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# CLONED RIBOPROBE FOR DETECTION OF A MYCOPLASMALIKE ORGANISM

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SUMMARY: A [$^{32}$P]-labeled single stranded-RNA probe (riboprobe) was constructed with plasmid vector pSP64 and used to detect and specifically identify an uncultured pathogenic mycoplasmalike organism in infected host. The riboprobe was more sensitive and reliable than complementary double stranded-DNA probe in detection of western X mycoplasmalike organism. When concentration of a double stranded-DNA probe was increased, nonspecific hybridization signal was observed with nucleic acid from healthy plants and from plants infected by other mycoplasmalike organisms. In contrast, sensitivity of detection with the complementary riboprobe was increased at elevated probe concentrations without nonspecific hybridization. 

INTRODUCTION: Mycoplasmalike organisms (MLOs) are unique prokaryotes that lack a cell wall. These pathogens are associated with several hundred plant diseases, including many that are responsible for widespread and severe economic losses in plant crops worldwide (1,2). Thus far, none of the MLOs has been obtained in pure culture in vitro. Failure to culture the MLOs has largely accounted for the lack of efficienct methods to detect and identify these pathogens. This circumstance has raised significant problems in the interstate and international movement of important plant germplasm, has impeded research on epidemiology and disease control, and has hindered understanding of the genetic relatedness among different pathogenic MLOs. Cloned DNA probes have recently been constructed and used for the detection of a few MLOs including western X (WX) disease MLO (3,4). Use of these double stranded (ds)-DNA probes has successfully detected infections in plants and insect vectors. However, we have noticed an increase of noise to

Abbreviations: MLOs, mycoplasmalike organisms; ds, double stranded; WX, western X; ss, single stranded; AY, aster yellows; VR, beet leafhopper transmitted virescence; EY, elm yellows; BB, tomato big bud; ORCH 1, an unknown orchard MLO; NaOAc; sodium acetate.

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signal ratio in hybridization reactions with ds-DNA probes in detection of MLOs when high concentrations of probes (>$10^6$ cpm/per ml of reaction solution) are used for hybridization. The development of new cloning vectors (5) has made it possible to transcribe in vitro inserted DNA into single stranded (ss)-RNA probes of high specific radioactivity. Several researchers have applied the asymmetric RNA probes in detection of target nucleic acids in various crude preparations (6,7,8). All results indicated that ss-RNA probes yielded lower noise to signal ratios than did ds-DNA probes and hybridized with target nucleic acids at higher efficiency than ds-DNA probes. These findings encouraged us to construct ss-RNA probes for detection of an MLO. We have subcloned a WX MLO disease-specific DNA fragment from plasmid pWX3 into cloning vector pSP64 and have transcribed in vitro a WX-specific ss-riboprobe. This riboprobe was found to be highly specific for detection of the WX-disease MLO in infected plant hosts and highly sensitive for differentiation of WX-disease MLO from other MLOs (9).

## MATERIALS AND METHODS

Sources of healthy and MLO-infected periwinkle plants. Diseased plants of periwinkle (Catharanthus roseus) were maintained by grafting in a white flowered clone of periwinkle grown in a greenhouse. Aster yellows (AY) infected periwinkle in this experiment was originally field collected at Beltsville and maintained in a greenhouse. Strains of MLOs were kindly provided separately in infected periwinkle plants by the following researchers: beet leafhopper transmitted virescence (VR) (George N. Oldfield, USDA, University of California, Riverside); western X (WX) (B.C. Kirkpatrick, University of California, Davis); elm yellows (EY) (Wayne Sinclair, Cornell University, Ithaca, New York); tomato big bud (BB) (James Dale, University of Arkansas,); an unknown MLO disease (ORCH1) (Sharon M. Douglas, Connecticut Agricultural Experimentation Station, New Haven, Connecticut).

Construction of DNA and RNA probes using plasmid pSP64. Plasmid pWX3 (4), which was pUC8 containing DNA fragments specific to WX disease, was used as the source of WX MLO-specific DNA. The plasmid pWX3 was digested with both Eco R1 and Hind III restriction endonucleases and the resulting fragments were fractionated by agarose gel electrophoresis. The WX MLO-specific inserts were electroeluted and then extracted with butanol to remove ethidium bromide. The aqueous phase containing inserts DNA was then extracted twice with a half volume of TE (10mM Tris-HCl pH8.0, 1mM EDTA) -saturated phenol and a half volume of chloroform-isoamyl alcohol (24:1), and once with an equal volume of ether. The insert DNA was precipitated by adding 1/10 volume of sodium acetate (NaOAc) and 2 volumes of ethanol and resuspended in sterile deionized $H_2O$. The inserts were ligated with Eco R1-and Hind III-digested plasmid pSP64 (Promega Biotec[1], Madison, Wisconsin) and used to transform competent Escherichia coli strain JM83 (10). Ampicillin-resistant colonies were screened by dot blot hybridization with [$^{32}$P]-labeled nick-translated plasmid pWX3. The presence of an insert in plasmid pSP64 corresponding to that in plasmid pWX3 was confirmed

[1]Mention of a commercial company or specific equipment does not constitute its endorsement by the U.S. Dept. of Agriculture over similar equipment or companies not named.

by restriction endonuclease digestion and agarose gel electrophoresis. [$^{32}$P]-labeled single-stranded RNA probe was synthesized in vitro using SP6 RNA polymerase and the linearized recombinant plasmid DNA template containing the WX-specific DNA fragment as described (Promega Biotec.).

Extraction of nucleic acid from periwinkle plants. Approximately 0.3g of plant tissue (midribs or young shoots) was placed in a precooled dry mortar in which liquid nitrogen was added to quickly freeze the sample. The frozen tissues were finely pulverized with a porcelain pestle. The pulverized samples were triturated, in Eppendorf Microfuge tubes with plastic mini-pestles, in 400 ul of extraction buffer (100 mM Tris, 50 mM EDTA, 500 mM Nacl, pH 8.0) containing 0.5% 2-mercaptoethanol, and 0.5% SDS. After centrifugation at 2000 rpm for 10 minutes in an Eppendorf microfuge, the supernatants were transferred to clean centrifuge tubes. The loose pellets were centrifuged again at 8,000 rpm for 10 minutes. The second supernatants were combined with the first and were heated at 65°C for 5 minutes. The supernatants were then centrifuged at 14,000 rpm for 5 minutes to remove the coagulated debris. The resulting supernatants were transferred to clean Eppendorf Microfuge tubes and extracted with 200 ul of TE-saturated phenol and 200 ul of chloroform-isoamyl alcohol. After centrifugation at 14,000 rpm for 5 minutes, the aqueous phase containing nucleic acid was transferred to a fresh tube and stored at 4°C or at -70°C for future use.

Dot blot hybridization. Nucleic acid samples prepared from periwinkle plants, unlabeled plasmid pSP64 containing an insert, and insert from plasmid pWX3 were denatured by adding 2N NaOH (2ul/100ul DNA sample) and incubating in a boiling water bath for 10 minutes. The nucleic acid samples were immediately cooled in an ice water bath and then neutralized by adding 2M Tris, pH7.0 (6ul/100ul DNA sample) and 1.5M NaOAc, pH 5.0 (2ul/100ul DNA sample). Two-fold dilutions of nucleic acid samples were made in 6X SSC (1X SSC=0.15M NaCl, 0.018 M NaOAc, pH7.0)

Three microliter aliquots of the original nucleic acid samples or DNA and/or their dilutions were spotted on a dry, clean nitrocellulose paper (pretreated in 10X SSC). The filters were air-dried and then baked at 80°C for 2 hr under vacuum. The filters were prehybridized for 1h at 65°C in a solution containing 6X SSC, 4X Denhardt's (1X=0.08% Ficoll, 0.08% polyvinylpyrollidone 0.08% bovine serum albumin), 0.5% SDS, and 150 ug/ml denatured salmon sperm DNA. For hybridization the solution was replaced with a similar solution containing denatured [$^{32}$P] labeled DNA or [$^{32}$P] labeled ss-RNA probe at concentrations of 3 X $10^5$ cpm/ml and $10^6$ cpm/ml for both probes. The [$^{32}$P] labeled DNA probe was alkali-denatured (1 ul 2N NaOH in 40 ul DNA sample) and incubated in a boiling water bath for 2 minutes, followed by immediate cooling in ice water. After overnight incubation at 65°C, the filters were washed for 15 minutes once at room temperature with an excess of 3X SSC containing 0.5% SDS, twice at 65°C with 3X SSC containing 0.5% SDS and twice at 65°C with 2X SSC containing 0.5% SDS. For filters receiving riboprobes, additional RNase treatment was performed as described (Promega Biotec). Before the RNase treatment, filters were rinsed three times in 2X SSC for 5 minutes. After incubation at room temperature for 15 minutes in 2X SSC containing 1 ug/ml RNase A, the filters were washed with 0.1X SSC containing 0.1% SDS at 50°C for 30 minutes. After final washing, filters were dried and exposed to X-ray film (Kodak XAR) for 24 h with an intensifying screen (MCI Optonix, Inc, Cedar Knolls, NJ).

RESULTS: DNA fragments specific to WX disease were excised from plasmid pWX3 using restriction endonucleases Eco Rl and Hind III. Three DNA fragments with 1360, 600, and 540 bp were generated (Fig. 1, see lane F). These fragments were purified by gel electroelution and were subcloned into

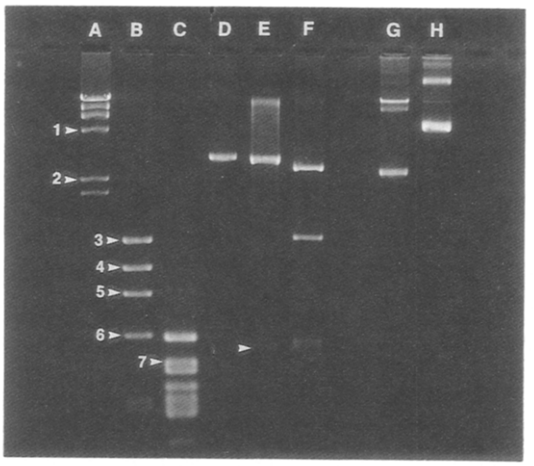


Fig. 1. Electrophoresis of cloning vectors and cloned DNA probes in a 2% agarose gel. Lanes A, B, and C, size markers (base pairs). Lane A, fragments of lambda DNA (digested with Hind III; 1:4355, 2:2322); lane B, DNA fragments of ØX174 RF (digested with Hae III, 3:1353, 4:1078, 5:872, 6:603); lane C, DNA fragments of pSTB14 (dimer of potato spindle tuber viroid in pUC9 digested with Hae III); lane D, plasmid pSP64 digested with Eco Rl and Hind III; lane E, cloned WX-DNA (in pSP64) digested with Eco Rl and Hind III. The lowest band (arrow) is the WX insert with a molecular weight of 540 bp.; lane F, plasmid pWX3 digested with Eco Rl and Hind III. Uppermost heavy band is plasmid pUC8; lower bands are WX DNA inserts of 1360, 600, and 540 bp, respectively; lane G, undigested cloned WX-DNA (in pSP64); lane H, undigested plasmid pWX3.

plasmid pSP64. A new clone with an insert (540 bp DNA fragment) in plasmid pSP64 was thus constructed and employed as a new DNA probe specific to WX. The riboprobe synthesized using the linearized new plasmid DNA template was single-stranded RNA with a sequence complementary to the 540 bp insert specific to WX disease.

Nitrocellulose filters spotted with nucleic acid preparations from healthy and WX diseased periwinkle plants were hybridized either with a [$^{32}$P] labeled DNA probe or a [$^{32}$P]-labeled riboprobe. Unlabeled plasmid pSP64 with insert (=DNA probe) and the insert from plasmid pWX3 were used as positive controls. When equal concentrations of probes (3 X $10^5$ cpm/per ml of reaction solution) were added to the hybridization solution, both the riboprobe and the DNA probe hybridized specifically with nucleic acid preparations from WX diseased plants, with the 2460 bp insert from plasmid pWX3 and with plasmid pSP64 containing the 540 bp WX insert but not with a nucleic acid preparation from healthy plants (data not shown). The limit of detection using homologous DNA (plasmid pSP64 with 540 bp insert) was 35 pg. Using a higher concentration of probes, at $10^6$ cpm/per ml, as little as 9 pg of homologous DNA could be detected by both riboprobe and DNA probe

(Fig. 2). The sensitivity of detecting WX specific DNA from diseased periwinkle thus increased 4 fold when the higher concentration of probe was used. However, the DNA probe under these conditions did hybridize nonspecifically with nucleic acid preparations from healthy plants (Fig. 2B1). In a separate experiment, using the same high concentration of probe the DNA probe reacted nonspecifically with nucleic acid preparations from AY, BB, EY, VR and ORCH1 and, to lesser degree with nucleic acid from healthy plants (Fig. 3). The riboprobe reacted specifically with nucleic acid preparations from WX diseased plant but did not react with nucleic acid preparations from healthy plants or from AY, BB, EY, VR, and ORCH1 diseased plants (Fig. 3).

DISCUSSION: Efficient detection of non-cultured MLOs by nucleic acid hybridization depends upon sensitivity and specificity of the hybridization probes used. DNA probes provide a relatively effective method for MLO detection in infected plants and insect vectors. However, the sensitivity and specificity of DNA probes are limited by their tendency to yield high noise to signal ratios, especially under low stringency hybridization conditions (7, Lee and Davis unpublished). This may be due, in part, to the tendency of ds-DNA probes for self-annealing and network formation. The latter may occur more readily when the probe hybridizes with

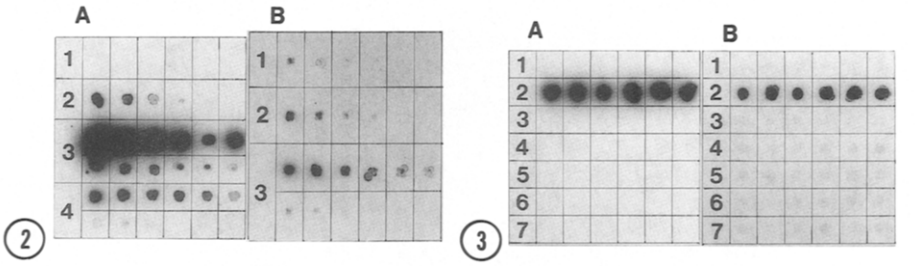


Fig. 2. Dot blot hybridization analyses of nucleic acid preparations using a WX specific [$^{32}$P] labeled riboprobe and a DNA probe (plasmid pSP64 plus insert). A, riboprobe; B, DNA probe. Nitrocellulose filters spotted with various nucleic acids were hybridized in solution containing $10^6$ cpm/ml of riboprobe or DNA probe. Dilution series of nucleic acid or DNA preparations. A1 and B1, healthy periwinkle (Cathatharanthus roseus); A2 and B2, WX- diseased periwinkle; A3, plasmid pSP64 with insert (=DNA probe); A4, insert excised from plasmid pWX3; B3, insert excised from plasmid pWX3. Dilution series of plasmid pSP64 with insert and insert DNA from plasmid pWX3 are in the following order: from left to right, 25 ng, 12.5 ng, 6.25 ng, 3.13 ng, 1.5 ng, 750 pg, 375 pg, 140 pg, 70 pg, 35 pg, 18 pg, 9 pg. Note non-specific hybridization of labeled DNA probe with healthy plant DNA.

Fig. 3. Hybridization of nucleic acid preparations from healthy and MLO diseased periwinkle with [$^{32}$P] labeled riboprobe and DNA probe. A, riboprobe; B, DNA probe. Nitrocellulose filters spotted with nucleic acids were hybridized in solution containing $10^6$ cpm/ml of riboprobe or DNA probe. Nucleic acid preparations: 1, healthy periwinkle; 2, western X; 3, aster yellows; 4, tomato big bud; 5, elm yellows; 6, beet leafhopper transmitted virescence; 7, ORCH1. Note non-specific hybridization of labeled DNA probe with healthy plant DNA and MLO DNA.

heterologous target DNA, permitting further non-specific binding of unhybridized probe to unmatched portions of the hybridized probe. Thus, an increase in sensitivity of DNA probe achieved by increasing the probe concentration can be obscured by an increase of noise to signal ratio and consequent decrease in specificity (see Fig. 2). The limitations of DNA probes can be overcome by using single-stranded RNA probes.

When single-stranded (ss) riboprobes are synthesized in vitro using SP6 RNA polymerase and the linearized recombinant plasmid DNA template, only the MLO-specific insert is labeled with [$^{32}$P]. Thus, a ss-riboprobe has high MLO-specific radioactivity, and is incapable of self-annealing. Since riboprobes cannot self-anneal, they are adaptable for a wide range of hybridization conditions in which high sensitivity and specificity can be achieved. The specificity of a riboprobe is further enhanced by the use of ribonuclease A to remove nonspecifically bound probe and to remove mismatched regions of RNA-DNA hybrids. The higher specificity of riboprobes compared to DNA probes was again demonstrated in our recent separate study of ss-RNA probes specific to the aster yellows disease, which is also caused by MLOs (Lee and Davis unpublished). A high sensitivity and specificity of probes is critically required for the efficient detection of MLOs in host tissues in which few target organisms are expected; ss-RNA probes are capable of fulfilling this requirement.

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