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Detection and Differentiation of the Mycoplasmalike Organism Association with Sugarcane White Leaf Disease Using Cloned Extrachromosomal DNA Probe

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ABSTRACT

DNA was isolated from sugarcane infected by sugarcane white leaf mycoplasmalike organism [SCWL-MLO]. The MLO DNA was separated from the host plant DNA by repeated CsCl-bisbenzimide buoyant density gradient centrifugation. Six bands of extrachromosomal DNA [E-DNA] presumably of plasmid origin were found by agarose gel electrophoresis which seem to be attributed to three different replicons. One of these replicons 2.5 kb in size was linearized with HindIII restriction enzyme and was cloned in *Escherichia coli*. A probe made from the insert hybridized with two bands of undigested DNA from diseased sugarcane corresponding to the homologous nicked and supercoiled forms of the cloned replicon. The probe hybridized with all 8 isolates examined collected in different areas of Thailand and reacted only slightly with E-DNA from bermudagrass and *Brachiaria* sp. showing white leaf symptoms. It did not hybridize with DNA from correspondingly plant material. Also, no hybridization was observed with DNA from plants infected by MLO associated with 18 different other plant diseases. The probe detected the DNA of the SCWL-MLO in 100 pg of purified SCWL-MLO and in 8 ng of total nucleic acids from infected sugarcane

Key word : mycoplasmalike organism, cloning, extrachromosomal DNA, plasmid, sugarcane white leaf disease.

INTRODUCTION

Mycoplasmalike organisms [MLOs] have been implicated as the causal agents of disease in several hundred plant species [McCoy *et al,* 1989]. Sugarcane white leaf [SCWL], a serious disease of sugarcane in Thailand, is associated with a MLO. Because the MLOs are not culturable *in vitro*, detection of the MLOs in plants and differentiation of the organisms are based on electron microscopic observations in the plant phloem, symptomatology, host plant ranges and insect vector relationships.

Efforts have been made to develop specific and more sensitive detection procedures by using serological techniques and nucleic acid hybridization. Polyclonal and monoclonal antibodies have been produced from MLO-infected plants for detection of MLOs in infected hosts using enzyme linked immunosorbent assay [ELISA] [Clark *et al,* 1983; Lin and Chen, 1985; Sinha and Benhamou, 1983]. Fragments of chromosomal DNA of MLO from MLO-infected plants and insects have been isolated and cloned [Bonner *et al,* 1990; Harrison *et al,* 1991; Kirkpatrick *et al,* 1987; Lee and Davis, 1988] and also plasmid DNA of MLO has been cloned to facilitate plasmid detection and characterization [Harrison *et al,* 1991; Kuske and Kirkpatrick, 1990].

In this paper we describe preparation and cloning of chromosomal DNA of MLO from infected sugarcane and report the use of a cloned plasmid DNA probe for detection of the SCWL-MLO in isolates from various locations and in homology studies.

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MATERIALS AND METHODS

DNA sources. DNA was isolated from sugarcane, bermudagrass, *Brachiaria* sp., and *Dactylocte nium* sp., all affected by the corresponding white leaf disease as well as from *Catharanthus roseus* affected by virescence, and sesame, sunhemp, and yardlong bean affected by the respective phyllody disease. All diseased plants as well as the corresponding healthy plants used as control were collected in Thailand.

Periwinkle-maintained MLO associated with the following diseases were included in this study : *Aquilagia* virescence from Germany, a leafhopper-borne virescence of *C. roseus* from Florida [USA] ,apple proliferation, European aster yellow, and primrose virescence from Germany, rape verescence from France, *Solanum maginatum* big bud from Ecuador, *C. roseus* virescence from Germany, *C. roseus* virescence from Peru, stolbur of red pepper from Yugoslavia, safflower phyllody from Israel. All these isolates were maintained by graft transmission at the Dossenheim Institute.

DNA extraction. DNA of healthy and infected plants were isolated as described by Kollar *et al* [1990]. In brief, the plant material was lyophilized and ground to fine powder. The tissue powder was suspended in ten volumes of extraction buffer [0.7M NaCl, 1% sodium dodecyl sulfate [SDS], 10 mM Tris-HCl, 20 mM EDTA, 0.3% dithiothreitol, pH 8.0] and was incubated at 55°C for 25 min. the lysate was extracted 3 times with chloroform: isoamyl alcohol [24: 1]. After centrifugation at 5,000 g, 20°C, for 15 min, the aqueous phase was recovered and one-tenth volume of 0.7 M NaCl and 10% cetyltrimethylammoniumbromide [CTAB] was added for every chloroform extraction performed. The nucleic acids were precipitated with one volume of 1% CTAB,50 mM Tris-HCl, and 10 mM EDTA, pH 8.0, at room temperature for 30 min. After centrifugation at 5,000 g, 20°C, for 20 min, the pellet was washed 3 times with 80% ethanol, dried under vacuum, and dissolved in TE buffer [10 mM Tris-HCl, 1 mM EDTA, pH 8.0]. For further purification to obtain MLO DNA, the dried pellet was dissolved in CsCl in 10 mM Tris-HCl, pH 8.0, refractive index 1.394.

Separation of MLO DNA from host plant. CsCl-bisbenzimide buoyant density gradient centrifugation was used to separate A+T rich MLO DNA from mixtures with plant DNA [Kollar *et al,* 1990]. After the first centrifugation [33,000 rpm in a Beckman 70.1 Ti rotor] at 20°C, for 65 h, the MLO DNA band above the host DNA was removed from each tube and the pooled fractions were recentrifuged [same codition as above] until host plant DNA was not visible. After final centrifugation the MLO DNA was extracted with CsCl-saturated isporopyl alcohol to remove the bisbenzimide. CsCl was removed by chromatography on Sepharose 4B[Pharmacia LKB] equilibrated with TE buffer, pH 8.0. The MLO DNA was pelleted by ultracentrifugation [45,000 rpm in a Beckman 70.1 Ti rotor] at 4°C for 24 h, and the pellet was dissolved in TE buffer.

Identification of number and physical forms of SCWL-MLO plasmids. Number and physical forms of the SCWL-MLO plasmids were identified from purified MLO DNA by using the two dimensional elec-trophoresis method described by Hintermann *et al* [1981]. The purified MLO DNA was run horizontally in 1% agarose gel in TAE buffer [50 mM Tris-base, 30 mM sodium acetate, 3 mM EDTA, pH 7.8]. After the first electrophoresis, the gel was stained with 1.3 mM ethidium bromide, photographed and was then electrophoresed in the second direction.

Construction of DNA probes. Cloning was performed by standard procedure [Harrison *et al,* 1991]. One of the plasmids 2.5 kb in size was excised from the gel and linearized with HindIII restriction endonuclease. The DNA was ligated into HindIII-digested plasmid Bluescript M 13, which qas used to transform *E. coli*, strain XL blue 1. Recombinant plasmids from ampicillin-resistant colonies were extracted and identified by agarose gel electrophoresis. They were screened for SCWL specificity by Southern blot hybridization with DNA both healthy and infected sugarcane.

Dot hybridization. Purified SCWL-MLO DNA and nucleic acid samples prepared from healthy and infected sugarcane were dot blotted [3 ul per spot] in two fold serial dilutions in TE [undiluted of 200 ng of MLO DNA and 1 ug of total nucleic acid from plants] on nylon membranes. DNA was denatured and neutralized by placing the membranes on filter paper saturated with denaturing solution [1.5 M NaCl, 0.5 M NaOH] and neutralizing solution [3 M sodium acetate, pH 5.5], respectively, for 5 min each. The membranes were air dried and then baked at 80°C for 2 h. Hybridization with digoxigenin-labeled probe was carried out according to the procedure recommended by the manufacturer [Boeringer-Manngeim]. The membranes were prehybridized at 68°C for 2 h in 5 x SSC (1 x SSC : 0.15 M NaCl, 0.015 M sodium

citrate, pH 7.0), 0.1% lauroylsarcosine, 0.02% SDS, 1% blocking agent and 50 ug/ml herring sperm DNA. For hybridization, the solution was replaced with a similar solution (2.5 ml per 100 cm^2 membrane) containing denatured digoxigenin-labeled DNA (10-50 ng/ml). The digoxigenin-labeled probes were denatured for 10 min at 95°C and chilled immediately on ice/NaCl for 3 min. After overnight hybridization at 68°C the membranes were washed twice at room temperature for 5 min with 2 x SSC containing 0.1% SDS. They were then rinsed at room temperature with a solution of 100 mM Tris-HCl, 150 mM NaCl, pH 7.5 before proceeding to color reaction.

Southern blot hybridization. HindIII digested and undigested DNAs from healthy and infected plants were electrophoresed in 1% agarose gel, denatured in 1.5 M NaCl + 0.5 M NaOH for 30 min, neutralized in 3 M sodium acetate, pH 5.5 for 30 min, transferred to nylon membranes as described by Sambrook *et al* [1989] and baked at 80°C for 2 h. The membranes were hybridized with digoxigenin-labeled probe as described above.

RESULTS

The DNA isolation from SCWL-infected sugarcane yielded about 100-150 ug of DNA per gram plant powder. About 0.1-0.5% MLO DNA was recovered from total DNA.

Six bands of plasmid DNA were found by two dimensional electrophoresis which seem to be attributed to three different replicons [Fig.1]. During the UV-treatment after the first electrophoretic step, supercoiled [SC] DNA nicked to form to open circular [OC] DNA. The newly formed OC DNAs had same mobility as the OC DNAs originally present in the nonradiated DNA preparation. The plasmid No2 [OC2 and SC2] was linearized with HindIII and RsaI restriction endonucleases [Fig.2.]

the transformation of *E. coli* cells with the ligation mixtures resulted in a total of 92 ampicillin-resistant colonies. Recombinant plasmids with inserts of 2.5 kb were detected in 12 colonies. In Southern hybridization, the inserts of all 12 recombenant plasmids reacted with DNA from infected sugarcane but not DNA from healthy sugarcane. One of recombinant plasmids, pSCWL2, was selelcted for further work. The pSCWL2 probe hybridized with two bands of undigested DNA from infected sugarcane corresponding to the homologous nicked and supercoiled forms of plasmid No2 [Fig. 3]. In dot hybridization, the probe detected the DNA of the SCWL-MLO in 100 pg of purified SCWL-MLO DNA and in 8 ng of total nucleic acid from infected sugarcane [Fig. 4].

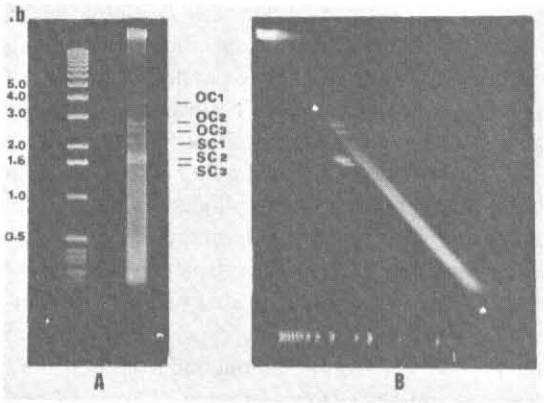

Figure 1 **Number and physical forms of SCWL-MLO plasmids after two steps of agarose gel electrophoresis [A and B]. There are two forms of plasmids, open circular [OC1, OC2, OC3] and supercoiled [SC1, SC2, SC3,] which corresponded to three plasmids respectively.**

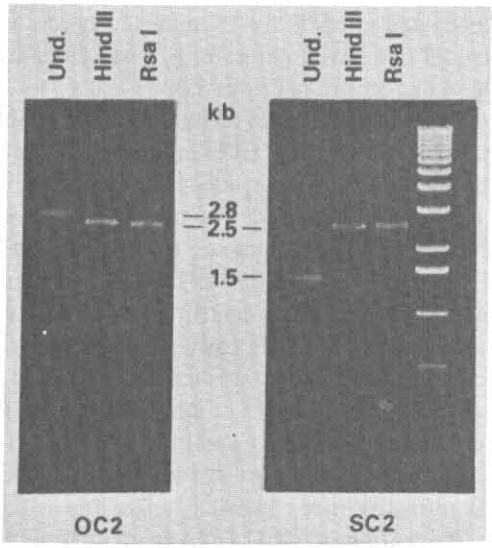

Figure 2 **Physical forms of SCWL-MLO plasmid 2, open circular [OC2: 2.8 kb] and supercoiled [SC2: 1.5 kb], were linearized with Hind III and Rsa I to linear form [2.5 kb].**

The pSCWL2 probe hybridized with all 8 isolates of the SCWL-MLO examined which were collected in different areas [Rayong, Kanchanaburi, Nakhon Pathom, and Prachuab Khiri Khan province] of Thailand. The probe hybridized slightly in Southern blots with two bands of undigested DNA from the

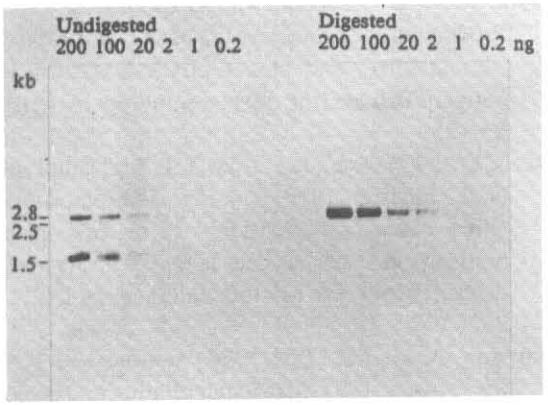

Figure 3 **Southern blot analysis of undigested and HindIII-digested DNA of the SCWL-MLO with probe pSCWL2.**

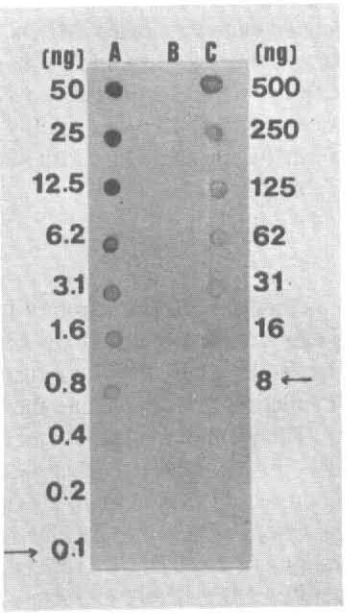

Figure 4 **Dot blot analysis of purified SCWL-MLO DNA [A:0.1-50 ng], total DNA from healthy [B: 1-500 ng] and total DNA from infected sugarcane [C:1-500 ng] with probe pSCWL2.**

Table 1 **Results of DNA hybridization between digoxigenin-labeled pSCWL2 probe and DNAs from other geographically and pathologically diverse MLOs**

Code	MLO	Source	Hybridization with probe
AAY	American aster yellow	USA	-
AKV	*Aquilegia* virescence	Germany	-
AT	Apple proliferation	Germany	-
AV	Aster yellow	Germany	-
AV2226	Aster yellow	Germany	-
BGWL	Bermudagrass white leaf	Thailand	+[a]
BRAWL	*Brachiaria* white leaf	Thailand	+[a]
COL	*Cuscuta odorata* latent	Germany	-
CVB	*Catharanthus* virescence	Geramany	-
CVL	*Catharanthus* virescence	Peru	-
CVT	*Catharanthus* virescence	Thailand	-
PRIVB	Primrose virescence	Germany	-
PRIVC	Primrose virescence	Germany	-
SAFP	Safflower phyllody	Israel	-
SBB	*Solanum* big bud	Ecuador	-
SCWL	Sugarcane white leaf	Thailand	+
SESP	Sesame phyllody	Thailand	-
STOL	Stol	Yugoslavia	-
SUNP	Sunhemp phyllody	Thailand	-
YLBP	Yardlong bean phyllody	Thailand	-

[a] : weak hybridized signal.

bermudagrass white leaf, the *Brachiaria* white leaf, and the *Dactyloctenium* white leaf MLOs. The bands observed corresponded to the homologous nicked and supercoiled forms of the cloned replicon. No hybridization did occur with DNA from healthy plants nor with DNA from the other MLO listed in Materials and Methods [Table 1].

DISCUSSION

The method for the extraction of total DNAs and subsequent isolation of MLO DNA periwinkle developed by Kollar *et al* [1990] was useful for the MLO DNA isolation from sugarcane showing white leaf symtom. The yield of total DNA from sugarcane with the SDS procedure was higher than with the CTAB procedure used [Kollar *et al,* 1990]. The estimated percent yield of SCWL-MLO DNA from gradient separations of total DNA extracts was lower than the yield of MLO DNA extracted from periwinkle or apple phloem.

After the discovery of extrachromosomal DNA in the maize bushy stunt MLO by Davis *et al* [1988], the similar incidence has been described in other plants infected with MLO [Davis *et al*, 1988; Harrison *et al,* 1991; Klein *et al,* 1990; Kuske and Kirkpatrick, 1990; Lee and Davis, 1988]. In this study, Southern hybridization analysis provided the first evidence for the presence of extrachromosomal DNA of the SCWL-MLO. The developed probe proved to be suitable for sensitive detection of the SCWL-MLO. The hybridization data revealed that the pSCWL 2 probe hybridized specifically to plasmid 2 DNA only. Thus, no nucleotide sequence semilarity exists between this plasmid and the other plasmids of the SCWL-MLO. Also, no hybridization occurred with DNA from 18 other MLOs. However, the probe reacted slightly with DNA from grasses showing white leaf symptom. The results indicate that the pSCWL 2 probe has a narrow detection range for the DNA of MLOs affecting monocotyledons. Further work is needed to determine the relationships among these various MLOs associated with white leaf symptom of monocotyledons.

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