Detection of Chrysanthemum Yellows Mycoplasmalike Organism by Dot Hybridization and Southern Blot Analysis

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ABSTRACT

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Chrysanthemum yellows (CY) mycoplasmalike organism (MLO) was detected in plants of *Catharanthus roseus* (periwinkle), *Chrysanthemum frutescens,* and *Chrysanthemum carinatum* by dot hybridizations of biotinylated cloned DNA probes to nucleic acid extracts from infected plants. Probes containing DNA fragments from an eastern U.S. aster yellows (AY) MLO or from a Connecticut periwinkle little leaf (O-1) MLO hybridized with nucleic acid extracted from CY-diseased but not healthy plants. Of five probes used in Southern blot analyses, one probe containing extrachromosomal DNA from O-1 MLO hybridized with extrachromosomal but not chromosomal DNA of CY MLO. Three probes containing chromosomal DNA of AY MLO and one containing chromosomal DNA of O-1 MLO hybridized with chromosomal DNA of CY MLO. The nucleotide sequence homologies between the probes and nucleic acid of CY MLO are evidence for genetic relatedness of a European MLO and American MLOs and provide diagnostic tools for CY MLO detection.

Uncultured cell wall-less prokaryotes called mycoplasmalike organisms (MLOs) are believed to be the cause of numerous plant diseases worldwide (7,17). Chrysanthemum yellows (CY), a disease associated with the presence of an MLO, has been reported in several species of chrysanthemum in Italy and The Netherlands (1,2). In laboratory studies, suscepts were found to include *Chrysanthemum frutescens* L., *Chrysanthemum carinatum* L., *Chrysanthemum coronarium* L., *Callistephus chinensis* (L.) Nees, *Lactuca sativa* L., *Tagetes patula* L., *T. signata* L., *Helichrysum bracteatum* (Venten.) Andr., *Catharanthus roseus* (L.) G. Don, *Trifolium repens* L., *Apium graveolens* L., *Daucus carota* L., and *Spinacia oleracea* L. (4).

Symptoms of CY include yellowing and stunting, virescence, phyllody, and formation of witches'-brooms. Conti and Mela (5) reported that *Euscelis incisus* Kbm and *E. lineolatus* Brullé transmit the causal agent under experimental conditions and that these leafhoppers occur in infected crops in the Italian Riviera. In recent years, the economic importance of the disease has increased, making it of interest to develop rapid means to detect the CY MLO in infected hosts and to investigate its possible relatedness to other MLOs.

Recent reports have indicated that cloned nucleic acid hybridization probes provide valuable means for the detection of MLOs (6,8,9,11,14). With some such probes, it has been possible to detect the presence of a broad array of North American MLO strains in different host plants (9,14). The existence of these probes makes it feasible to initiate studies of possible genetic relatedness of North American and European MLOs and to investigate the detection of some European MLOs.

In this communication, we report results from work with the CY MLO. The findings demonstrate the detection of the MLO in infected plants by nucleic acid hybridizations employing nonradioactive, biotinylated cloned DNA probes, indicate the presence of extrachromosomal DNA in CY MLO, and suggest a genetic relatedness of CY MLO with MLO strains from North America.

MATERIALS AND METHODS

Sources of MLOs. The hosts of CY MLO used in this work—*Catharanthus roseus, Chrysanthemum frutescens,* and *Chrysanthemum carinatum*—were initially inoculated in the laboratory by means of the leafhopper vector *E. incisus,* which had acquired the MLO from CY-diseased *Chrysanthemum carinatum.* The disease agent was then routinely maintained in these plants in an insect-proof greenhouse by either grafting or insect transmission.

The other MLOs used were maintained by grafting in greenhouse-grown *Catharanthus roseus* plants. Aster yellows (AY) MLO was collected in the field in Beltsville, MD (14). Dwarf strain of aster yellows (DY) MLO was kindly provided by B. C. Kirkpatrick (University of California, Davis). Periwinkle little leaf (O-1) MLO was collected in the field in Connecticut (7). Tomato big bud (BB) MLO and blueberry stunt (DB) MLO were kindly provided by James Dale (University of Arkansas, Fayetteville).

Hybridization procedures. MLO-specific cloned DNA probes developed in previous work (8,9,14) were labeled by nick translation with biotin-7-dATP. Probes consisted either of recombinant plasmids (p) composed of plasmid vector pSP64 and cloned MLO DNA fragment or of cloned DNA insert (I) alone. Probes used were pAY18, pAY19, and pAY27, which contained AY MLO chromosomal DNA, and CN40I and CN42I, which contained cloned O-1 MLO chromosomal and extrachromosomal DNA, respectively.

For dot hybridizations, nucleic acid was extracted from 0.3 g of tissue (leaf and/or stem unless stated otherwise) from an infected or healthy plant as described elsewhere (8,9,14), denatured by addition of 3 µl of 2 N NaOH to 50 µl of nucleic acids and heating in boiling water for 10 min, and neutralized by addition of 3 µl of 2 M TRIS and 1.0 µl of 1.5 M sodium acetate, pH 7.0. Aliquots (3 µl each) of this nucleic acids sample were diluted with 6× saline sodium citrate (SSC) (1× = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) in a twofold dilution series and were applied to clean nitrocellulose membranes. The membranes were baked under vacuum at 80 C for 2 hr. Each dot of undiluted sample contained about 7 g of nucleic acids.

Prehybridizations, hybridizations, washes, filter blocking, and probe detection procedures were performed according to the BluGENE Nonradioactive

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Nucleic Detection System (Cat. No. 8279SA, Bethesda Research Laboratories, Gaithersburg, MD). After a prehybridization for 4 hr at 42 C in the presence of 50% formamide, 5× SSC, 5× Denhardt's solution (1× = 0.08% Ficoll, 0.08% polyvinylpyrrolidone, 0.08% bovine serum albumin), 250 mmol of sodium phosphate, and 0.5 mg of denatured salmon sperm DNA per milliliter, hybridization was performed for 16 hr under the same conditions, except that the formamide concentration was 45% and the hybridization solution also contained 5% dextran sulfate and 0.2 mg of biotin-labeled DNA probe per milliliter.

Filters were then washed at room temperature for 3 min twice in 2× SSC containing 0.1% sodium dodecyl sulfate (SDS) and twice in 0.2× SSC containing 0.1% SDS and then at 50 C for 15 min twice in 0.16× SSC containing 0.1% SDS. Filter blocking was performed (BluGENE System) for 1 hr at 64 C in a buffer (0.1 M TRIS, pH 7.5) containing 3% bovine serum albumin (Pentex: Bovine Albumin Fraction V, Miles Scientific, Naperville, IL) and 0.15 M NaCl. Visualization of hybridized biotinylated probe was performed using Streptavidin-alkaline phosphatase conjugate and nitro blue tetrazolium plus 5-bromo-4-chloro-3-indolyl phosphate (BluGENE System) (sensitivity in our work was 1 pg of homologous DNA).

For Southern blot analyses, nucleic acid was extracted from plants by the procedure of Dellaporta et al (10), electrophoresed in 0.7% agarose gel, stained with ethidium bromide for photography, destained with deionized water, denatured by addition of 1.5 M NaCl plus 0.5 M NaOH for 30 min, and neutralized for 30 min at room temperature in 1.5 M NaCl plus 0.5 M TRIS, pH 7.5. Nucleic acid was then transferred from gel to nitrocellulose membranes (16,20). Membranes were baked, prehybridized, and hybridized with labeled probes.

RESULTS AND DISCUSSION

The presence of CY MLO in infected plants could be detected with both recombinant plasmid probes and probes consisting of cloned MLO DNA alone. In dot hybridizations, nucleic acid from CY MLO-infected but not healthy plants hybridized with biotinylated probes containing cloned DNA from AY and O-1 MLOs. Positive hybridizations were observed in dots containing nucleic acid from the equivalent of less than 300 μg of plant tissue.

***Catharanthus roseus* infected with CY MLO.** Results from dot hybridizations with probes CN40I, CN42I, and pAY18 are shown in Figs. 1 and 2. Hybridization signals were observed with probe CN40I for nucleic acid extracted from plants infected by CY, O-1, BB, and AY MLOs but not healthy *Catharanthus roseus* plants (Fig. 1). Probes CN42I and pAY18 hybridized with nucleic acid from plants infected by CY, O-1, BB, AY, DY, and DB MLOs but not healthy plants (Fig. 2). Probes pAY19 and pAY27 also detected the presence of CY MLO in experimentally infected plants of *Catharanthus roseus* (*data not shown*).

The results confirm previous results in the detection of MLOs other than the CY MLO (8,9,14). The clear hybridization signals obtained in the present work indicate that CY MLO can be detected in periwinkle by hybridizations with either O-1 or AY MLO DNA and with either recombinant plasmid or cloned MLO insert DNA. The results further illustrate the feasibility of dot hybridizations with biotinylated probes for detecting MLOs, as reported earlier (8,9,14).

Chrysanthemum infected with CY MLO. Because chrysanthemums are economically important hosts of the CY MLO, we attempted to detect the MLO in this plant. Results from dot hybridizations with probe pAY27 are shown in Fig. 3. Positive hybridization signals were obtained with nucleic acid samples from leaves of experimentally infected plants of *Chrysanthemum carinatum*. In other tests, positive hybridization signals were also obtained with nucleic acid extracted from roots of infected plants and from the insect vector *E. incisus* carrying the CY agent (*data not shown*). No hybridization signal was observed with nucleic acid from healthy plants or insects. In a few cases, the presence of plant components in nucleic acid samples resulted in a slightly darkened dot on the nitrocellulose, but this darkening was readily distinguished from colored hybridization signals. The detection of MLO infection in *Chrysanthemum* provides a valuable tool for studying the epidemiology of the CY disease and for monitoring the elimination of the pathogen from planting stock.

Southern blot analyses. Nucleic acid was extracted from MLO-infected and healthy plants of periwinkle by the method of Dellaporta et al (10) and electrophoresed in 0.7% agarose. The results are shown in Fig. 4A and 4C. A prominent band corresponding to chromosomal DNA occurs with each extract.

Figure 4B illustrates results from Southern hybridizations with probe CN42I. This probe gave no signal of hybridization with chromosomal DNA of CY MLO or AY MLO but did hybridize with bands of extrachromosomal DNA from both MLOs. No signals were observed with DNA from healthy plants. These results indicate that CY MLO and AY MLO contain extrachromosomal DNA and that the O-1 MLO extrachromosomal DNA in probe CN42I shares sequence homologies with these DNAs. The hybridizations with extrachromosomal DNA from AY MLO confirm earlier findings of extrachromosomal DNA in this MLO and of its sequence homology with probe CN42I (8,13). The results with CY MLO are the first to indicate the presence of extrachromosomal DNA in this prokaryote.

Figure 4D illustrates results from Southern hybridizations of probe pAY27 to undigested DNA in extracts from healthy plants or from plants infected with AY or CY MLO. This probe hybridized with chromosomal DNA of

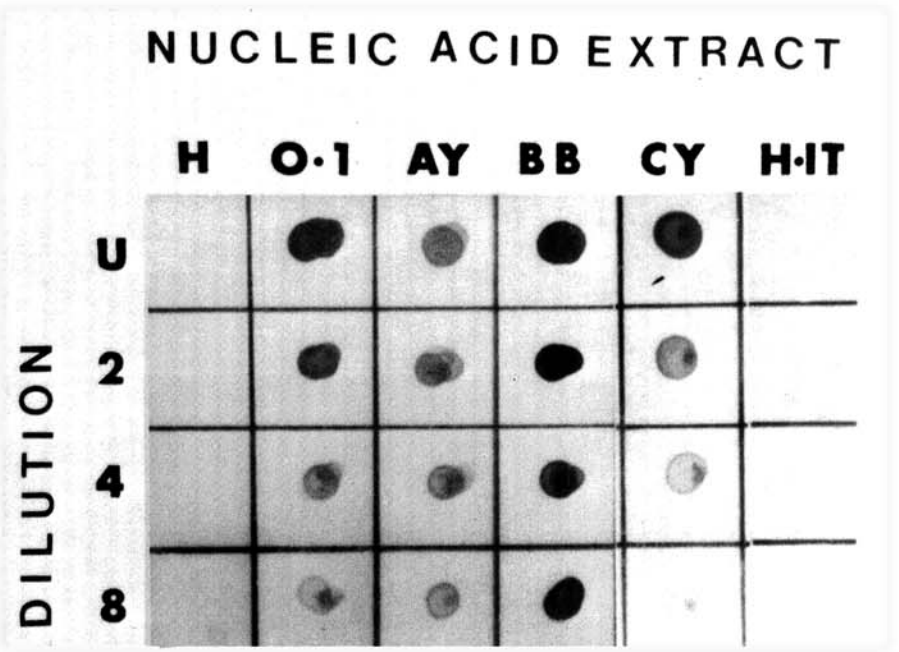

Fig. 1. Dot hybridizations of biotinylated cloned DNA probe CN40I to nucleic acid preparations extracted from healthy *Catharanthus roseus* (periwinkle) plants or from periwinkle plants infected by mycoplasmalike organisms. H, healthy; H-IT, healthy-Italy; CY, chrysanthemum yellows; O-1, periwinkle little leaf; AY, aster yellows from Maryland; BB, tomato big bud; U, undiluted; 2, 4, and 8, reciprocals of dilutions.

AY MLO, in agreement with other work (14), and with chromosomal DNA of CY MLO. Probes pAY19 and CN40I also hybridized with chromosomal DNA of CY MLO (*data not shown*). The nucleotide sequence homology among AY MLO DNA, O-1 MLO DNA, and the chromosome of CY MLO indicates that these MLOs are genetically related.

These findings demonstrate a relationship between a European MLO and North American MLOs. This relationship involves genetic relatedness at the level of chromosomal DNA and nucleotide sequence homology between extrachromosomal DNA of these MLOs. Probes containing cloned sequences of either chromosomal or extrachromosomal DNA from a North American MLO detected infection of plants by a European MLO. The demonstrated feasibility of detecting an MLO in chrysanthemum using biotinylated DNA probes provides a methodology of practical interest in chrysanthemum culture.

"Strains" of MLOs have been dis-

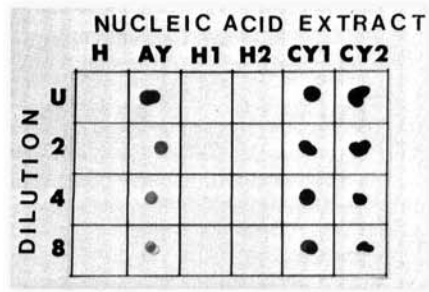

Fig. 3. Detection of chrysanthemum yellows (CY) mycoplasmalike organism (MLO) in *Chrysanthemum* by dot hybridizations of biotinylated cloned DNA probe pAY27. H, healthy *Catharanthus roseus;* AY, aster yellows-infected *Catharanthus roseus;* H1 and H2, healthy *Chrysanthemum* plants; CY1 and CY2, CY MLO-infected *Chrysanthemum carinatum* plants; U, undiluted nucleic acid extract; 2, 4, and 8, serial twofold dilutions of extract.

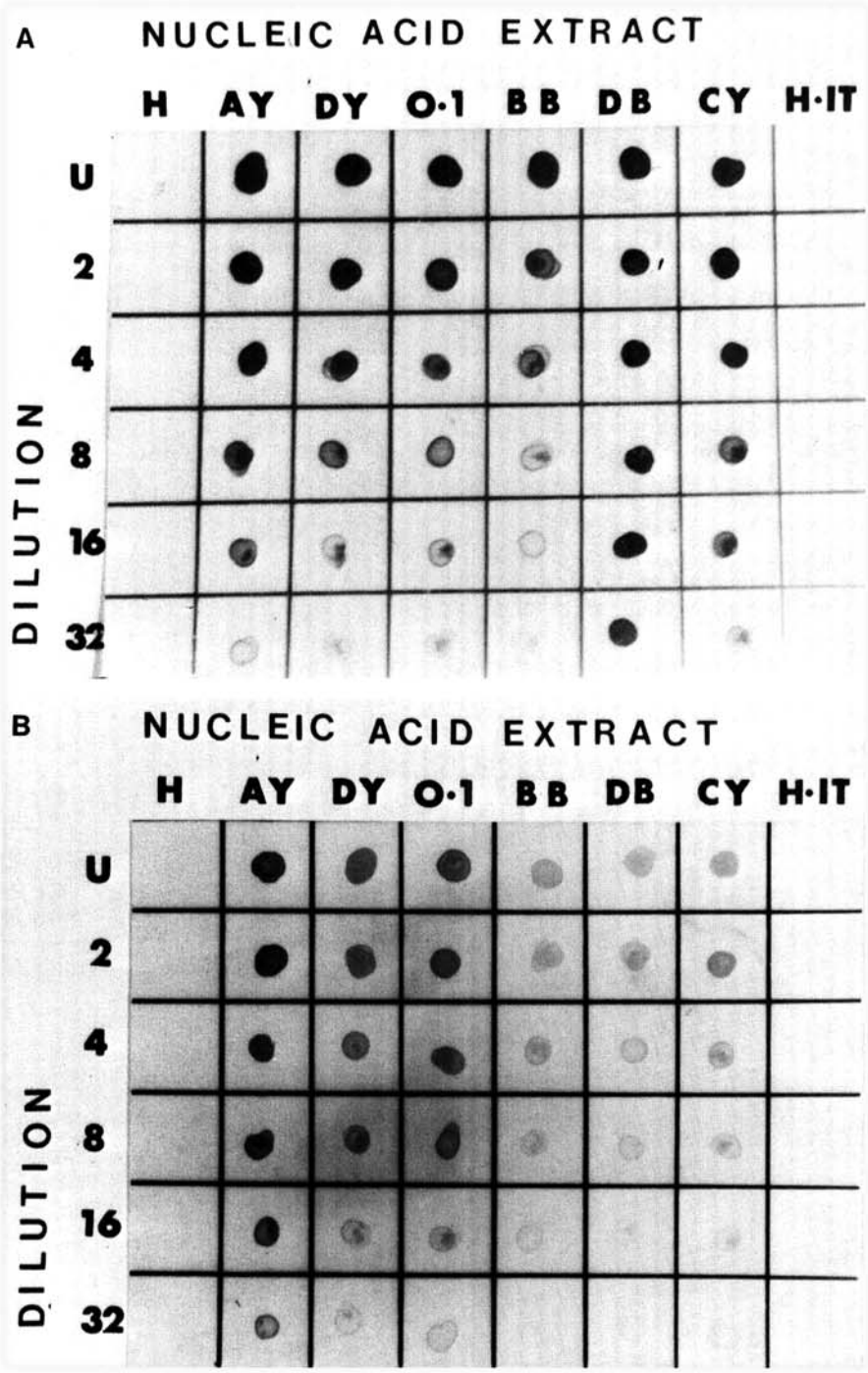

Fig. 2. Dot hybridizations of biotinylated cloned DNA probe CN42I **(A)** and pAY18 **(B)** to nucleic acid preparations extracted from healthy *Catharanthus roseus* (periwinkle) plants or from periwinkle plants infected by mycoplasmalike organisms. H, healthy; H-IT, healthy-Italy; AY, aster yellows from Maryland; O-1, periwinkle little leaf; BB, tomato big bud; CY, chrysanthemum yellows; DY, dwarf aster yellows; DB, blueberry stunt; U, undiluted; 2, 4, 8, 16, and 32, reciprocals of dilutions.

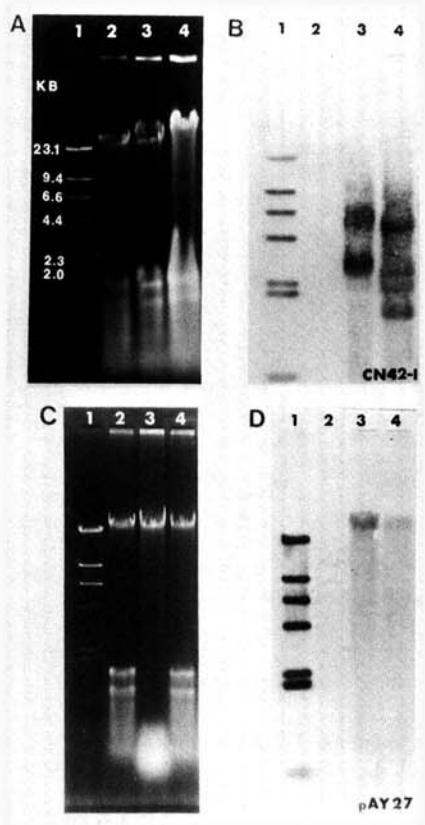

Fig. 4. Southern blot analysis of undigested DNA extracted from healthy *Catharanthus roseus* (periwinkle) plants or from periwinkle plants infected with mycoplasmalike organisms (MLOs). Nucleic acid extracted from plants was electrophoresed in 0.7% agarose gel, stained with ethidium bromide, photographed **(A** and **C)**, and destained, then transferred to nitrocellulose membranes and hybridized with biotin-labeled probes consisting of cloned DNA of periwinkle little leaf MLO (probe CN42I) **(B)** or of recombinant plasmid containing DNA from aster yellows (AY) MLO (probe pAY27) **(D)**. Lane 1, biotinylated lambda DNA-*Hin*dIII fragments; lane 2, nucleic acid extract from healthy plants; lane 3, extract from plants infected with AY MLO; lane 4, extract from plants with chrysanthemum yellows.

tinguished principally on the basis of biological properties such as the symptoms they induce in plant hosts, the specificity of their transmission by insect vector species, and the range of plant host species susceptible to infection (21,22). Shiomi and Sugiura (18) divided MLO-induced plant diseases in Japan into three groups on the basis of host range and symptoms. Conti et al (4) suggested that the biological characteristics of CY MLO resemble those of MLOs in strain group I of Shiomi and Sugiura (18). This group contains Japanese AY MLO and other MLOs infecting plants including *A. graveolens, Callistephus chinensis, Chrysanthemum coronarium, D. carota, L. sativa,* and *S. oleracea.* The CY MLO is known to infect all six of these species (4). American AY MLO also infects these plants and induces symptoms similar to those induced by the group I strains (12). American AY MLO is transmitted by *Macrosteles* species, as are MLOs in strain group I of Shiomi and Sugiura (18), but it is not known whether or not CY MLO is transmitted by *Macrosteles.*

In the future, serological procedures (3,15,19) and the use of nucleic acid hybridization probes should help to clarify the relatedness of CY MLO and other MLOs. These approaches should also facilitate the application of phytosanitary regulations and the production of MLO-free planting stock.

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