Cluster-Specific Polymerase Chain Reaction Amplification of 16S rDNA Sequences for Detection and Identification of Mycoplasmalike Organisms

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ABSTRACT

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Oligonucleotide primers were designed for polymerase chain reaction amplification of 16S rDNA from mycoplasmalike organisms (MLOs) that belong to the aster yellows (AY) MLO strain cluster. Primer pairs designated F0R1, F1R1, F2R1, and F4R1 primed the amplifications of DNA sequences of approximately 1,440, 1,320, 1,300, and 660 bp, respectively, when templates consisted of DNAs extracted from plants infected by MLOs known to be members of the AY cluster. No amplifications were observed when templates consisted of DNA extracted from healthy plants or from plants infected by non-AY MLO cluster strains. The polymerase chain reaction primer pairs enable detection of MLOs associated with several different plant diseases and permit rapid and specific identification of their affiliations with AY MLO.

Although long presumed to be viruses, the causal agents of aster yellows (AY) and numerous other diseases of plants are now believed to be wall-less prokaryotes termed mycoplasmalike organisms (MLOs) (8,9,11). AY MLOs infect a very wide range of susceptible plant species (2,11,12), but an inability to isolate the pathogens in pure culture hinders progress in understanding the AY pathogen and disease. Major problems in understanding and managing diseases caused by AY MLOs arise from difficulties in achieving rapid and sensitive pathogen detection and accurate disease diagnosis. Pathogen identification has been especially problematic. For decades, identification and classification of AY and other MLOs have been based principally on the determination of biological characteristics, such as specificity of transmission by insect vectors, range of plant species susceptible to infection, and nature of symptoms induced in infected plants (2,14), but these approaches are labor-intensive, time-consuming, and can lead to erroneous identifications.

Since the first development of cloned MLO DNA probes (10), increasing attention has been devoted to molecular methods for MLO detection and identification. Cloned DNA probes have been applied in dot hybridizations and restriction fragment length polymorphism (RFLP) analyses to investigate MLOs associated with several diseases (for a review, see 14). Previously, we proposed a scheme for the classification of MLOs into a system of genomic clusters on the basis of results from dot hybridizations with cloned DNA probes (3–5,7,13,16,17). The classification system was further supported by data from RFLP analyses of MLO chromosomal DNA (15,18). In those studies, we found that several MLO strains closely related to AY MLO could be classified together in a single genomic cluster, termed the AY MLO strain cluster, and that this cluster was composed of strains of no fewer than three separate genomic types.

Recently, in a separate approach, we have also classified MLOs on the basis of RFLP analyses of polymerase chain reaction (PCR)-amplified 16S rDNA sequences (19). The MLO groups identified by this method coincided with the strain clusters delineated in our earlier MLO classification scheme. Analysis of 16S rDNA confirmed phylogenetic relationships of MLOs within the AY MLO cluster and distinctness among clusters, which validated the classifications. In addition, we have found it possible to design primers for cluster-specific PCR amplification of rDNA, which enables simultaneous MLO detection and identification of cluster affiliation of the detected MLO. In this report, we describe the design and use of oligonucleotide primers for cluster-specific amplification of 16S rDNA from MLOs in the AY MLO cluster. A related abstract has been published (20).

MATERIALS AND METHODS

MLOs. We investigated amplification of 16S rDNA from MLOs representing several genomic clusters as indicated in Table 1. AY MLOs were the same strains as those used in a previous study of the AY MLO cluster (15). American aster yellows (AY = AY1) MLO was originally collected from a naturally diseased periwinkle (*Catharanthus roseus* (L.) G. Don) plant from a field in Beltsville, Maryland (13). The following additional MLOs were received from the indicated researchers, who kindly provided each strain separately in periwinkle tissue: tomato big bud (BB), James Dale, University of Arkansas, Fayetteville; periwinkle little leaf (O-1 = CN1, O-13 = CN13) (4), Sharon M. Douglas, Connecticut Agricultural Experiment Station, New Haven; severe AY (SAY2), dwarf aster yellows (DAY), and Tulelake aster yellows (TLAY2), A. H. Purcell, University of California, Berkeley; clover phyllody (CPh) (1) and Canada peach X-disease (CX), L. N. Chiykowski, Agriculture Canada, Ottawa; beet leafhopper-transmitted virescence (VR), G. N. Oldfield, University of California, Riverside; ash yellows (AshY) (7) and elm yellows (EY1), W. A. Sinclair, Cornell University, Ithaca, NY; western X-disease (WX), B. C. Kirkpatrick, University of California, Davis; potato witches'-broom (PWB), a strain of Alberta aster yellows (AY27), a strain of New York aster yellows (NYAY), and clover proliferation (CP), C. Hiruki, University of Alberta, Edmonton, Canada; Italian chrysanthemum yellows (CY2), A. Bertaccini, University of Bologna, Italy; strains of Oklahoma aster yellows (OKAY3 and OKAY1), J. Fletcher, Oklahoma State University, Stillwater; and a New Jersey strain of aster yellows (NJAY), T.-A. Chen, Rutgers University, New Brunswick, NJ. MLO strains were maintained in a greenhouse where white flowered clones of healthy periwinkle were graft-inoculated with tissues from periwinkle plants singly infected by each of the MLOs.

PCR and primers. Primers for PCR were designed on the basis of a 16S ribosomal (r) RNA sequence from an *Oenothera*-infecting MLO (MIAY) (21). The nucleotide sequences of the primers are

TABLE 1. Summary of results from polymerase chain reaction amplification of 16S rDNA from mycoplasmalike organisms (MLOs)

Template DNA (MLO strain)[a]	Genomic cluster (type)[b]	DNA amplification with primer pair[c]				
		F0R1	F1R1	F2R1	F3R1	F4R1
AY1	Aster yellows MLO cluster (I)	+	−	+	+	+
DAY		+	W	+	+	+
SAY2		+	−	+	+	+
TLAY2		+	−	W	+	W
OKAY3		+	−	+	+	+
CY2		+	−	+	+	+
NYAY		+	−	+	+	+
BB	Aster yellows MLO cluster (II)	+	−	+	+	+
OKAY1		+	−	+	+	+
AY27		+	−	+	+	+
CN13		+	−	+	+	+
CN1		+	−	W	+	+
NJAY		+	−	+	+	+
CPh	Aster yellows MLO cluster (III)	+	−	+	+	+
EY1	Elm yellows MLO cluster	−	−	−	−	−
WX	X-Disease MLO cluster	−	−	−	W	−
CX		−	−	−	−	−
AshY	Ash yellows MLO cluster	−	−	−	−	−
VR	Beet leafhopper-transmitted virescence MLO cluster (T)[d]	−	−	−	−	−
CP	Clover proliferation MLO cluster (T)	−	−	−	−	−
PWB		−	−	−	−	−

[a] Template DNA was extracted from healthy periwinkle plants or from plants singly infected by MLOs.
[b] Genomic cluster affiliations and subcluster types are based on data in references 4–7 and 13–20.
[c] + = Amplification of a DNA sequence was observed; − = no amplification of DNA was observed; W = faint DNA band in gels. Reaction mixtures containing primer pairs F0R1, F1R1, F2R1, F3R1, and F4R1 yielded amplification of DNAs of about 1,440, 1,320, 1,300, 970, and 660 bp, respectively.
[d] Tentative cluster classification.

presented in Table 2. For PCR, each primer pair consisted of primer r16R1 plus one of the other primers given in Table 2.

Samples of nucleic acid for use as templates in PCR were prepared from healthy periwinkle plants and from periwinkle plants singly infected with various MLOs, as previously described (22). PCR amplifications of DNA were carried out in reaction mixtures totaling 50 μl each as previously described (22). The PCR was performed in a thermal cycler (Perkin-Elmer Cetus, Norwalk, CT) as follows: each of the first 34 cycles consisted of denaturation, 1 min at 94 C; annealing, 2 min at 50 C; and extension, 3 min at 72 C. In the last cycle, the extension step was 10 min at 72 C, and the reaction mixture was then held at 4 C. PCR products were analyzed by electrophoresis in 1.2% agarose gels followed by staining with ethidium bromide and examination under UV transillumination. The size standard 1-kb DNA ladder used in gels was obtained from GIBCO BRL (Gaithersburg, MD). The presence of the MLO DNA template in nucleic acid extracts was verified by parallel PCR with the use of oligonucleotides, as reported elsewhere (19), to prime amplifications of 16S rDNA from all MLOs tested.

TABLE 2. Nucleotide sequences of primers designed for aster yellows mycoplasmalike organism cluster-specific amplification of 16S ribosomal DNA

Primer designation[a]	Nucleotide sequence
r16F0 (F0)	5′ CTG GCT CAG GAT TAA CGC TGG CGG C 3′
r16F1 (F1)	5′ AAG ACG AGG ATA ACA GTT GG 3′
r16F2 (F2)	5′ ACG ACT GCT GCT AAG ACT GG 3′
r16F3 (F3)	5′ CCT AAT GAA TAA GCC CCG GCT AAC TAT GTG 3′
r16F4 (F4)	5′ TGG TAG TCC ACG CCG TAA 3′
r16R1 (R1)	5′ TTC CCT CTT CTT GCG AAG TTA GGC CAC CGG 3′

[a] Primers were used in pairs in polymerase chain reaction mixtures. Each mixture contained primer r16R1 plus one of the remaining primers. Abbreviated designations are in parentheses.

RESULTS AND DISCUSSION

Representative results from PCR amplifications of 16S rDNA are presented in Figures 1 and 2. All experiments were repeated at least twice with the same results. When primers F0R1, F1R1, F2R1, or F4R1 were used, DNA amplifications were observed only for reaction mixtures that contained template DNA extracted from plants infected by MLOs previously assigned to the genomic AY MLO cluster (Table 1). These MLOs included AY, DAY, SAY2, TLAY2, OKAY3, CY2, NYAY, BB, OKAY1, AY27, CN13, CN1, NJAY, and CPh. Bands representing DNAs of approximately 1,320, 1,300, 970, and 660 bp were observed in gels in which reaction mixtures contained primer pairs F1R1, F2R1, F3R1, and F4R1, respectively (Fig. 1). Figure 2 illustrates results from gel electrophoretic analyses of PCR products from reactions in which templates consisted of DNAs extracted from plants infected by non-AY cluster MLOs and primer F2R1 or F3R1 was used. No DNA amplifications were observed when these two primer pairs were used (or when any of the other primer pairs in this work were used) and when the template consisted of DNA extracted from healthy plants or from plants infected by EY1, CX, VR, CP, PWB, or AshY MLOs. However, a very faint band, representing a DNA of about 970 bp, was observed in gels when oligonucleotide pair F3R1 was used to prime PCR in which the template was DNA from WX MLO-infected plants.

In a related study, oligonucleotide primers were designed for PCR amplifications of segments of 16S rDNA from all MLOs (19). In that work, restriction analyses of PCR products yielded classifications corresponding to those proposed earlier on the basis

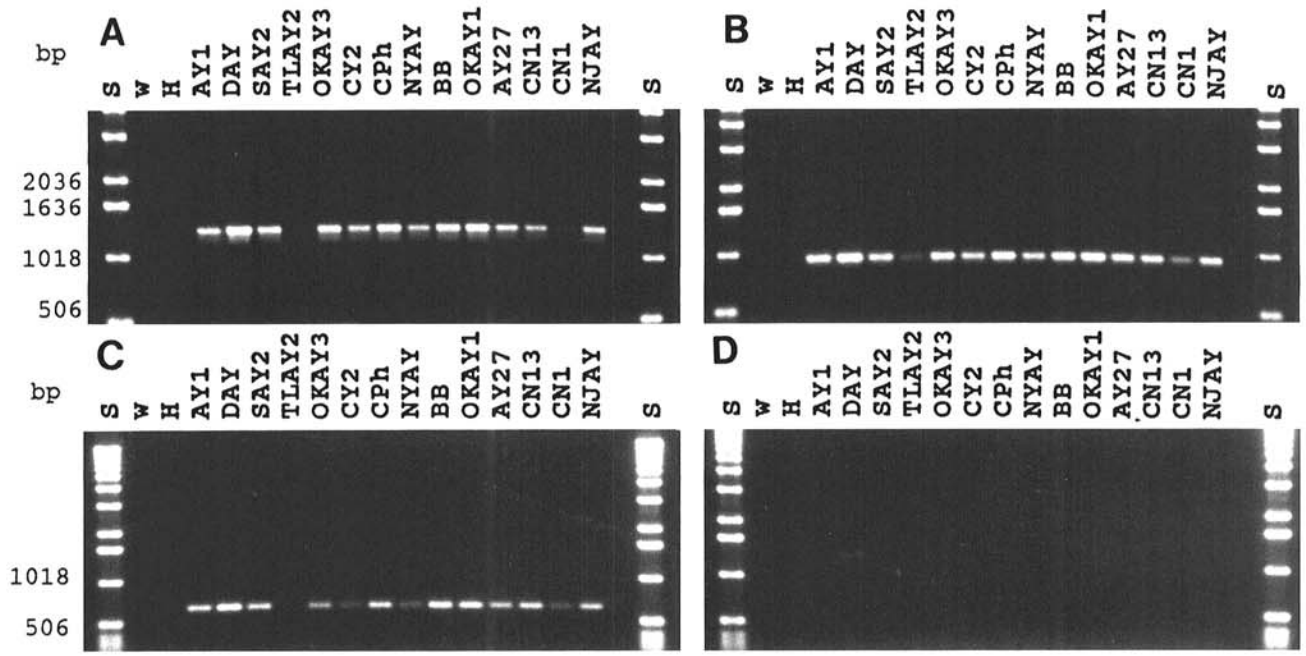

Fig. 1. Polymerase chain reaction amplifications of 16S rDNA from mycoplasmalike organisms (MLOs) in the aster yellows MLO cluster with primer pair **A,** F2R1, **B,** F3R1, **C,** F4R1, or **D,** F1R1. The template consisted of DNA extracted from healthy (H) plants of periwinkle (*Catharanthus roseus*) or from periwinkle plants singly infected by Maryland aster yellows (AY1), western dwarf aster yellows (DAY), severe aster yellows (SAY2), Tulelake aster yellows (TLAY2), Oklahoma aster yellows 3 (OKAY3), chrysanthemum yellows (CY2), a New York aster yellows (NYAY), tomato big bud (BB), Oklahoma aster yellows 1 (OKAY1), an Alberta aster yellows (AY27), Connecticut periwinkle little leaf 1 and 13 (CN1, CN13), a New Jersey aster yellows (NJAY), and clover phyllody (CPh) MLO. W, the reaction mixture contained deionized water in place of the template DNA; S, 1-kb DNA ladder. The DNA bands observed for TLAY2 with the F2R1 and F4R1 primers, CN1 with the F2R1 primer pair, and DAY with the F1R1 primer pair were fainter than those observed for other MLO-primer combinations.

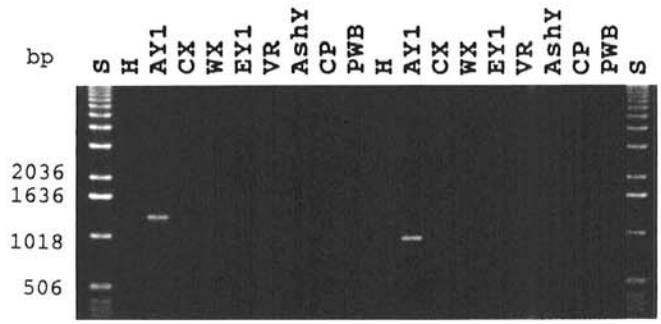

Fig. 2. Agarose gel electrophoretic analysis of products from polymerase chain reaction amplifications of 16S rDNA with primer pair F2R1 (left) or F3R1 (right). The template consisted of DNA from healthy (H) periwinkle plants or plants singly infected by aster yellows (AY1) or non-AY cluster MLOs. EY1 = elm yellows in periwinkle; WX = western X-disease; CX = Canada peach X-disease; AshY = ash yellows; VR = beet leafhopper-transmitted virescence; CP = clover proliferation; PWB = potato witches'-broom; and S = 1-kb DNA ladder. DNA bands were observed for AY1 template DNAs when either primer pair was used. A very faint band, representing a DNA of about 970 kb but not seen in the figure, was observed for WX template DNA when the F3R1 primer pair was used. No DNA bands were observed when the templates were DNAs of other MLOs or of healthy plants.

of dot hybridizations (3–5,7,13,16–18) and RFLP analyses of chromosomal DNA (17,18). In the present study, several oligonucleotide pairs were designed to prime AY MLO cluster-specific amplification of 16S rDNA. In all cases, the sizes of the DNA sequences amplified conformed approximately to the sizes expected on the basis of primer sequence position in 16S rDNA as published earlier (21). Under our conditions, DNA from all strains in the AY MLO cluster yielded amplifications of MLO-specific DNA when primer pair F0R1, F2R1, F3R1, or F4R1 was used in reaction mixtures. We are encouraged to conclude that DNA amplifications primed by the oligonucleotide pairs F0R1, F2R1, and F4R1 are limited to MLO strains that are members of the AY MLO cluster, since all AY MLO strains yielded DNA bands and no non-AY cluster MLOs yielded bands when these primers were used. Primer pair F1R1 was also specific for AY cluster MLOs, although amplification is apparently limited to DAY (and presumably very closely related strains).

Under less stringent conditions of primer-template annealing than those employed in this study, DNA amplification might not be limited to AY cluster MLOs. For example, weak amplification of a DNA sequence observed in WX MLO suggests low homology between primer pair F3R1 and WX MLO DNA. However, DNA amplifications with the other four primer pairs were limited to template DNA from plants infected by MLOs previously assigned to the AY MLO cluster, and primer pairs F0R1, F2R1, and F4R1 appear to permit AY cluster-specific and clusterwide amplification of 16S rDNA. Conceivably, tentative assignment of an unknown MLO to the AY MLO cluster could be made on the basis of a specific DNA amplification, under the PCR parameters that we used, when reaction mixtures contain F0R1, F2R1, or F4R1. It may be useful to employ at least two of these primer pairs in separate reactions when an unknown MLO is tested. Mutually confirming results from restriction analyses of 16S rDNA (19) and cluster-specific PCR would be expected if a strain's cluster assignment is correct.

The sequences of our oligonucleotide primers are based on a published sequence for the 16S rRNA gene of MLO strain MIAY from Michigan (21). When reaction mixtures contained primer pair F1R1, only DNA from strain DAY yielded DNA amplification. This result is consistent with the possibility that strain MIAY may be most closely related to strain DAY among the MLO strains we tested.

It is now recognized that a genomic cluster of AY MLO-affiliated strains can be identified by DNA-based methods (4–6,13,15,16,19). The AY MLO cluster contains a variety of MLO strains, including some previously termed AY MLO and others that have gone by different names (15). The broad plant-host

range of known strains in the AY MLO cluster is suggestive of widespread natural occurrence of diseases caused by these MLOs. The availability of rapid and sensitive means to detect these pathogens in their hosts and to identify them as AY MLOs should facilitate disease diagnosis, implementation of phytosanitary regulations, programs to develop disease-free plants, and efforts to understand natural disease spread.

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