TECHNICAL SHEET No. 33

Virus Detection: *Tomato yellow leaf curl virus* (TYLCV)

Methods: Variability analysis of TYLCV species (strains)

General

Virus detected: TYLCV species (strains) from tomato, pepper and bean samples
Methods: Variability analysis of TYLCV species (strains)

Developed By

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Goals

To develop a method for the rapid detection of TYLCV species (strains) from a large number of samples; and assignment of isolates into TYLCV (Israel) or TYLCSardiniaV species.

Introduction

Tomato-infecting whitefly transmitted geminiviruses (begomoviruses) are a complex of strains and species. Based on sequence comparison, the various TYLCV isolates or different species can be grouped according to a geographically based scheme (Zeidan et al., 1998). 1. From the Middle East (Israel, Egypt, Jordan, Lebanon, Northern Saudi Arabia) (TYLCV) (Navot et al., 1991). 2. From Southwest Europe (Italy) (*Tomato yellow leaf Sardinia virus*, TYLCSarV) (Kheyr-Pour et al., 1991). 3. From tropical Africa (Senegal, Tanzania), and 4. From Southeast and East Asia (Thailand, China). TYLCV from the Caribbean Islands and from the Southeast USA originated from the Middle East. All these isolates, except TYLCV from Thailand, have a monopartite genome. This virus is also called *Tomato yellow leaf curl Thailand virus*. Some related whitefly-transmitted viruses infecting tomato are also called *Tomato leaf curl virus*, ToLCV, and have been found in India and Australia. The ToLCV isolates have monopartite genome, except of ToLCV from Northern India. For some of the tomato-infecting begomoviruses there is evidence of recombination (Chatchawankanphanich and Maxwell, 2002; Navas-Castillo et al., 2000). Information about geminiviruses can be found on the Web at Gemininet (http://www.danforthcenter.org/iltab/geminiviridae).

Materials and Methods

Nucleic acid extraction

5 mg of tomato leaf tissue was ground with 500 µl of extraction buffer (100 mM Tris-HCl, 50 mM EDTA, 0.5 M NaCl, 10 mM Beta-mercaptoethanol) vortexed and allowed to stand at 65°C for 10 min. After adding potassium acetate (5 M, pH 8), the mixture was incubated on ice for 10 min and clarified by centrifugation at 13,000 rpm for 20 min at 4°C. An equal volume

of isopropanol was added to the supernatant, and then the solution was incubated for 10 min at –20°C and centrifuged for 10 min at 13,000 rpm. The pellet was resuspended in sterilized water and subjected to RNase treatement followed by phenol/chloroform/isoamyl alcohol (25:24:1) and chloroform/isoamyl alcohol extraction. After precipitation with three volumes of absolute alcohol for 30 min at –20°C and centrifugation for 10 min at 13,000 rpm, DNA was washed with 70% ethanol and resuspended in steriled water.

Dot blot hybridization

200 ng of extracted DNA from each sample was first added to an equal volume of formaldehyde (15%) then heated at 65°C for 20 min. Using a dot blot apparatus (BioRad) under vacuum, DNA was transferred to a nylon filter (membrane) previously washed in 20X SSC (150 mM NaCl, 15 mM sodium citrate). The membrane was air dried at room temperature and stored until used.

A random primed digoxygenine-dUTP DNA probe spanning a TYLCV genome region of choice was used. The labelling reaction was carried out according to manufacturer's instructions (Bohringer). 1 µg of DNA was denatured at 100°C for 10 min, then 2 µl of hexanucleotides, 2 µl of dNTP (10 mM each) and 1 µl of Klenow DNA polymerase fragment were added. The mixture was incubated for one hour at 37°C and 2 µl of a stopping reaction (200 mM EDTA, pH 8) added to stop the reaction. The labelling probe was precipitated using 2.5 µl of 4 M LiCl, 1 µl of glycogene and 75 µl of absolute ethanol.

The membrane was incubated for one hour at 42°C, then the denatured probe (100°C for 10 min) was added and both were incubated at 65°C overnight. The membrane was then washed twice with 2X SSC, 0.1% SDS for 5 min at room temperature and twice with the same solution for 15 min at 42°C. After another wash with buffer I (100 mM Tris-HCl, pH 7.5, 150 mM NaCl) for 1 min, the membrane was saturated for 30 min with buffer II (buffer I plus 0.5% blocking agent), washed again with buffer I and incubated for 30 min with buffer I containing an alkaline phosphatase-conjugated dUTP-specific IgG diluted at 1/5000. The membrane was washed twice for 15 min with buffer I, then for 2 min with buffer III (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$) and finally incubated in buffer I supplemented with NBT and BCIP according to manufacturer's instructions. Color development was stopped by washing the membrane in 50 ml Tris-EDTA and drying.

PCR - RFLP of the coat protein gene of TYLCV (species/strains)

Degenerate primers allowing the amplification of the coat protein gene were designed by analyzing sequences of TYLCV (Israel) from Egypt (NC 001996.1), Portugal (AF105975), Spain (AF071228), and the sequence of TYLCSardiniaV from Sardinia (X61153) (M. K. Nakhla, University of Wisconsin-Madison). These primers should thus anneal with both types of genomes: TYLCV (Israel) and TYLCSarV.

PCPv (PTYCPv369) (viral sense)
5'ACGCCCG(T/C)CTCGAAGGTTCG 3'
PCPc (PTYCPc1023) (complementary sense)
5' GTACA(T/A)GCCATATACAATAACAAGGC 3'

PCR amplification was done with 150 ng of total extracted DNA of each selected sample. PCR conditions were: one cycle of 3 min at 94°C followed by 35 cycles of 50 sec at 94°C, 50 sec at 55°C and 1 min at 72°C, then a final extension step of 10 min at 72°C.

Amplified fragments of about 673 bp were subjected to electrophoresis in a 1% agarose gel and stained with 0.5 µg/ml of ethidium bromide.

PCR fragments were column purified according to Qiagen Kit. DNA was then digested with the restriction endonuclease *Ava*II, which was expected to cut TYLCSarV differently from TYLCV (Israel) (Accotto et al., 2000). Digestion products were analyzed by electrophoresis on a 1.6% agarose gel.

Results and Discussion

Analysis of samples by dot blot hybridization under high stringency provided a reliable tool for both identification and quantification of TYLCV. The intensity of the signal is positively correlated with the virus accumulation in the infected plant. This assay offers a reliable tool to test a large number of samples rapidly.

Using degenerate primers of the coat protein gene, PCR fragments can be amplified from either Israeli or Sardinian viral genome. Analysis by PCR-RFLP using AvaII allows the assignment of TYLCV isolates into one of the two species: TYLCV (Israel) isolates produce a restriction pattern of about 370 and 300 bp while that of the TYLCSarV isolates consistently have three fragments with approximate sizes of 360, 160 and 150 bp.

Our ultimate goal is to develop a simple, rapid and reliable method for diagnosis and typing of TYLCV isolates.

References

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