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DNA Probe and Nucleic Acid Hybridization for Plant Virus Detection

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ABSTRACT

The DNA probe for detection of tomato yellow leaf curl virus (TYLCV) was constructed by using the oligolabelling method. Cloned TYLCV-DNA component A in a Bluescript vector was linearized by cutting at EcoRI site, denatured and primed with random hexanucleotide primer. The complementary DNA strand, labeled with ^{32}P, was completely synthesized by Klenow polymerase filling-in reaction. The cDNA probe was denatured and used in virus detection by nucleic acid hybridization technique. The probe obtained had sensitivity at 1 picogram level of template DNA, and reacted well with viral DNA either in crude extract or in DNA preparation of infected tomato tissues. The probe can also detect viral DNA from squash blotted samples of infected tissues and viruliferous whitefly vector.

INTRODUCTION

There are a number of methods which are simple and sensitive for rapid plant virus detection. Several techniques based on immunological reactions are practical for routine diagnostic work and permit the detection of small amount of virus particle or viral protein (Stewens, 1983). The method of choice for detecting plant virus agents is nucleic acid hybridization (Paulakitis, 1988). This assay allows the rapid detection of viral nucleic acid in a large number of individual plant samples. The sensitivity of the technique permits the detection of plant virus which occurs in plant at very low concentration or being restricted for its distribution in plant cells. In case of tomato yellow leaf curl virus (TYLCV), it was difficult to detect virus in infected tomato plants (Thongrit *et al.*, 1986). The practical method was the immunoelectron microscopy, but it was shown to be a tedious work and time consuming if hundreds of samples were to be diagnosed.

In this paper, we described the method for the construction of DNA probe to be used in the detection of tomato yellow leaf curl virus (TYLCV), a geminivirus, by using DNA:DNA hybridization reaction on the filter. This is the first report on using DNA probe for plant virus detection in Thailand.

MATERIALS AND METHODS

Template DNA

Tomato yellow leaf curl virus (TYLCV) was purified from infected tomato tissues and viral single stranded DNA was extracted from virus particles as previously reported (Attathom *et al.*, 1989). Circular double stranded DNA of TYLCV was synthesized from extracted ssDNA by annealing with synthesized primers containing base sequences similar to hairpin loop region of tomato golden mosaic virus DNA (Hamilton *et al.*, 1984). The completion of double strand was done by Klenow polymerase filling-in reaction. Double stranded DNA was cloned by ligation into Bluescript plasmid at EcoRI site. Analysis of cloned DNA by agarose gel electrophoresis and restriction enzyme mapping revealed two components of TYLCV-DNA with equal size of about 2.7 kilobasepairs. Full length cloned DNA of component A (Fig. 1) was used as a template for construction of DNA probe.

Construction of DNA probe

The component A of TYLCV cDNA was oligonucleotide primed and labeled with ^{32}P following the method of Hodgson and Fisk (1987). In the reaction mixture, 300 ng of dp (N) random primer was

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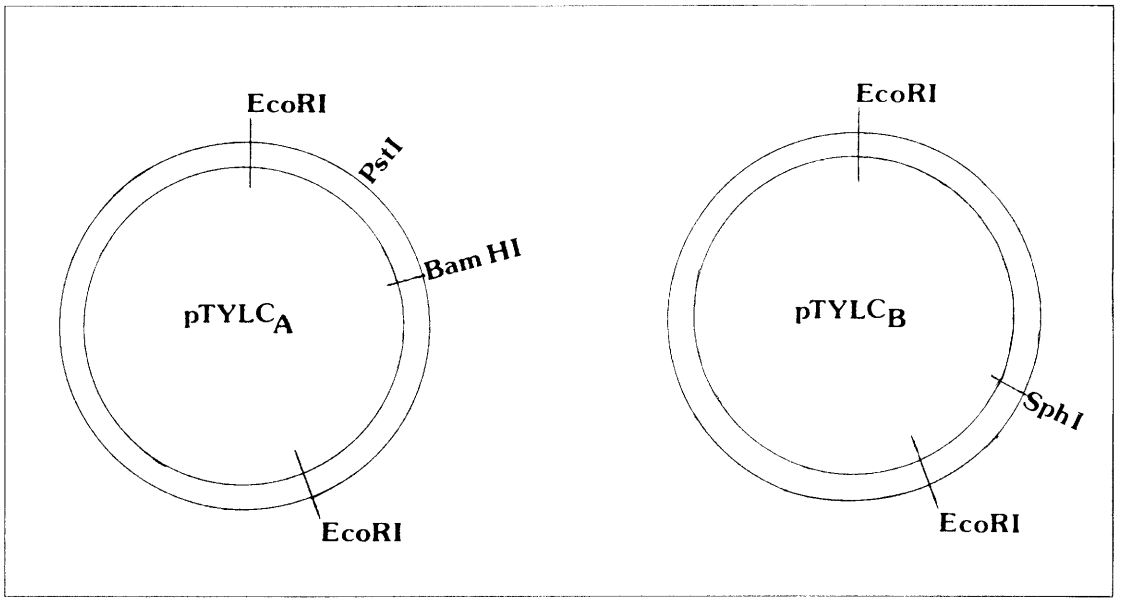

Fig. 1 Simplified restriction maps of cloned TYLCV DNA component A (left) and B (right).

combined with 500 ng of template DNA in 10 µl of TE buffer (10 mM tris, pH 7.5, 1 mM EDTA). After immersing a reaction tube in a 80°C water bath for 2 min and left cool to room temperature, the mixture was added with 4 µl of 10x oligolabelling buffer (0.5 M tris, pH 6.9, 0.1 M magnesium sulfate, 1 mM dithiothreitol), 0.6 mM each of dCTP, d GTP and dTTP, 4 µl (40 µCi) of α-^{32} P-dATP (3000 µCi/m mole), and 5-10 units of Klenow polymerase, to make 40 µl final volume. After 30 min at 37°C, the reaction was added with 160 µl of STE (TE with 0.1 M NaCl) to bring the volume to 200 µl. The oligolabelling mixture was purified by using spun-column chromatography (Maniatis *et al.*, 1982). The eluent was collected and determined for the incorporation percentage of α-^{32} P-dATP by a liquid scintillation counter. The probe was immersed in a boiling water bath for 2 min prior to hybridization.

Sample preparation

DNA samples were prepared by three different methods as followed ;

Method I. A minipreparation method described by Dellaporta *et al.* (1983) was used. Leaf tissues of 0.5 g were ground to powder in liquid nitrogen and added with 15 ml of extraction buffer (100 mM tris, pH 8.0, containing 50 mM EDTA, pH 8.0, 500 mM NaCl, 10 mM mercaptoethanol). After adding 1 ml of 20% SDS, the suspension was mixed thoroughly by vigorous shaking and incubated at 65°C for 10 min. It was then added with 5 ml of 5M potassium acetate, shaked vigorously and incubated at 0°C for 2 min. After pelleting out the precipitate, the supernatant was added with 10 ml of isopropanol and incubated at -20°C for 30 min. DNA was pelleted and dried on paper towel for 10 min. DNA pellet was redissolved with 0.7 ml of 50 mM tris buffer, 10 mM EDTA, pH 8.0. The suspension was spun in a microfuge to remove insoluble debris, and supernatant was transferred to a new eppendorf tube. DNA was pelleted by adding 75 µl of 3M sodium acetate and 500 µl isopropanol, and centrifuged. DNA pellet was washed with 80 % ethanol, dried and redissolved in 200 µl of 100 mM tris buffer, 10 mM EDTA, pH 8.0.

Method II. This method is used to prepare DNA from large number of samples of infected tomato tissues. Healthy and diseased tomato leaf tissues were ground each in one ml of 0.1 M tris buffer, pH 8.0 and centrifuged to remove plant debris. To each 250 µl of supernatant, an equal volume of denature solution containing 67 µl of 3 M NaOH, 50 µl of 0.1 M EDTA and 133 µl of distilled water was added. DNA solution was boiled for 10 min in a water bath and immediately chilled on ice. Ten fold diltions of denatured DNA were made from 1/10 up to 1/1000000, and these DNA samples were used for DNA probing.

Method III. This squash blotting method (Navot *et al.*, 1989) was performed for obtaining rapid DNA prepa-

ration either from infected leaf tissue or from whitefly, the insect vector. Tomato tip leaves were squashed onto a nylon membrane using a sterile glass rod and added with 10 µl of denatured solution (0.125 NaOH, 0.125X SSC). Whitefly adults collected from healthy and infected tomatoes were squashed either singly or group of five onto the nylon membrane by the same procedure. The membrane was air dried before subjected to hybridization.

DNA hybridization

Dot blotting was performed according to NEN™ protocol (Anonymous, 1985). Prehybridization was at 65°C in a water bath with shaking for 3 hours followed by hybridization at 65°C in a water bath with shaking for 15 hours (Perbal, 1988). After washing and air dried, the filter was subjected to autoradiography at -80°C for 96 hours.

RESULTS AND DISCUSSION

Sensitivity of TYLCV-DNA probe

The ^{32}P-oligolabeled TYLCV probe obtained in this experiment showed about 8-15 % incorporation of a radioisotope after 30 min reaction. This probe can detect as low as one picogram of template DNA (component A), or one nanogram of component B (Fig, 2). DNA samples extracted by minipreparation method reacted strongly with the probe. When DNA preparation obtained from 0.5 g leaf tissue and resuspended in 100 µl TE buffer was diluted ten fold to 1/10, 1/100, 1/1000, 1/10000, and 1/100000, positive results were detected in all dilutions. The same results were also obtained when crude sap of plant tissues was used for hybridization test. Healthy leaf tissue showed negative result for all tests.

Detection of viral DNA in squash blotted tomato leaf tissues

TYLCV-DNA was detected by the probe of component A in squashed samples from infected tomatoes but not from healthy ones (Fig. 3). When inoculated tomato seedlings were determined for the presence of viral DNA at one week interval post inoculation, it was observed that viral nucleic acids were found in the second week post inoculation and increased up to six weeks (Fig. 4). There were previ-

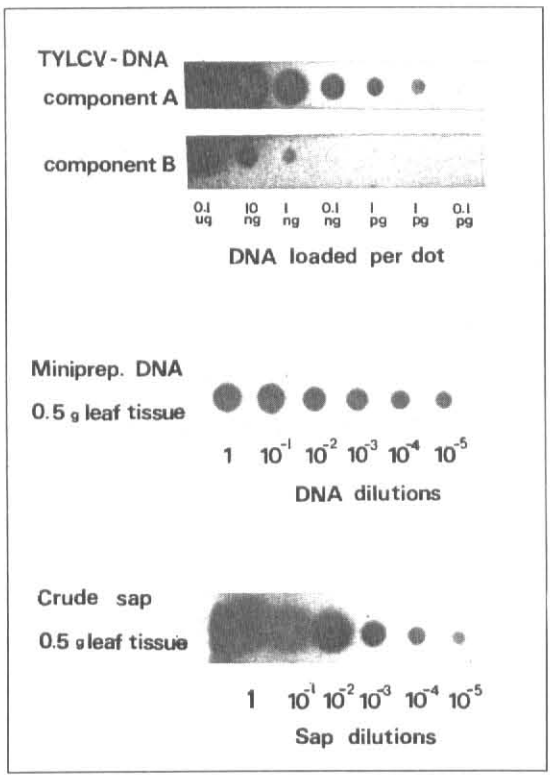

Fig. 2 Autoradiographs of tomato yellow leaf curl virus (TYLCV) DNA showing the sensitivity of (TYLCV) DNA probe by filter hybridization.

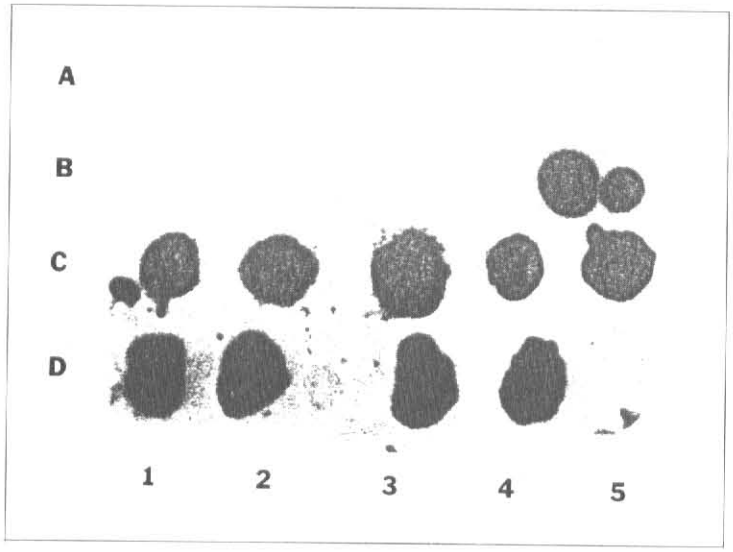

Fig. 3 Detection of tomato yellow leaf curl virus (TYLCV) DNA from tomato tissues by squash blot method. Row A, B; healthy toamto, Row C; diseased leaf tissues, Row D; diseased petiole tissues, B 4-5; positive control of crude sap from diseased plant, D 5; negative control of crude sap from healthy plant

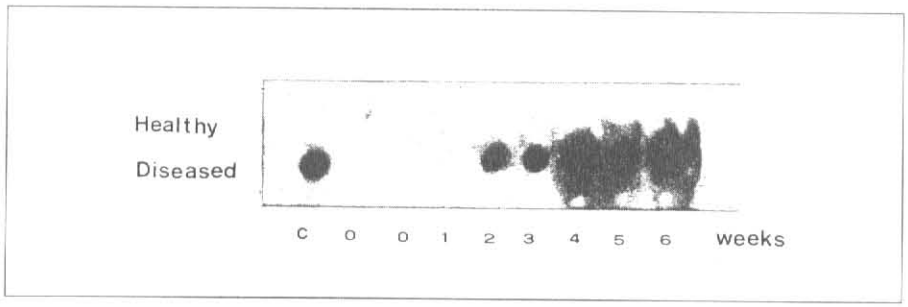

Fig. 4 Quantitation of tomato yellow leaf curl virus (TYLCV) DNA at one week intervals after graft inoculation. Detection was made by using DNA probe. C = control using crude saps from healthy and diseased plants.

ous reports of other geminivises such as African cassava mosaic virus (ACMV) and been golden mosaic virus (BGMV) for the detectable viral nucleic acids in inoculated plants within 14 and 9 days, respectively (Robinson *et al.*, 1984; Aozaki *et al.*, 1988).

Detection of TYLCV-DNA from squashed viruliferous whitefiles

Positive reactions were obtained only from the whiteflies that were fed on TYLCV infected tomato plants. Positive detection percentages were about 50 and 100 for single and five whiteflies per dot, respectively (Fig. 5). Although the virus-vector relationship has not yet determined, this squash-blot method and the developed oligolabelling prove should provide a practical routine diagnosis for viral nucleic acid in a single insect sample.

This is the first time in Thailand that cDNA probe was constructed and used for detection of TYLCV, a geminivirus infecting tomato plants. Nucleic acid hybridization on filter and simplified sample preparation made the diagnosis very rapid and higher efficient compared to previously used techniques such as an immunobinding assay (Poolpol, 1986). It will be very interesting to use this technique in various experiments to study about etiology, ecology and epidemiology of TYLCV which may contribute to an effective control measure for the destructive disease of tomatoes in this country.

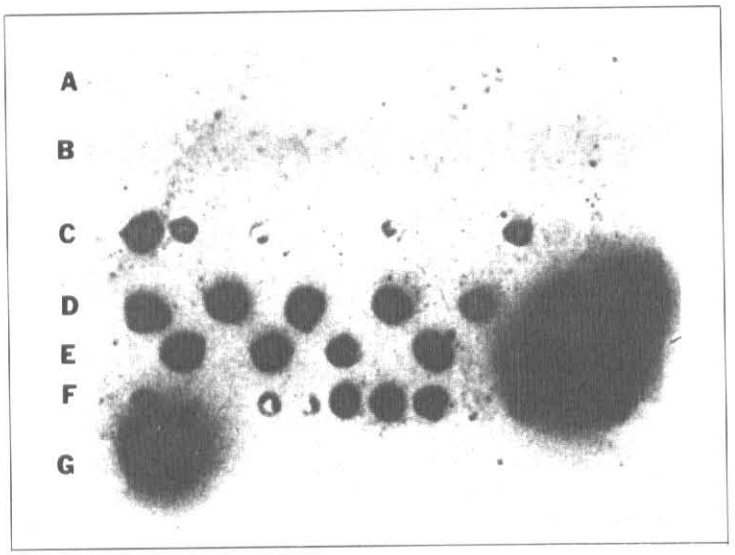

Fig. 5 Autoradiograph of tomato yellow leaf curl virus (TYLCV) DNA in viruliferous whiteflies. Row A; non-viruliferous, one whitefly per dot, Row B; non-viruliferous, 5 whiteflies per dot, Row C, F; viruliferous, one whitefly per dot, Row D, E; viruliferous, 5 whiteflies per dot. Right corners of Row D, E and left corner of Row G; positive control using TYLCV component A.

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