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Development of Cloned DNA Probes for Phytoplasma Associated with Loofah Witches' Broom

C. P. KUAN[1,2] and H. J. SU[2]

Authors' addresses: [1]National Laboratories of Foods and Drugs, Department of Health, Executive Yuan, 161-2, Kuen Yang Street, Nankang, Taipei 115, Taiwan; [2]Department of Plant Pathology and Entomology, National Taiwan University, Taipei 107, Taiwan, Republic of China (correspondence to C. P. Kuan)

With 2 figures

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Abstract

DNA was isolated from periwinkle (*Catharanthus roseus*) infected with a phytoplasma that originated in loofah witches' broom affected by loofah. Cloned DNA inserts from six LfWB-phytoplasma specific recombinant plasmids were not only labelled with digoxigenin, but also used as probes. Probes hybridized with DNA derived from LfWB-phytoplasma affected periwinkle and loofah, but not with DNA from healthy plants or plants infected with phytoplasmas associated with elm yellows, red bird cactus, peanut witches' broom, paulownia witches' broom, Ipomoea obscura witches' broom, aster yellows (two isolates), and sweet potato witches' broom obtained with DNA from different phytoplasmas experimentally maintained in periwinkle. The probes could detect LfWB-phytoplasma DNA with as little as 16 ng and 32 ng of DNA from periwinkle and loofah, respectively. The method proposed herein provides a means for specifically detecting and identifying of loofah witches' broom phytoplasma, as well as confirming the notion that this phytoplasma represents a distinct strain cluster.

Zusammenfassung

Entwicklung von geklonten DNA-Proben für Phytoplasma assoziiert mit Luffa Witches' Broom

DNA wurde aus Immergrünpflanzen (*Catharanthus roseus*) isoliert, die mit einem aus Luffa witches' broom befallenen Luffapflanzen (*Luffa cylindrica*) gewonnenen Phytoplasma infiziert waren. Geklonte DNA-Inserts aus sechs LfWB-phytoplasmaspezifischen Rekombinantplasmiden wurden nicht nur mit Digoxigenin markiert sondern wurden auch als Proben angewandt. Proben hybridisierten mit der DNA, die aus LfWB-Phytoplasma infizierten Immergrün-bzw. Luffapflanzen stammte, nicht aber mit DNA aus gesunden Pflanzen oder aus Pflanzen, die mit Phytoplasmen, die mit elm yellows, red bird cactus, peanut witches' broom, paulownia witches' broom, Ipomoea obscura witches' broom, aster yellows (zwei Isolate) sowie sweet potatio witches' broom assoziiert sind, befallen waren. Die Proben konnten LfWB-Phytoplasma-DNA mit 16 ng und 32 ng DNA aus Immergrün-bzw. Luffapflanzen detektieren. Die hier beschriebene Methode ermöglicht sowohl die spezifische Ermittlung und Identifizierung des luffa witches' broom Phytoplasmas als auch die Bestätigung der Vermutung, daß dieses Phytoplasma eine eigene Stammgruppe darstellt.

Introduction

The vegetable sponge of *Luffa cylindrica* is an important food crop in Taiwan. Loofah witches' broom (LfWB) was first discovered on loofah (*Luffa cylindrica* Roem.) in the Pingtung area in Taiwan (Yang et al., 1974). Phytoplasma (Mycoplasmalike organism) associated with Loofah witches' broom (LfWB-phytoplasma) resides in sieve tubes of cucurbitaceous host plant and unevenly in a low population. Electron microscopic studies point toward a mycoplasmal aetiology for the disease (Chung et al., 1975; Chen et al., 1979). LfWB phytoplasma, similar to other phytoplasmas, has not been cultured. A recent study developed and applied monoclonal antibodies against LfWB phytoplasmas to LfWB-phytoplasma (Cheon and Su, 1987). Cloned DNA probes were also used in that work to detect phytoplasmas in plant and insect tissue by using nucleic acid hybridization assays. Moreover, in the same study, probe hybridizations were used to differentiate phytoplasmas (Bertaccini et al., 1990; Hibben et al., 1992), to monitor their distribution within a host plant (Bonnet et al., 1990; Kuske and Kirkpatrick, 1992), and to provide information concerning genetic relatedness among phytoplasmas from various hosts and geographic locales (Deng and Hiruki, 1991; Lee et al., 1991). Other investigations recently used polymerase chain reaction (PCR) as a highly sensitivite method for detecting DNA (Deng and

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Hiruki, 1990; Schneider et al., 1993). Furthermore, PCR has been used for pathogen detection in plant hosts and vector insects (Harrison et al., 1991; Davis et al., 1992).

This work isolates and performs molecular cloning of LfWB-phytoplasma DNA from diseased plants, in an attempt to develop cloned DNA probes specific for LfWB-phytoplasmas, and to characterize LfWB-phytoplasma specific DNA probes. The probes are then applied for detection and differentiation of LfWB-phytoplasma. Using these specific probes also allows for LfWB-phytoplasmas to be identified in the ecology of these organisms.

Materials and methods

Plant material

Loofah naturally infected with LfWB phytoplasma was collected from fields in the Pingtung area. Periwinkle (*Catharanthus roseus*. (L.) G. Don) infected with LfWB phytoplasma originally obtained by transmission through dodder (*Cuscuta australis* R. Brown) was maintained and propagated in periwinkle by side-grafting as described previously (Cheon and Su, 1987). Plants affected by several other phytoplasma-associated diseases were maintained in periwinkle or collected from fields. These included periwinkle with Loofah witches' broom, red bird cactus witches' broom, elm yellows, peanut witches' broom, paulownia witches' broom, Ipomoea obscura witches' broom, aster yellow from California, aster yellow from Belstrille, and sweet potato witches' broom was provided by I. L. Yang (Taiwan Agricultural Research Institute, Wufang).

Separation of LfWB-phytoplasma DNA from host DNA

The procedure used for extraction of total DNA from LfWB phytoplasma-infected periwinkle plants was according to the methods of Koller et al. (1990). Cesium chloride (CsCl)-bisbenzimide density gradient centrifugation was used to separate phytoplasma DNA from host plant DNA (Bonnet et al., 1990; Kollar et al., 1990; Harrison et al., 1991).

Molecular cloning of phytoplasma DNA and screening of recombinants

Approximately 1 μg of CsCl-purified LfWB-phytoplasma DNA was digested with DraI restriction endonuclease (Boehringer Mannheim GmbH, Mannheim, Germany). Resulting fragments were ligated with SmaI-degested, and the dephosphorylated Bluescrit plasmids were used to transform compentet cells of *Escherichia coli* JM 109 according to the procedures described by Sambrook et al. (1989). To tentatively identify recombinants containing cloned fragments of phytoplasma DNA, plasmid DNA was extracted from selected white colonies by alkaline lysis method (Sambrook et al., 1989) and monitored for appropriate size by cleavage with BamHI (Boehringer Mannheim) and PstI (Boehringer Mannheim). The recombinant plasmid were screened by dot hybridizations using digoxigenin-11-dUTP (Boehringer Mannheim) labelled total DNA extracts from healthy or LfWB-phytoplasma-infected periwinkle as probes. Each DNA preparation was denatured by boiling for 10 min, immediately cooled on ice for 5 min, and then spotted onto Magna Graph nylon membranes (Micron Seps., Westboro, MA, USA) using a micropipette. Membranes were air-dried and cross-linked with the blotted DNA with a UV-crosslinker (Spectronics Corporation, Wesburg, NY). Membranes were given two moderate stringency washes (30 min each) at room temperature in 2X SSC containing 0.1% SDS, and twice again at 55°C with 0.1X SSC containing 0.1% SDS. After the final wash, membranes were air-dried, and signal detection was performed using digoxigenin nucleic acid detection kit (Boehringer Mannheim) following supplier's instructions. Sizes of the cloned inserts in each selected recombinant were determined by agarose gel electrophoresis (1.5%).

Specificity and sensitivity of cloned DNA probe

To determine probe specificity, two sets of membranes were tested as replicates. One membrane was washed under moderate stringency (described previously), and the other was washed under high stringency (last two washes with 0.1X SSC containing 0.1% SDS at 68°C instead of 55°C). Each membrane was spotted with undigested DNA samples (200 ng of total DNA extracts per spot) from healthy periwinkle and Loofah plants, LfWB-phytoplasma infected periwinkle and Loofah, and plants with eight other previously-mentioned phytoplasma-associated diseases, and then hybridized with each of the cloned LfWB-phytoplasma DNA probes, as previously described. To determine the detection sensitivity of the cloned LfWB-phytoplasma DNA probes, individual digoxigenin-labeled LfWB-phytoplasma DNA probes were hybridized with blots consisting of total DNA extracts from LfWB-phytoplasma infected periwinkle and Loofah. Each sample DNA was applied to membranes as serial twofold dilution in TE buffer (pH 8.0). Membranes were evaluated under high stringency.

PCR amplification

Primers (97F2 and 97R2) for PCR were designed on the basic of the LfWB-phytoplasma DNA insert in probe LfWB97. The following primers were used in this study: 97F2, CCCTGAAAATGCCGAAG; 97R2, GCAAAAGGAATGGGAAACAC. Inserts were sequenced with T7 and T3 forward and reverse primers with Taq polymerase (Prism Ready Reaction Dye Primer Cycle Sequencing Kit: Applied Biosystems). The sequences obtained were analysed on SeqEd version 1.0.3 software (Applied Biosystems). Reaction mixture was overlaid with 25 μl of mineral oil. The PCR was performed in a GeneAmp PCR system 480 (Perkin-Elmer, Norwalk, CT, USA) thermal cycler. After initial denaturation for 5 min at 94°C, 35 cycles of amplification were carried out as follows: denaturation for 1 min at 94°C, annealing for 30 s at 60°C, and extension for 1 min at 72°C. The final extension step was lengthened by 10 min, and then the reactions were held at 4°C. PCR products were analysed by electrophoresis in a 1.5% agarose gel, stained with ethidium bromide, and visualized with a UV transilluminator.

Results

Cloned DNA probe

450 transformant colonies of *E. coli* were obtained in the cloning of LfWB-phytoplasma DNA from periwinkle plants infected with the LfWB-phytoplasma. 122 recombinant plasmids were identified by dot hybridization to specifically react with labelled total DNA from LfWB-phytoplasma infected periwinkle, but not with that from healthy periwinkle DNA. Six of those recombinant plasmids were selected for further characterizing on the basis of restriction analysis and size of the cloned DNA inserts. The insert sizes in the six recombinant plasmids ranged from 0.3 to 1.2 kb. Insert DNA fragments excised from recombinant plasmids are pLfWB97, pLfWB47, pLfWB223, pLfWB38. Next, the cloned inserts of the six plasmids were labelled with digoxigenin and employed as probes in dot hybridizations with total nucleic acid extracted from periwinkle, both healthy and infected with other phytoplasmas, hybridizations at 55°C and 68°C yielded the same results.

Specificity and sensitivity of probe

Three probes hybridized with nucleic acid from the infected plant; no hybridization signals close by were detected with nucleic acid from healthy plants. Table 1 summarizes the results from dot hybridization. No dot hybridization signal appeared between any probe and DNA from healthy host plants; among which included periwinkle and loofah. Three probes, LfWB47, LfWB223, LfWB97, hybridized with DNA extracts from LfWB-phytoplasma infected periwinkle and loofah specifically, but not with DNA extracts from plants infected with any of the other eight phytoplasmas (EY, RBC, PnWB, PaWB, IPWB, AY-Cal, AY-Bel, SPWB) that were examined. Meanwhile, the remaining two probes, pLfWB38 and pLfWB65, only hybridized with DNA extracts from LfWB-phytoplasma infected periwinkle and loofah and with DNA extracts from elm yellows and red bird cactus witches' broom under both high- and moderate-stringency wash conditions.

In sensitivity tests involving dot hybridizations under high-stringency wash conditions, LfWB-phytoplasma specific probes detected the presence of phytoplasma DNA in as little as 16 ng of total DNA extracted from LfWB-infected periwinkle as in 32 ng of DNA from diseased loofah. Figure 1 illustrates representative hybridization results from this type of experiment.

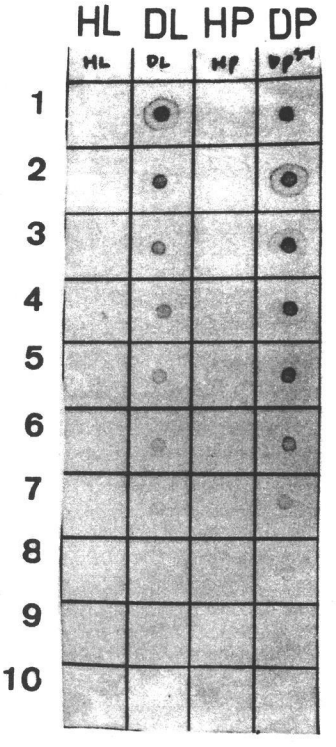

Fig. 1 Sensitivity test of the labelled probes pLfWB97 by dot hybridization in two-fold serial dilutions. Total DNA extracts from periwinkle (DP) and loofah (DL) infected with LfWB-MLO. Healthy periwinkle (HP) and loofah (HL) served as controls. 1, lug; 2, 500 ng; 3, 250 ng; 4, 125 ng; 5, 62.5 ng; 6, 31.25 ng; 7, 15.62 ng; 8, 7.81 ng; 9, 3.90 ng; 10, 1.95 ng

Table 1
Specificity of LfWB-MLO DNA probes characterized by dot-blot hybridizations of DNA preparations from various MLO infected plants with labelled recombinant plasmids

DNA probe	DNA of MLOs[b]											
	LfWBL	LFWBP	EY	RBC	PNWB	PaWB	IPWB	AY-Cal	AY-Bd	SPWB	HL	HP
pLfWB223	+	+	−	−	−	−	−	−	−	−	−	−[a]
pLfWB47	+	+	−	−	−	−	−	−	−	−	−	−
pLFWB444	+	+	−	−	−	−	−	−	−	−	−	−
pLfWB97	+	+	−	−	−	−	−	−	−	−	−	−
pLfWB38	+	+	+	+	−	−	−	−	−	−	−	−
pLfWB65	+	+	+	+	−	−	−	−	−	−	−	−

[a]: + positive hybridization; −, negative hybridization; [b]: Kinds of MLOs *Catharanthus roseus* (Periwinkle); LFWBL, Loofah witches' broom MLO in loofah; LFWBP, Loofah witches' broom MLO in periwinkle; EF, Elm yellows; EBC, Red bird cactus witches' broom; PNWB, Peanut witches' broom MLO; PaWB, Paulownia witches' broom; IPWB, Ipomoea obscura witches' broom; AY-CAL, Aster yellow MLO of California strain; AY-BEL, Aster yellow MLO of Belstrille strains: SPWB, sweet potato witches' broom; HP, healthy periwinkle; HL, healthy loofah.

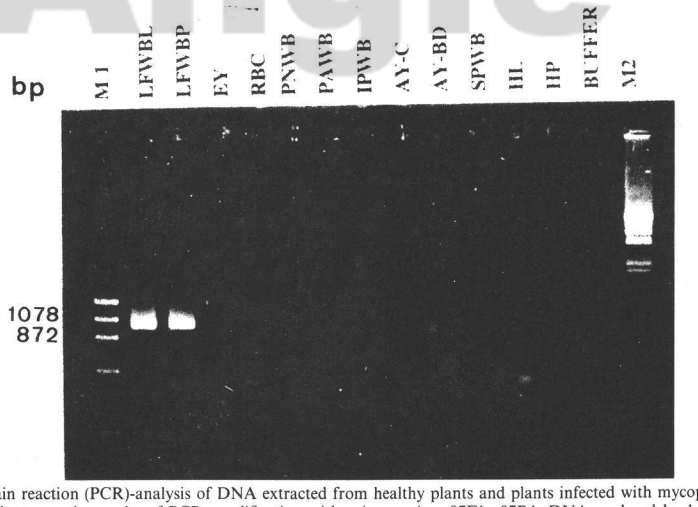

Fig. 2 Polymerase chain reaction (PCR)-analysis of DNA extracted from healthy plants and plants infected with mycoplasmalike organisms (MLOs). Reaction products are the results of PCR amplification with primer pairs p97F1-p97R1. DNA analysed by 1.5% agarose gel electrophoresis after staining with ethidium bromide, and the bands were visualized with ultraviolet light. Each strain description is the same as those in Table 1. Lane M1, molecular weight standards (in base pairs): 1353, 1078, 872, 603, 310, 281+271 bp from top band to bottom band. Lane M2, lambda DNA Hind III and EcoRI fragments

PCR amplification of LfWB-phytoplasma DNA

To sequence the cloned DNA and according to the sequence data, we designed oligonucleotide primers that were synthesized using total DNA from LfWB-phytoplasma infected periwinkle and loofah DNA as templates. After conducting 25–40 cycles with primer pairs 97F2+R2, a distinct DNA fragment of about 0.95 kb was produced using total DNA from LfWB-MLO infected periwinkle and loofah DNA as templates. No specific PCR product was obtained in all other PCR reactions with the DNA templates from healthy plants and from other phytoplasma-affected plants after electrophoresis when 35 cycles were conducted (Fig. 2).

Amplifying phytoplasma DNA analysis confirmed the identity of the LfWB phytoplasma DNA fragments by hybridization DNA probe. Both probes, LfWB97 and LfWB223 detected LfWB phytoplasma in all diseased loofahs and periwinkles examined herein. Representative results obtained with the probe were specific to LfWB phytoplasma. A similar pattern of results was obtained when the hybridization test was used on the same plant samples except that pLfWB38 reacted with LfWB phytoplasma. However, EY phytoplasma and RBC phytoplasma also exhibited positive signal results was obtained from LfWB as from loofah and periwinkle. No hybridization was obtained with nucleic acid extracted from similar tissues of healthy loofah.

Discussion

Cloning phytoplasma DNA has allowed for sensitive probes to be developed, capable of detecting the presence of various phytoplasmas in infected plants and insect tissues (Kirkpatrick et al., 1987; Lee and Davis, 1988; Kusk et al., 1991; Davis et al., 1992). Dot blot hybridization with DNA extraction from loofah infected with LfWB-phytoplasma and the amplified fragments, pLfWB97, pLfWB47 and pLfWB223 as probes reveals specific hybridization to LfWB-phytoplasma DNA signal. However, no nonspecific hybridization with sap from healthy plants is detected. DNA probes against loofah phytoplasma developed herein are useful in detecting the loofah disease and also in differentiating phytoplasmas associated with other diseases. Our primary concern in developing DNA probes to detect the loofah phytoplasma is their relative sensitivity and specificity. Herein, detection sensitivity varies according to the plant host tested, which is particularly evident after screening field-grown infected loofah and greenhouse-grown periwinkle. Results obtained from the probes indicate that our cloned DNA can consistently detect loofah phytoplasma DNA to 16 ng of DNA from periwinkle and 32 ng of DNA from loofah with dot hybridization. In addition, variations between samples are interpreted to reflect the differences in phytoplasma titers in different infected host tissues. Specificity tests, indicate that probe LfWB38 cross-hybridized with DNA preparations from elm yellows and red bird cactus witches' broom is a trend similar to the results of LfWB38 and LfWB65 assays. Probe LfWB38 exhibits cross-hybridization with DNA of EY-phytoplasma and RBC-phytoplasma at high stringency with a decreased hybridization signal, which is particularly weak in RBC-phytoplasma DNA. The possibility exists, however, that one or more of these probes may hybridize with nucleic acid of as yet untested phytoplasmas, different from LfWB-phytoplasma but having a region of DNA homologous with that of the LfWB38 DNA probe.

LfWB, EY, the same group consisting of the phylogenetically distinct phytoplasma taxa. (Gundersen et al., 1994). This finding suggests that LfWB-phytoplasma were more closely related to EY- and RBC-phytoplasma than to other phytoplasmas. PCR techniques have recently been applied in detecting or classifying phytoplasma pathogens by developing primers from 16S rRNA sequence (Lim and Sears, 1989; Ahrens and Semuller 1992; Nakashima et al., 1993; Schneider et al., 1993) or from sequences of specific cloned phytoplasma DNA fragments (Davis et al., 1988, 1990). Findings presented herein provide a basis for the possible feasibility of not only detecting LfWB-phytoplasmas in any plant, including alternate hosts of the phytoplasmas, but also investigating symptomless phytoplasma infections in loofah plants. The sensitive and reliable phytoplasma detection provided by the LfWB-phytoplasma DNA probes and further development of LfWB-phytoplasma specific PCR, amplification along with electrophoretic analysis of the LfWB-phytoplasma DNA fragment can be routinely applied in ecological studies including insect vectors and possible alternative host plants. Moreover, this study demonstrates that the DNA probes and DNA amplification method proposed herein is quite effective in detecting LfWB-phytoplasma and other pathogenic phytoplasmas.

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