Comparison of Monoclonal Antibodies, DNA Probes, and PCR for Detection of the Grapevine Yellows Disease Agent

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ABSTRACT

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Two monoclonal antibodies (MAbs) specific for a grapevine yellows mycoplasmalike organism (GY-MLO) were produced by fusing mouse myeloma cell lines NS1/1 to spleen cells of mice immunized with partially purified GY-MLO from diseased periwinkles. Using enzyme-linked immunosorbent assays (ELISAs) and indirect immunofluorescence (IF) staining, the MAbs were shown to be specific for the GY-MLO. Although both ELISA and IF tests were satisfactory with diseased periwinkles, serological detection of GY-MLO in diseased grapevines was not always reliable because of the extremely low titer of GY-MLO in the phloem and the interference of plant pigments in dot blot immunoassays. We developed specific DNA probes and primers for use in polymerase chain reaction (PCR) for the detection of the GY-MLO. An enriched preparation of GY-MLO chromosomal DNA was obtained by bisbenzimide-CsCl buoyant density gradient centrifugation of the total DNA extracted from diseased periwinkles. The enriched DNA was cloned into pUC19 and used to transform *Escherichia coli* DH5α cells. Two recombinant plasmids (pGYD-1 and -2) reacted specifically to GY-MLO DNA and contained 9.0- and 1.6-kb inserts, respectively. Based on the partial sequence of the cloned genomic DNA fragment GYD-2, three oligonucleotides (oligos 1, 2, and 3) were designed and synthesized for use as primers in PCR. Using these specific DNA probes in dot hybridizations, positive results were observed with 10 ng of total DNA from GY-MLO-infected periwinkles. On the other hand, PCR could detect GY-MLO when only 10^{-2} pg of DNA from the same source was used as template. The labeled DNA probes were used to successfully detect GY-MLO in grapevine samples collected from Italy and the United States. Using oligos 1 and 2, a 550-bp DNA fragment was amplified from crude DNA extracts of infected periwinkles and grapevines. Employing oligos 1 and 3, a 600-bp DNA fragment was amplified only from infected periwinkles, not from diseased grapevines. No PCR products were detected when DNAs from healthy periwinkles or grapevines were used as templates. Results from PCR suggested that related but distinct strains of GY-MLO must exist, causing similar yellows symptoms in grapevines. An inverse relationship also was observed between symptoms of yellows and results from both dot hybridization and PCR. Almost all wild grapevine (*Vitis riparia*) samples collected near vineyards in New York showed a positive association with GY-MLO. Although none of the *V. riparia* samples showed GY symptoms, they may serve as an important alternative host for the GY causal agent.

Flavescence dorée (FD) is one of the most serious diseases of grapevines (*Vitis vinifera* L.). In 1957, Caudwell first reported FD in France (6), and since then, grapevine yellows (GY) disease showing symptoms similar to those of FD has been found in Italy, Germany, Australia, and the United States (5,16,29,33). Reports have associated mycoplasmalike organisms (MLOs) with the disease (17,28), but the evidence is sketchy, apparently due to the extremely low titer of the microorganisms in infected grapevines. The causal agent of FD is believed to be transmitted naturally to grapevines by the leafhopper *Scaphoideus titanus* Ball (38). In Italy, the occurrence and spread of GY does not seem to be correlated with the presence of *S. titanus* (R. Osler, L. Carraro, E. Refatti, and N. Loi, *unpublished data*).

In France, FD-MLO was transmitted from infected grapevines to broadbeans (*Vicia faba* L.) by *S. titanus* collected from FD-affected vineyards (8). The MLOs were then experimentally transmitted from broadbeans to periwinkles (*Catharanthus roseus* (L.) G. Don) by the leafhopper *Euscelidius variegatus* Kirschbaum, which is not a natural vector of FD. In Udine, Italy, GY-MLO was transmitted directly from infected grapevines to periwinkles by dodder in the greenhouse (L. Carraro, *unpublished data*). Exhibited symptoms on the two experimentally transmitted MLO-infected periwinkles were quite different. The French periwinkles showed yellowing, early abscission of leaves, slow growth, and small flowers, whereas the periwinkle symptoms of northern Italy exhibited small leaves and phyllody (3). Recently, MLOs associated with grapevine yellows occurring in other parts of Italy were transmitted to periwinkles by dodder. At least two different symptoms were noted (11). In the United States, GY has been found in New York (33) and Virginia (R. C. Pearson, *unpublished data*). The disease symptomatology closely resembles that of FD in France and GY in Italy, and *S. titanus* has been found in the American vineyards (30). However, because of a lack of methods to detect and identify the MLOs associated with the disease, there is only circumstantial evidence to indicate that the American GY disease is a form of European GY (30). Moreover, it is impossible to know whether the grapevine yellows diseases of various regions of the world are caused by the same MLOs or whether the same leafhopper vector is responsible for spreading the disease.

We report the development of several reliable methods for the detection of the GY disease agent in both infected periwinkles and grapevines. These include monoclonal antibodies (MAbs) specific against GY-MLO, DNA probes derived from cloned GY-MLO DNA fragments, and oligonucleotide primer sets designed according to the sequence data of a cloned GY-MLO DNA fragment for the amplification of GY-MLO DNA by polymerase chain reaction (PCR).

MATERIALS AND METHODS

Diseased plants. Periwinkles (*C. roseus*) infected with the GY-MLO were obtained by dodder (*Cuscuta campestris*) transmission in Italy from grapevines (*V. vinifera* cv. Chardonnay) naturally infected with GY and provided by L. Carraro, Udine, Italy. The diseased periwinkles were propagated by grafting and were main-

tained in the greenhouse. Periwinkle plants infected with various other MLOs and maintained in our greenhouse also were used in this study. Grapevines with or without GY symptoms were collected from *V. vinifera* vineyards in the Friuli region of northern Italy and from the Finger Lakes region of central New York during 1991 and 1992. Healthy grapevines were generated from White Riesling seeds (provided by R. C. Pearson) and were maintained in an insect-controlled greenhouse.

Antigen preparation. Leaves of GY-infected periwinkle showing typical symptoms were collected, rinsed with tap water, and surface-sterilized with 0.5% bleach. Midribs were excised and incubated in a macerating enzyme solution (0.8% cellulase, 0.2% hemicellulase, and 0.2% pectinase in isolation medium) (19) at 4 C overnight. Vascular tissues were isolated according to Lee and Davis (25). Tissues were ground in isolation medium, and the homogenate was subjected to two cycles of differential centrifugation (2,000 *g* for 10 min and 20,000 *g* for 40 min). The pellet was suspended in isolation medium, and further separation of MLOs from plant organelles was carried out according to the method of Jiang and Chen (19). Finally, the pellet containing partially purified GY-MLO was resuspended in phosphate-buffered saline (PBS, pH 7.0), sonicated, and used as immunogen to immunize BALB/c mice.

Immunization and hybridoma production. BALB/c mice were immunized on day 1 by a 0.2-ml intraperitoneal injection of a mixture containing 20 μg of partially purified GY-MLO in 100 μl of PBS and an equal volume of Freund's complete adjuvant. The second, third, and fourth injections were done similarly at 7-day intervals, except that incomplete Freund's adjuvant was used in the mixture. Forty-two days after the first injection, a boost injection was applied intravenously with 50 μg of immunogen, and fusion was performed 3 days later. The procedure for cell fusion was adapted from the method of Lane (24).

Screening and antibody isotyping. Hybridomas secreting antibodies specific to GY-MLO were screened by indirect enzyme-linked immunosorbent assay (ELISA) with biotinylated anti-mouse IgG and IgM conjugates as secondary antibodies (20). The hybridomas producing specific antibodies were selected for further monocloning by limiting dilution (27). The immunoglobulin classes and subclasses of the MAbs were determined by indirect ELISA (9).

Antibody production. Ascitic fluids in mice were produced by injecting about 10^6 hybridoma cells into the peritoneal cavity of each pristane-primed BALB/c mouse. After 2–3 wk, ascitic fluids were collected from the mice every 2 days with a syringe. MAbs also were collected from hybridoma culture supernatants after cell numbers had reached $5–7 \times 10^6$ cells per milliliter.

Specificity of MAbs. The specificity of MAbs was determined by comparison with other yellows disease agents in indirect ELISA. Periwinkle plants infected with MLOs associated with apple proliferation, ash yellows, the Maryland strain of aster yellows, the New Jersey strain of aster yellows, eastern X-disease, elm yellows, loofah witches'-broom, peanut witches'-broom, paulownia witches'-broom, and sweet potato witches'-broom were used in this study.

Immunofluorescence (IF) staining. Freehand cross sections of midribs, leaf petioles, and young stems of both diseased and healthy periwinkle plants were made and fixed in cold acetone for 1–2 min. The fixed sections were dried and incubated with culture supernatant of MAb GYM-1 for 1 h at 37 C. The remaining steps of the IF-staining procedure were as described by Chen and Jiang (9).

Dot blot immunoassay. The veins and petioles of grapevine leaves were ground in PBS (1:5, w/v) containing 1% polyvinylpyrrolidone (PVP). The homogenate was clarified by low-speed centrifugation (500 *g* for 10 min). The supernatant was sonicated and used as antigens. Each antigen solution (50–200 μl) was blotted onto a 0.2-μm nitrocellulose membrane (Schleicher & Schuell Inc., Keene, NH) with the S & S Minifold apparatus (Schleicher & Schuell Inc.). Saturation of the free binding sites was done with PBS containing 1% nonfat dry milk and 0.5% bovine serum albumin (BSA) at 37 C for 60 min. After blocking, the membranes were incubated with MAbs in culture supernatant at 37 C for 1 h. After three successive washings, 10–15 min each in a PBS-Tween solution (PBS containing 0.05% Tween 20), the blots were incubated at 37 C with biotinylated goat anti-mouse IgG and IgM for 40 min, rinsed with PBS again, and incubated with streptavidin-peroxidase conjugates at 37 C for 20 min. Finally, the blots were rinsed and developed in a mixture containing 3 mg of 4-chloro-1-naphthol per milliliter of PBS and 0.01% H_2O_2 at room temperature for 20 min. The reactions were stopped by rinsing the blots with distilled water.

Isolation of GY-MLO DNA. Total DNA was extracted by following the procedure described by Doyle and Doyle (15), with minor modifications. Leaf midribs (20 g) were cut from GY-MLO-infected periwinkle leaves and ground to a fine powder in liquid nitrogen. The tissue powder was suspended in 120 ml of preheated (60 C) CTAB buffer (100 mM Tris-HCl [pH 8.0], 1.4 M NaCl, 20 mM EDTA, and 2% hexadecyltrimethylammonium bromide) and incubated at 60 C for 30 min with occasional shaking. The mixture was filtered through two layers of cheesecloth and was extracted once with two-thirds volume of chloroform-isoamyl alcohol (24:1, v/v). After centrifugation at 4,300 *g* for 10 min, the aqueous phase was transferred to a clean tube, and nucleic acids were precipitated by isopropanol. The MLO DNA was separated from host-plant DNA by cesium chloride-bisbenzimide buoyant density gradient centrifugation (22,39). After centrifugation at 20 C for 44 h, a putative GY-MLO DNA band was visualized by UV light and collected by piercing the centrifuge tube wall with a syringe. The MLO DNA-enriched fraction was extracted twice with an equal volume of CsCl-saturated isopropanol, diluted with 3 volumes of distilled water, and the DNA was precipitated by adding isopropanol at −20 C overnight. After centrifugation and drying, the DNA pellet was resuspended in TE buffer (10 mM Tris, 1 mM EDTA [pH 8.0]).

Cloning of GY-MLO DNA and screening of recombinants. About 100 ng of CsCl-purified GY-MLO DNA was digested with *Eco*RI at 37 C for 3 h and ligated into *Eco*RI-digested pUC19, using a 5:1 insert/vector ratio, at 14 C for 16 h. One-tenth of the ligation mixture was used to transform *Escherichia coli* DH5α competent cells (GIBCO BRL Life Technologies, Inc., Gaithersburg, MD) following the manufacturer's instructions. Two recombinant plasmids containing DNA fragments (GYD-1 and -2) specific to GY-MLO were selected by differential hybridization with ^{32}P-labeled CsCl-purified GY-MLO DNA and total DNA from healthy periwinkles as probes. The probes consisted of inserts, GYD-1 and -2, from the two recombinant plasmids labeled with the random-primers DNA-labeling system (GIBCO BRL, Life Technologies, Inc.). The recombinant plasmids, composed of plasmid vector plus cloned MLO DNA fragment, were isolated by the boiling method (36), and the insert sizes were determined by *Eco*RI digestion and agarose gel electrophoresis. The inserts were excised from the gel and eluted using a Geneclean kit (BIO 101, Inc., La Jolia, CA). The recovered inserts were used for analysis of enzyme digestion or were labeled as probes for detection of GY-MLO DNA in diseased plants.

Dot blot hybridization. Crude nucleic acids were extracted from midribs and petioles of both healthy and diseased periwinkles or grapevines following the procedures described for isolation of GY-MLO DNA but omitting the buoyant density gradient centrifugation. DNA samples were resuspended in 6× SSC (1× SSC is 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0), denatured by boiling for 10 min, and immediately chilled on ice for 5 min. Denatured DNA samples, either undiluted or diluted in twofold serial dilutions in 6× SSC, were blotted onto a nylon membrane (Biotrace RP, Gelman Science Inc., Ann Arbor, MI) with the S & S Minifold apparatus (Schleicher & Schuell Inc.). Membranes were air-dried, baked at 80 C for 2 h, prehybridized at 65 C for 3 h in a solution containing 5× SSC, 10× Denhardt's solution (1× = 0.02% Ficoll, 0.02% PVP, and 0.02% BSA), 0.1% SDS (sodium dodecyl sulfate), and 500 μg of denatured salmon sperm DNA per milliliter, and hybridized with a similar solution containing the denatured ^{32}P-labeled DNA probes (GYD-1 or both GYD-1 and -2) for 16 h at 65 C. After

hybridization, membranes were washed in high-stringency wash conditions, consisting of two washes in 2× SSC with 0.1% SDS at room temperature (15 min per wash) and two washes in 0.1× SSC with 0.1% SDS at 65 C (30 min per wash), and were autoradiographed for 24 h.

Subcloning and sequencing. According to the results of enzyme-digestion analysis, GYD-2 was cut by *Acc*I and *Eco*RI, and one of the fragments was recovered from a gel and subcloned into plasmids pUC18 and pUC119. Recombinant plasmid DNA used as template for DNA sequencing was purified by CsCl density gradient centrifugation. DNA sequencing was performed by the dideoxy method, using ^{35}S-labeled dATP and a sequenase kit (United States Biochemical Corporation, Cleveland, OH), following the protocols suggested by the manufacturer.

TABLE 1. ELISA values (OD_{490}) of grapevine yellows mycoplasmalike organism (MLO) monoclonal antibodies reacting with healthy and MLO-infected periwinkle preparations

Mycoplasmalike organisms	Monoclonal antibodies	
	GYM-1	GYM-2
Grapevine yellows	0.650[a]	0.411
Apple proliferation	0.046	0.042
Ash yellows	0.041	0.064
Aster yellows (MD)[b]	0.007	0.030
Aster yellows (NJ)[c]	0.031	0.014
Eastern X-disease	0.038	0.009
Elm yellows	0.045	0.049
Loofah witches'-broom	0.056	0.036
Paulownia witches'-broom	0.022	0.021
Peanut witches'-broom	0.042	0.015
Sweet potato witches'-broom	0.046	0.036
Healthy periwinkle	0.060	0.020

[a] Optical density readings greater than 0.1 are considered positive.
[b] The Maryland strain of aster yellows MLO.
[c] The New Jersey strain of aster yellows MLO.

PCR primers design. Based on a partial sequence of the subcloned DNA fragment, three oligonucleotides were designed, synthesized (Applied Biosystem DNA synthesizer, Foster City, CA), and used as two primer sets in PCR. One primer pair, oligos 1 and 2, directed the amplification of an approximate 550-bp fragment of GY-MLO DNA. Another PCR primer pair, oligos 1 and 3, amplified an approximate 600-bp fragment of GY-MLO DNA.

DNA amplification. Nucleic acids used as templates were isolated from both healthy and diseased periwinkles or grapevines following the procedure described in dot blot hybridization. The amplification was carried out in a 25-μl PCR mixture containing 200 ng of template DNA, 250 μM each of dATP, dCTP, dGTP, and dTTP, 1.0 μM of each primer of a pair, 25 mM Tris (pH 8.0), 5 mM $MgCl_2$, 50 mM NaCl, 100 μg of BSA (GIBCO BRL) per milliliter, and 1.25 U of *Taq* DNA polymerase (Promega Corp., Madison, WI). The reaction mixture was overlaid with 25 μl of mineral oil, and the PCR was performed for 30 or 35 cycles in a Hybaid thermal reactor (National Labnet Co., South Plainfield, NJ). Thermocycling conditions were as follows: initial denaturation for 2 min at 94 C; and then 30 or 35 cycles of denaturation for 1 min at 94 C, annealing for 1 min at 50 or 55 C, and extension for 3 min at 72 C. The final extension step was extended by 20 min at 37 C. PCR products were analyzed by electrophoresis in a 1% agarose gel, stained with ethidium bromide, and visualized with a UV transilluminator (Fotodyne Inc., New Berlin, WI).

RESULTS

MAbs against GY-MLO. Mouse myeloma cells P3/NSI/1-Ag4-1 were fused with spleen cells from mice immunized with par-

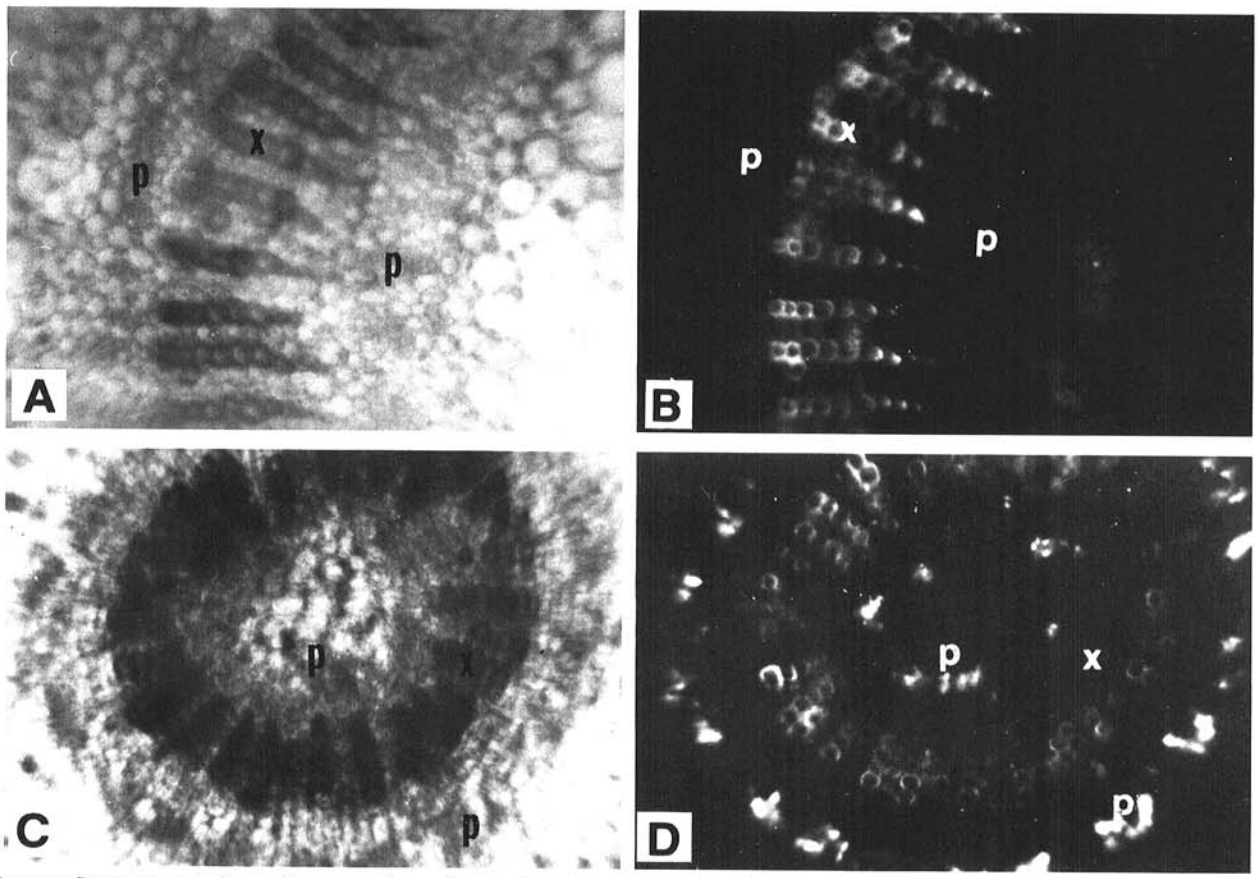

Fig. 1. Immunofluorescence staining of cross sections of grapevine yellows mycoplasmalike organism-infected periwinkles with monoclonal antibody GYM-1. The same section was photographed under bright field and UV light. **A,** Healthy section under bright field and **B,** the same section under UV light showing no fluorescence in the phloem; **C,** diseased periwinkle section under bright field and **D,** the same section under UV light showing typical apple-green fluorescence (white areas in the photo) in the phloem areas. p, phloem; x, xylem.

tially purified GY-MLO. A total of more than 2,100 hybridomas was obtained from three fusions. After screening by differential ELISA, only two stable hybridoma clones that secreted antibodies specific to GY-MLO, but not to healthy periwinkles, were selected and monocloned. From these two clones, two monoclonal hybridoma cell lines were selected and designated as GYM-1 (10H12A12) and -2 (2B6C12). Several hybridoma cell lines, though producing anti–GY-MLO antibodies initially, did not survive subculturing.

Antibody characteristics. Antibodies or ascitic fluids from the two selected monoclonal hybridoma cell lines were characterized for immunoglobulin isotypes, indirect ELISA titer, and specificity. The immunoglobulin isotypes of the two MAbs were determined using indirect ELISA. MAbs GYM-1 and -2 belonged to isotypes IgG2a and IgG3, respectively. Antibody dilution end points of cell culture supernatants were 625 for GYM-1 and 125 for GYM-2 in indirect ELISA. However, antibody titers in ascitic fluids were greater than those in the cell culture media: 100,000× for GYM-1 and 10,000× for GYM-2.

Two methods, indirect ELISA and IF staining, were used to determine specificity of MAbs. In indirect ELISA, GYM-1 and -2 reacted specifically with GY-MLO-infected periwinkles but did not cross-react with antigens prepared from healthy periwinkles or from periwinkles infected with nine other MLOs (Table 1). When both MAbs were used in IF staining, a very bright green fluorescence was observed only in the phloem elements of GY-MLO-infected periwinkles. Sections from healthy periwinkles did not show the fluorescein isothiocyanate (FITC) fluorescence (Fig. 1). Under UV light (9), a yellow-brown autofluorescence also was observed on the secondary cell walls of xylem elements, but this could be easily distinguished from the typical FITC fluorescence (Fig. 1)

Cloning of GY-MLO chromosomal fragments. Over 400 transformants were obtained and screened by differential colony hybridization with ^{32}P-labeled total DNA from healthy periwinkles and CsCl-purified GY-MLO DNA. The majority of the recombinant colonies hybridized with DNAs from both diseased and healthy periwinkles. Two transformants that hybridized with DNA from diseased, but not from healthy plants, were selected. Southern blot hybridization analysis confirmed that the two clones contained DNA specific to GY-MLO (data not shown). They were designated pGYD-1 and -2.

Characterization of cloned inserts. The molecular sizes of the cloned inserts were estimated by digesting the two recombinants with *Eco*RI and electrophoresing them in a 0.8% agarose gel. The pGYD-1 plasmid contained a 9.0-kb insert, and the pGYD-2 plasmid contained a 1.6-kb insert. The specificity of the two cloned inserts was determined by dot blot hybridization in which both inserts hybridized with total DNA extracted from GY-MLO-infected periwinkles but not with total DNA extracted either from healthy periwinkles or from periwinkles infected with seven other MLOs (Table 2). When the GYD-1 insert was used as a probe to detect GY-MLO, it could detect GY-MLO DNA in as little as 10 ng of total DNA isolated from infected periwinkles (Fig. 2A). Both DNA inserts were excised from the recombinant plasmids and digested with *Acc*I, *Bam*HI, *Bgl*II, *Cla*I, *Eco*RV, *Hin*dIII, *Nde*I, *Pst*I, and *Xba*I restriction endonucleases. GYD-1 was digested by *Bgl*II, *Eco*RV, and *Hin*dIII, whereas GYD-2 was digested by *Acc*I, *Eco*RV, and *Nde*I.

Sequence and primer characteristics. One of the *Acc*I-*Eco*RI fragments of GYD-2 was subcloned into pUC18 and pUC119 plasmids. Recombinant plasmids (GYD-2-1 and -2-2) were isolated with CsCl density gradient centrifugation and sequenced. According to the partial sequence data, three oligonucleotides were designed. Oligo 1 (50% A+T): 5′-TACTGCTGCGCGAC-GTCTAATT-3′; oligo 2 (55% A+T): 5′-CCAAGCTGTGACT-GTCTTTA-3′; and oligo 3 (80% A+T): 5′-GACATTCGATTA-ACATTTTATTGATAAATT-3′ were synthesized and used as primers in PCR to detect GY-MLO DNA. To determine the spe-

TABLE 2. Dot hybridization analyses of ^{32}P-labeled grapevine yellows mycoplasmalike organism (MLO) DNA probes to DNA extracts from healthy and MLO-infected periwinkle plants

Mycoplasmalike organisms	DNA probes[a]	
	GYD-1	GYD-2
Grapevine yellows	+	+
Apple proliferation	−	−
Ash yellows	−	−
Aster yellows (MD)[b]	−	−
Aster yellows (NJ)[c]	−	−
Elm yellows	−	−
Paulownia witches'-broom	−	−
Sweet potato witches'-broom	−	−
Healthy periwinkle	−	−

[a] +, positive hybridization; −, no hybridization signal.
[b] The Maryland strain of aster yellows MLO.
[c] The New Jersey strain of aster yellows MLO.

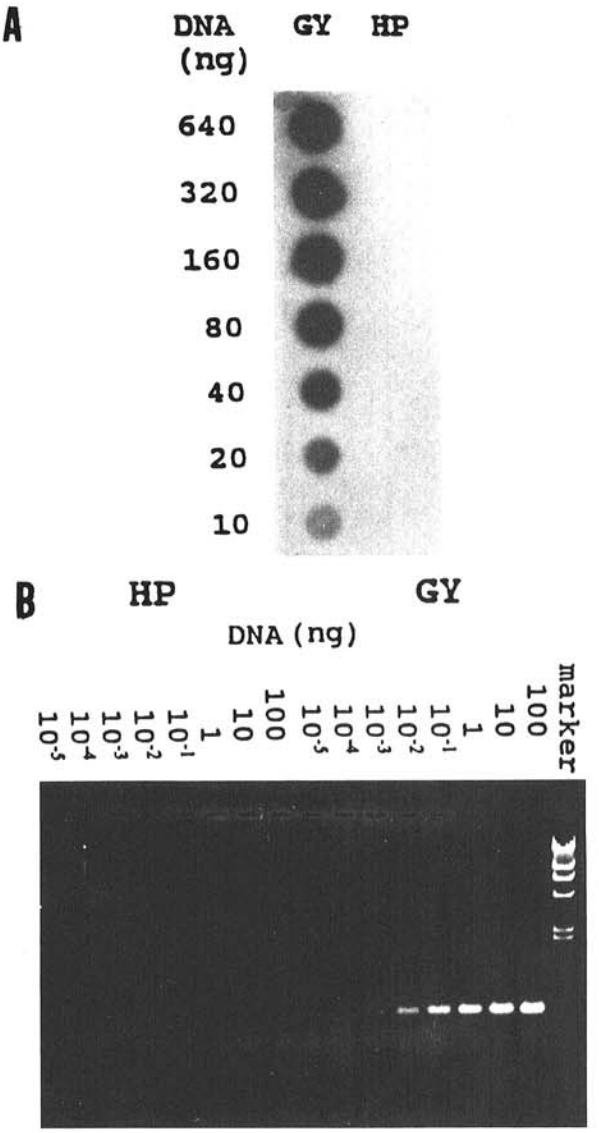

Fig. 2. Detection sensitivity of probe and primers for grapevine yellows mycoplasmalike organism (GY-MLO). **A,** ^{32}P-labeled DNA fragment GYD-1 in dot blot hybridization and **B,** oligos 1 and 2 in polymerase chain reaction. Nucleic acids were extracted from both healthy (HP) and diseased (GY) periwinkles and subjected to serial twofold dilution for dot blot hybridization and 10-fold dilution for polymerase chain reaction (PCR). In **B,** a DNA fragment corresponding to 550 bp was amplified from all dilutions of GY DNA but no PCR product was observed when healthy periwinkle DNA was used as a template. Marker was lambda DNA digested with *Hin*dIII, from top to bottom band: 23.1, 9.4, 6.6, 4.4, 2.3, 2.0, and 0.56 kb.

cificity of these primers for detecting GY-MLO DNA, two primer sets (oligos 1 and 2 and oligos 1 and 3) were used in PCR amplification with MLO DNAs from other yellows diseases. Both primer sets reproducibly amplified only the DNA fragments from nucleic acids extracted from GY-MLO-infected periwinkles. No PCR products could be demonstrated when DNAs from healthy periwinkles and seven other MLO-infected periwinkles were used as templates (Fig. 3) The sensitivity of the primer pair in detecting GY-MLO also was determined. Total DNAs from GY-MLO-infected and healthy periwinkles were serially diluted 10-fold, started from 100 ng, and amplified by the primer pair oligos 1 and 2. As little as 10^{-5} ng of DNA from GY-infected plants could be detected, but no PCR product was observed when healthy periwinkle DNA was used as a template (Fig. 2B).

Detection of GY-MLO in grapevine samples. Grapevine samples with or without GY symptoms were collected from the Friuli region of Italy and the Finger Lakes region of New York during 1991 and 1992. Both ELISA and IF tests, which had been successful in detecting GY-MLO in periwinkle, failed to show the presence of the microorganisms in diseased grape phloem cells. As an alternate, we used the GYM-1 MAb in dot blot immunoassays in which repeated addition of antigen preparation must be applied to the wells to concentrate it in a spot on the membrane. Although positive reactions could be observed with some samples (data not shown), the yellow-brown pigments from grapevine samples often interfered with the results. This interference made negative reactions difficult to recognize, particularly with samples collected during the fall.

In dot hybridization, total nucleic acids were extracted from approximately 0.3 g of diseased grapevine leaf veins by the CTAB procedure and were blotted onto nylon membranes. Results showed that distinct positive signals appeared in some grapevine samples collected from both areas (Table 3). Grapevine samples collected in the Finger Lakes region of New York during the growing season fell into two categories composed of those with and without yellows symptoms. The vines with symptoms gave weak hybridization signals or none at all. The vines without symptoms gave visible or strong hybridization signals. Another interesting result was that GY-MLO could be detected in three wild grapevine plants (*Vitis riparia* Michx.) that were collected near vineyards in New York. These wild grapevines did not exhibit yellows symptoms.

Because the titer of GY-MLO was extremely low in infected grapevine and because the dot hybridization results did not correlate with the symptomatology, a further increase of detection sensitivity was needed. PCR was used to detect GY agents in the grapevine samples. Three oligonucleotides were synthesized and used as primers. Using oligos 1 and 3, a 600-bp DNA fragment was consistently amplified from diseased periwinkles. However, this fragment could not be amplified from diseased grapevines collected in New York and Italy (data not shown). When oligos 1 and 2 were used as primers, a 550-bp DNA fragment was amplified from both diseased periwinkles and diseased grapevine samples (Fig. 4). This result indicated that oligos 1 and 2 were more useful for GY-MLO detection in grapevines. Thus, oligos 1 and 2 were used exclusively in our disease diagnosis, and the results, as shown in Figure 4. and Table 4, paralleled those from dot blot hybridization.

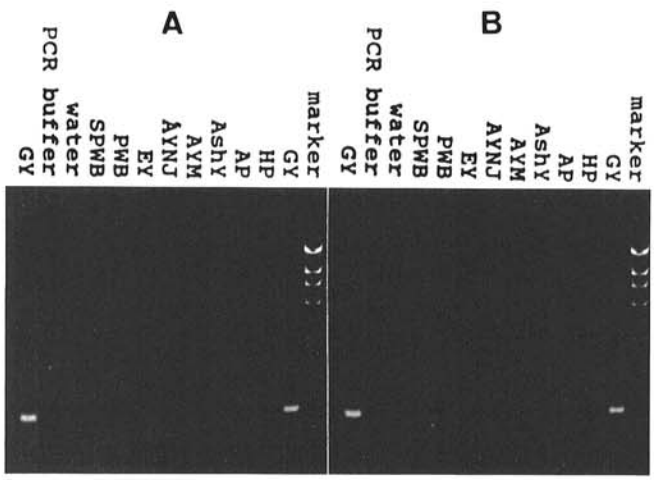

Fig. 3. Specificity of primers for detecting grapevine yellows mycoplasmalike organism (GY-MLO) in polymerase chain reaction (PCR): **A,** oligos 1 and 2 and **B,** oligos 1 and 3. Nucleic acids extracted from healthy and MLO-infected periwinkle plants were used as templates. HP, healthy periwinkle; AP, apple proliferation; AshY, ash yellows; AYM, the Maryland strain of aster yellows; AYNJ, the New Jersey strain of aster yellows; EY, elm yellows; GY, grapevine yellows; PWB, paulownia witches'-broom; SPWB, sweet potato witches'-broom. In **A and B,** a 550- and 600-bp DNA fragment was amplified only from DNA extracted from GY-MLO-infected periwinkles. No PCR products were observed when DNAs from healthy and other MLO-affected periwinkles were used as templates. Marker was lambda DNA *Hin*dIII fragments.

DISCUSSION

A major limitation to obtaining etiological and epidemiological information for GY has been the lack of rapid, sensitive, and specific methods for pathogen detection and disease diagnosis. The GY agent, like other MLOs, is still unculturable, and thus, diagnosis of the disease has relied primarily on symptom expression. Observation of MLOs in phloem tissue of diseased grapevines is extremely difficult. A previous report (28) and our experiences (T. A. Chen, *unpublished data*) using transmission electron microscopy with GY indicated that the concentration of the causal microorganism in diseased plants was very low. Sections taken from grapevines showing typical GY symptoms often showed collapsed phloem cells (31,33,34) and accumulation of electron-dense substances that would obscure any MLOs if they were indeed present. Both polyclonal and monoclonal antibodies have been used in MLO detection and identification and have shown promising results (9,10,20,27). Another approach is the application of recombinant DNA technologies. Using cloned DNA fragments as probes in nucleic acid hybridization has provided a sensitive and reliable method for detecting several MLO diseases (2,12,18,21,26). DNA probes also have been used to differentiate among MLO strains (23). In this study, we successfully developed two MAbs and two DNA probes specific for some GY-MLOs. Our results demonstrated that both the immuno- and nucleic acid probes can be used to detect GY-MLOs not only in periwinkles but in grapevine samples from two different geographic areas. The existence of common epitopes and DNA sequence homology within GY-MLOs from Italy and New York suggested that MLOs associated with GY diseases from these two areas are related.

The successful transmission of MLOs associated with grapevines showing GY symptoms to periwinkles has, without doubt, facilitated our studies. One could still question the possibility

TABLE 3. Summary of results from dot blot hybridization analyses of grapevine samples probed with ^{32}P-labeled GYD-1 and GYD-2

Location and date of sampling	No. of samples with symptoms/symptomless	No. of positive samples[a]
Finger Lakes, NY[b]		
Jun. 1991	1/6	0/1, 5/6
Jul. 1991	1/4	0/1, 2/4
Aug. 1991	6/6	0/6, 6/6
Sept. 1991	5/12	1/5, 9/12
Friuli, Italy[c]		
Sept. 1991	5/6	1/5, 5/6
Oct. 1991	5/6	0/5, 4/6

[a] Number of positive samples/number of samples with disease symptoms and number of positive samples/number of samples without symptoms, respectively.

[b] Samples collected from region in New York consisted of *Vitis vinifera* cv. White Riesling and *Vitis riparia.*

[c] Samples collected from region in Italy consisted of *Vitis vinifera* cv. Chardonnay.

that some of the MLOs transmitted to periwinkles were not the major pathogens. When dealing with an unculturable plant pathogen, such a question must be considered, especially because MLO strains transmitted from grapevines to periwinkles in Europe exhibited different symptoms. At present, it is not clear whether the discrepancy of symptomatology on periwinkles is due to strain differences among GY-MLO or is the result of contamination through methods employed for transmission. Our results, however, clearly showed that the probes derived from MLOs in our periwinkles were useful in detecting MLOs specifically associated with diseased grapevines. Further studies are needed to answer some of these questions.

During the course of this study, we realized that the sensitivity of the probes was important for practical use in disease detecton. The IF test with our MAbs was specific and rapid for diagnosing infected periwinkles; however, it was useless for diagnosing grapevines, perhaps because the titer of GY-MLO was so low and its distribution was so uneven in the plant tissues. The DNA probe has greatly increased the sensitivity of detection. Nevertheless, in dot blot hybridization analysis, 10 ng of total DNA from infected periwinkles was required for positive detection of GY-MLO. At least 2,000- to 3,000-fold of total DNA from diseased grapevines was needed for positive signals. In addition, dot blot hybridization is very time-consuming.

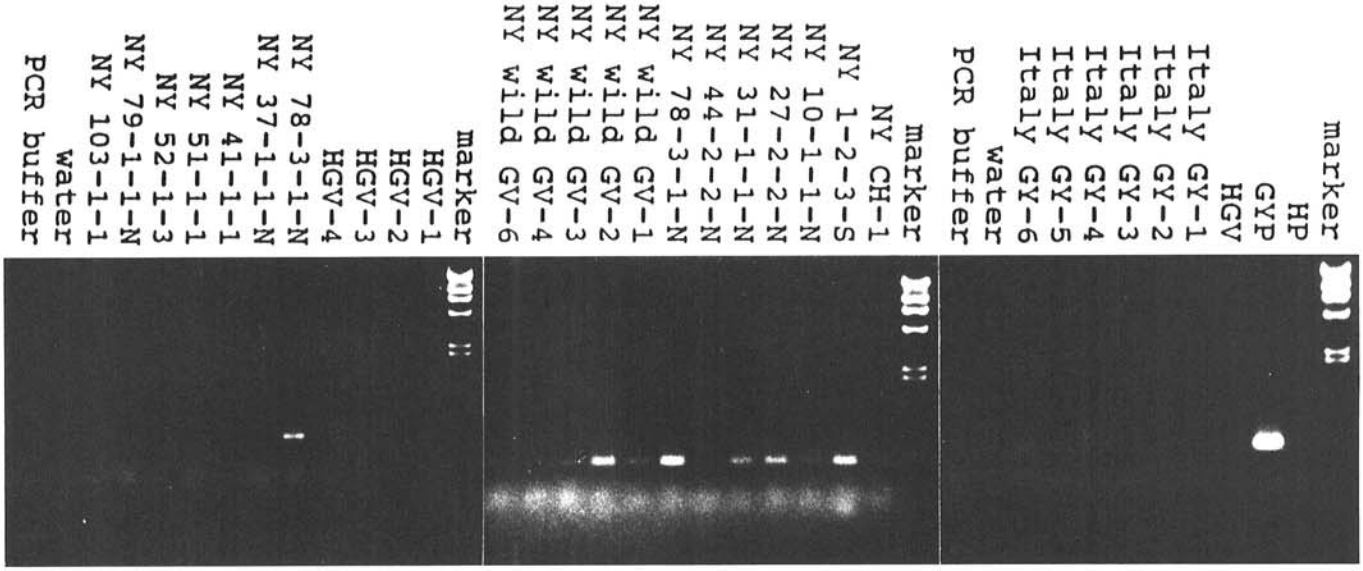

Fig. 4. Detection of grapevine yellows mycoplasmalike organism (GY-MLO) DNA in nucleic acids extracted from samples collected from the Friuli region of Italy and the Finger Lakes region of New York with oligos 1 and 2 in polymerase chain reactions (PCR). Healthy periwinkle (HP) and healthy grapevine (HGV) seedlings generated from White Riesling seeds were used as the negative control. GY-MLO-infected periwinkle (GYP) was used as the positive control. These samples were collected during September 1992 and were the same as those in Table 4. All samples were tested in the same PCR and run on three gels. All wild vines and samples, 1-2-3-S, 27-2-2-N, 31-1-1-N, 37-1-1-N, and 78-3-1-N were symptomless; the remaining plant samples showed symptoms. Marker was lambda DNA *Hin*dIII fragments.

TABLE 4. Summary of grapevine yellows symptoms and results from polymerase chain reactions (using oligos 1 and 2) of grapevine samples collected from Italy and New York

Location and date	Sample no.	Species or cultivar	Symptom[a]	PCR amplification[b]
Finger Lakes, NY	Wild vines 1	*Vitis riparia*	−	++
Sept. 1992	Wild vines 2	*V. riparia*	−	++++
	Wild vines 3	*V. riparia*	−	+
	Wild vines 4	*V. riparia*	−	+
	Wild vines 6	*V. riparia*	−	−
	CHI	Chardonnay	+++	+
	1-2-3-S	Chardonnay	−	++++
	10-1-1-N (33)	Chardonnay	++++	+
	27-2-2-N	White Riesling	−	++++
	31-1-1-N	White Riesling	−	+++
	37-1-1-N	White Riesling	−	−
	41-1-1	White Riesling	++	−
	44-2-2	White Riesling	+++	−
	51-1-1	White Riesling	++++	−
	52-1-3	White Riesling	+	−
	78-3-1-N	White Riesling	−	++++
	79-1-1-N	White Riesling	+	−
	103-1-1	White Riesling	+++	−
Friuli, Italy	1	Chardonnay	+++	−
Sept. 1992	2	Chardonnay	+++	−
	3	Chardonnay	+++	−
	4	Chardonnay	+++	+
	5	Chardonnay	+++	+
	6	Chardonnay	+++	+

[a] Disease severity was rated by visual estimation of the symptoms. −, no symptoms; +, mild symptoms, about 10% of leaves showing symptoms; ++, moderate symptoms, 10–40% of leaves showing symptoms; ++, moderate symptoms, 10–40% of leaves showing symptoms; +++, severe symptoms, 40–70% of leaves showing symptoms; and ++++, very severe symptoms, over 70% of leaves showing symptoms.
[b] −, no visible band; +, faint band; ++, clear band; +++, strong band; and ++++, very strong band (Fig. 4).

In view of these drawbacks, the PCR method was chosen as an alternative technique. PCR involves primer-directed amplification of target DNA with a thermostable DNA polymerase (35) and needs only a few DNA templates. Thus, it provides a significant advantage for detecting target organisms in low concentrations. This study showed that our primer set (oligos 1 and 2) was able to detect GY-MLO in as little as 10^{-2} pg of total DNA from infected periwinkle plants. In terms of sensitivity, PCR increased the detectability of GY-MLO one million times compared with the sensitivity of the cloned DNA fragments.

Primers designed for PCR can be derived either from cloned MLO-specific DNA fragments (13) or from the 16S ribosomal RNA gene (rDNA) (1). In the latter case, DNA fragments originating from host plants and MLOs might be amplified simultaneously, and the sizes of both amplified fragments could be similar. An additional analysis with restriction enzyme digestion would be necessary to distinguish the target DNA products from those of host-plant origin. In comparison, primers derived from specifically cloned DNA fragments would be more specific in amplifying the target MLO DNA. Our results have substantiated the fact that no PCR product was amplified from healthy plant DNA after 35 cycles. A specific band was obtained only from either infected periwinkles or infected grapevines.

The discrepancy between the PCR results of the two primer sets (i.e., oligos 1 and 2 or 1 and 3) suggested that the GY-MLOs from periwinkles and those from grapevines are closely related but not identical. The results agreed with the data from dot immunoassay and dot hybridization. The GY-MLOs in periwinkles originated in and were transmitted from a diseased Chardonnay grapevine in Friuli, Italy, and the organism has been propagated in periwinkle for more than 6 yr. Nevertheless, one cannot rule out the possibility that minor gene mutation of this MLO in periwinkle has occurred during this time.

In previous reports, necrosis and collapse of sieve elements of infected plants were associated with several MLO diseases, such as lethal yellowing of palm (40), elm phloem necrosis (4), pear decline (37), and X-disease of stone fruits (14). In these distorted phloem elements of diseased plants, very few MLOs can be detected. This also is true in the case of GY and may explain the inverse relationship between disease symptoms and positive signals obtained in our dot blot hybridization and PCR experiments. Apparently, the typical symptoms of GY disease are the results of damaged phloem tissues in which the conditions also are unsuitable for MLOs. Conversely, some grapevines without visible symptoms may harbor high titer of MLOs in the sieve tubes.

The presence of GY-MLO in symptomless wild *V. riparia* grapevines in New York is very interesting. Maixner et al (30) reported the common occurrence of *S. titanus*, the FD vector, completing its life cycle on *V. riparia* in the same New York locations from which collections were made for this study. Furthermore, they reported 13% of leafhoppers gave a positive reaction when tested with polyclonal antibodies prepared against FD in France (30). However, they were unable to explain this percentage of presumably infected leafhoppers occurring in vineyards in which very few vines exhibited symptoms of GY. Our data suggest many of the symptomless *V. vinifera* and *V. riparia* vines in and around those vineyards are infected, perhaps explaining the previous New York findings. *V. riparia* is common in hedgerows and stream beds of southern Canada and is common from Iowa to the Great Lakes states bordering Canada (32). If they were highly tolerant to the GY-MLO, they could serve as an important alternate host for the organism. Both FD and GY diseases are relatively new in Europe. Thus, it is possible that the GY-MLO is endemic in eastern North America and has been transferred to Europe via the introduction of American grapevine for use in disease resistance and rootstock-breeding programs, as has been proposed by Caudwell (7).

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