The high similarity coefficient ($F = 0.94$) derived from RFLP analyses between the CP and PWB MLO strains suggests that the genomic sequence arrangements of these two MLOs are similar. We anticipate that further in-depth investigation aimed towards establishing an MLO classification scheme will reveal that the CP and PWB MLOs are strains of the same taxon. In contrast, the RFLP patterns exhibited by AshY, CPh, and VR MLOs were distinct from one another and from those exhibited by CP and PWB MLOs. The low similarity coefficients between the CP MLO and those MLOs ($F = 0.06$ to 0.14) suggest that genomic
organization in the AshY, CPh, and VR MLOs differs significantly from that in CP and PWB MLOs, even though they do have some nucleotide sequence homology. Thus, we conclude that the phylogenetic relationship between CP MLO and those of AshY, CPh, and VR is not of the same rank as that between CP and PWB MLOs. We infer the relationship between CP and PWB MLOs on the basis of results with probes containing DNA from a single strain of CP MLO. Other field strains of CP MLO may differ from the strain we used, but this strain has been considered representative of CP MLOs by us. If, as we propose, CP and PWB MLOs are essentially strains of the same MLO, variation among field strains of CP MLO (e.g., from clover) would be expected to be as great as the difference between CP and PWB MLOs.

The relatively slow pace of etiological and epidemiological studies of uncultured MLOs and their associated diseases can be attributed in part to the previous lack of rapid and accurate means for MLO detection and identification. The application of cloned MLO DNA probes in dot hybridizations and RFLP analyses provides an alternative means to identify MLOs in place of reliance solely on the traditional and often time-consuming biological characterizations. The present study provides direct evidence revealing an MLO strain relationship that was suggested over two decades ago (6) on the basis of similarities in properties such as range of plant host species, disease symptoms induced in plants, and occurrence of the MLOs in the same geographic region. Genetic analyses employed in this study revealed that CP and PWB MLOs are very closely related strains.

ACKNOWLEDGMENTS


REFERENCES