TECHNICAL SHEET No. 32

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

Method: Non-Radioactive Technique for Dot Blot Hybridization

General

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Method: non-radioactive technique for dot blot hybridization

Developed by

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Goals

To detect and distinguish between TYLCV species by a non-radioactive hybridization.

Advantages

The method can be used in many laboratories, since it is a non-radioactive technique. Also, it is a quick and reliable method.

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:
http://www.danforthcenter.org/iltab/geminiviridae
http://gemini.biosci.arizona.edu/
http://life.bio2.edu/ICTVdB/29030043.htm
http://www.im.ac.cn/vide/descr840.htm

Materials and Methods

Summary

1. Tissue samples are collected and stored in manner so that they don't decompose (E.G., cut young leaves in very small pieces (1 mm strips) and then let dry for at least 48 hours on filter paper).
2. Membrane spotting: Sample is ground in a buffer and debris removed by an appropriate method (see Dellaporta/heat technique). Small amount (5 µl) is spotted on a neutral nylon membrane, and then dried for about a half hour.
3. Lysing: prior to hybridization, membranes are treated with denaturing solution to form ssDNA, which binds to the nylon membrane and will be available for hybridization with a nucleic acid probe.
4. Fixing: UV-cross linker can be used to cross link the DNA to the membrane.
5. Preparing the probe and adding it to the membrane: This is the hybridization step. The membrane and labeled probe are placed in a hybridization solution. The hybridization conditions can vary so that the DNA probe will hybridize with the DNA of the test organisms when the percent DNA similarity is 65-75% (low stringency) or greater than 90% (high stringency). Low stringency would be used to detect any begomovirus in the sample and high stringency conditions would be used if differences in geminiviral species were being detected.
6. Washing: membranes are washed in a mild salt/detergent solution to remove any probe that was not hybridized to the test DNA.
7. The film is exposed for a time period from hours to days.

DNA Extraction Sardinian – Species Specific Primer (J. Potter et al., 2003)

1. Extract DNA from sample in extraction buffer: Plant tissues to be tested are: TYLCV positive control, unknown sample, tissue from non-infected tomato.
2. Obtain a small amount of tissue (2 cork borer for fresh or frozen tissue, 5 mg for dry tissue) and place in a microfuge tube.
3. Resuspend sample in 500 µl of Dellaporata buffer.
4. For dry tissue, leave on ice for 20 min with 500 µl Dellaporta.
5. Grind using a Kontes pestle.
6. Incubate at 65ºC for 5-10 min.
7. Spin for 10 min at 14,000 rpm.
8. Remove supernatant to new tube amd discard tissue debris.
9. Dilute 1:10 and 1:100 for PCR. Use without dilution for hybridization.
10. Store at –20ºC.

Dellaporta Buffer: total of 50 ml	
150 µl BME (beta mercaptoethanol)	Fisher BP 176-100
5 ml 1 M Tris pH 8.0	Fisher BP 152-5
5 ml 0.5 EDTA pH 8.0	Fisher BP 120-500
5 ml 5 M NaCl	Fisher S 271-10
34 ml dds H_2O	

Membrane Lysing

1. In a large glass tray, saturate 3 layers of Whatman (Cat# 3030917) 3 mm paper with 0.5 N NaOH. (20 ml 5M NaOH +180 ml H_2O). Remove any excess.
2. Lay membranes DNA side up on saturated paper, create no bubbles or folds. Leave for 5 min (saturate paper again before lysing another set of membranes).
3. Transfer membrane to a tray with 1 M Tris pH 7.4, leave for 5 min with agitatation.
4. Transfer membranes to a tray with 2X SSC (20 ml 20X SSC + 180 ml H_2O) (20X SSC: dissolve 175.3 g of NaCl + 88.2 g sodium citrate in 800 ml H_2O, adjust pH to 7.0 with 10 N NaOH. Adjust the volume to 1 liter with H_2O, autoclave).
5. Leave for 5 min agitating occasionally.
6. Transfer membranes to tray with 95% ethanol. Leave for 5 min, agitating occasionally. Ethanol will get cloudy, replace if lysing many membranes.
7. Air dry membranes (1/2 hour).

DNA Fixation on Membrane

There are two methods:

1. UV cross-link DNA by selecting optional cross-link on machine or by exposing to UV light with trans-illumination device for 3 min.
2. Bind the DNA to the nylon membrane by baking for 2 h at 80ºC.

Preparation of DNA probes

One of the easiest ways to prepare a DNA probe is to use the viral amplified fragment from a PCR reaction with appropriate primers and target DNA.

<u>*General Probe*</u>: For a general probe (about 500 bp) for Eastern Hemisphere begomoviruses, sequences of several viruses were aligned and the most conserved region of the coat protein (*CP*) gene determined. Primers were designed that would amplify this highly conserved region, which corresponds to the 3' end of the *CP* gene, from TYLCV (Israel) isolate (Navot et al., 1991):

1. PTYCPv (PTYv369) (viral sense) 5'- ACGCCCGCTCGAAGGTTCG-3'
2. PTYCPc (PTYc1023) (comp. sense) 5'-CGTCAGCCATATACAATAACAAGGC-3'

A full-length, infectious clone (pEG1) of the TYLCV from Egypt was generally used as the target DNA.

<u>*TYLCV (Israel)-specific probe*</u>: The viral species-specific probe was the intergenic region (common region, ori region), which is the most variable among different begomovirus species. Two different sets of primer pairs could be used for TYLCV (Israel) (Navot et al., 1991):

One primer set was designed by Navas-Castillo et al. (1999):

1. MA-30 (viral sense): 5' GAGCACTTAGGATATGTGAGG 3' (anneals at nt 2566-2586)
2. MA-31(comp. sense): 5' AGTGGATCCCACATATTGC 3' (anneals at nt 152-170)

One primer set was designed by M. K. Nakhla (University of Wisconsin-Madison):

1. PTYv21 (viral sense): 5' GTTGAAATGAATCGGTGTCCC 3'
2. PTYc287 (comp. sense): 5' TTGCAAAGACAAAAAACTTGGGACC 3'

(Number in primer name corresponds to nt position in genome.)

TYLCSarV-specific probe: The primer set was designed by Navas-Castillo et al. (1999) for the Sardinian species of TYLCV, TYLCSardiniaV (Kheyr-Pour et al., 1991):

1. MA15: 5'-GGATCCCACATATTG-3'
2. MA14: 5'-TGCATTTATTTGAAAACG-3'

PCR Conditions

The thermocycler used was Thermocycle PTC-0200 DNA Engine (Alpha unit). Program: (94ºC, 45 sec, 55ºC,1:00 min, 72ºC, 1:45 min) x 30 cycles, followed by (94ºC, 1:00 min, 55ºC, 1:00 min, 72ºC, 5:00 min) x 1 cycle, then 4ºC.

PCR reaction mixture

H_2O 12.9 µl	Primer(+) 10 µM 1.0 µl
Buffer (Taq) 10X 2.5 µl	Primer (-) 10 µM 1.0 µl
Mg++ (Taq) 10 X 2.5 µl	dNTP's 10X 2.5 µl (each dNTP at 2.5 mM)
Taq DNA polymerase 0.1 µl	DNA sample 2.5 µl

Total: 25 µl	

Target DNA could be either DNA extracted from infected plants as describe above or plasmid DNA with an appropriate insert. Other DNA extraction methods outlined in Rojas et al. (1993) could be used.

Cleaning the DNA - DNA precipitation from PCR reaction

Combine the volume from two 50-µl PCR reactions, measure the volume and adjust to 400 µl. Add 0.1 volume of 3 M sodium acetate, (about 40 µl in 400 µl), add 2.5 volume 100% ETOH (about 1 ml), vortex and incubate at – 80ºC for 30 min or –20ºC for 4 h, spin at 14K for 15 min, keep pellet and add 70% ETOH spin at 14K remove ETOH, dry in speed vac, resuspend in 50 µl H_2O, shake for 5 min, run argarose gel to see how much DNA you have.

Considerations:

- The concentration of salt in the probe DNA to be labeled should be as low as possible and not exceed 50 mM. This is the reason for the precipitation step above.
- The DNA concentration should be accurately determined and adjusted to 10 ng/µl before labeling.
- Maintaining the DNA in single-stranded form is required to ensure a good labeling efficiency.
- Enzyme labeled probes should not be denatured prior to addition to the hybridization buffer.

Preparation of Labeled Probe

KIT: AlkaPhos Direct labeling reagents from Amersham Pharmacia Biotech UK limited, Cat number: RPN 3680. CRR-star detection reagent Cat number: 3682 from Amersham.

1. Preparation of cross-linker working solution from kit: 10 µl of cross-linker working solution are need for each probe; thus for 3 probes, the total is 30 µl. Take an 8 µl cross-link tube (black tube in kit) + 32 µl H_2O supplied = 40 µl cross link working solution. (This cross-linker working solution can be saved at 2-8ºC for later use).
2. Dilute the DNA to a concentration of 10 ng/µl using the water supplied (10 µl = 100 ng DNA).

3. Place 10 µl of the dilute DNA sample in a microfuge tube and denature by heating for 5 min in a vigorously boiling water bath.
4. Immediately cool the DNA on ice for 5 min. Spin briefly in microfuge to collect the contents at the bottom of the tube.
5. Add 10 µl of reaction buffer (red tube in kit) to cooled DNA. Mix thoroughly, but gently.
6. Add 2 µl labeling reagent (yellow tube). Mix thoroughly, but gently.
7. Add 10 µl of the cross-linker working solution. Mix thoroughly. Spin briefly in microfuge to collect the contents at the bottom of the tube.
8. Incubate the reaction for 30 min at 37ºC.
9. The probe can be used immediately or kept on ice for up 2 h. For long-term storage probes may be stored in 50% (v/v) glycerol at –15ºC for up to six months.

Hybridization

1. Pre-heat the required volume of prepared Alk. Phos direct hybridization buffer to 55ºC. The volume of buffer should be equivalent to 0.25 ml/cm^2 of membrane (generally, about 23-25 ml per hybridization tube).
2. Place the blot (membrane) into the hybridization buffer and pre-hybridize for at least 15 min at 55ºC in shaking water bath or hybridization oven.
3. Add the labeled probe to the buffer used for the pre-hybridization step (directly to the hybridization tube). Typically use 5-10 ng of probe/ml of buffer. (100-200 ng of probe for 20 ml of hybridization solution).
4. Hybridize at 55ºC overnight in shaking bath or hybridization oven.

Post-Hybridization Stringency Wash

1. Primary wash in hybridization oven:
   a. Pre-warm the primary wash buffer at 55ºC for general probe (low stringency) or 60-65ºC for species-specific probe (high stringency).
   b. Place blots in 1-2 ml/cm (30-40 ml) wash buffer.
   c. Wash for 2X 20 min.
2. Second wash:
   a. Prepare second wash buffer (100 ml in tray: 5 ml of 20X stock + 95 ml H_2O + 200 µl $MgCl_2$)
   b. Wash bath at room temperature for 2X 10 min, gently agitating.

Solutions for Hybridization

A) Hybridization Buffer: Store in aliquots at -20ºC (Add NaCl to Hyb buffer to give final solution of 0.5 M. Add blocking reagent to give a final concentration of 4 %. Mix at room temp for 1-2 hours on magnetic stirrer until clear.)

- Hyb Solution 500 ml bottle (in the kit).
- NaCl 14.61 g
- Blocking reagent 20 g (Kit)

B) Primary Wash: Make to a volume of 500 ml, can be stored 1 week at 4ºC.

- Urea 60 g
- SDS 0.5 g
- 0.5 M NaH_2PO_4 50 ml*
- NaCl 4.35 g
- 1.0 M $MgCl_2$ 0.5 ml
- Blocking reagent 1 g (kit)

* monobasic sodium phosphate, adjust to pH 7.0 with NaOH.

C) Secondary Wash: Add above to 450 ml dH_2O, adjust pH to 10.00 with NaOH. Store up to 4 months at 4ºC.

500 ml 20X Stock:

- Tris base 60.5g
- NaCl 56 g

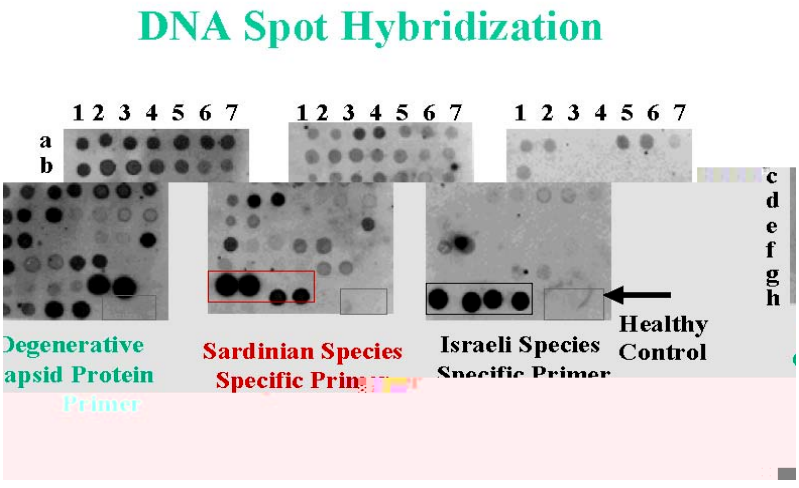
Secondary Wash, 500 ml Working Solution:

- Dilute 1:20, (20X stock, secondary wash stock) 25 ml
- dH_2O 475 ml
- 1M $MgCl_2$ 1 ml

Signal Detection with CDP-Star

Note: wear powder-free gloves or rinse gloved hands with water.

1. Drain excess secondary wash buffer from the blots by touching the corner of the blots against a clean surface, place the blots (sample side up) on clean non-absorbent, flat surface, eg, a tray. Do not allow the blots to dry out.
2. Pipette detection reagent (in the kit, CDP star) on to the blots, about 30-40 µl/cm^2 (2-4 ml/blots, enough to cover the whole blot) and leave for 2-5 minutes at room temperature. Drain excess reagent from membranes before next step.
3. Wrap the blots in Saran Wrap or place in detection bag. Place the blots DNA side up in the film cassette.
4. Switch off the lights and place a sheet of autoradiography film (Ex. ECL film, Hyper-film) on the top of the blots (shiny side of the film up). Close the cassette and expose for 4 h at room temperature. The DNA side of the filter (wrapped in saran Wrap) must be placed next to the film for maximum sensitivity.
5. Remove the film and develop, you can also expose a second film for 24 hours.



Results

The example to the right is from tomato samples collected in Morocco and hybridized with the general probe (CP gene), TYLCSarV-species probe, and TYLCV (Israel)-species probe (PTYv21/PTYc287, primers). Three identical membranes were prepared. None of the probes hybridized with the DNA extracted from symptomless (non-virus infected) tomatoes, and the general probe hybridized with most samples.

Many tomato samples had mixed infections and more tomatoes were infected with TYLCSarV than TYLCV.

Discussion

This non-radioactive method has been tested in laboratories in San Carlos University, Guatemala, Agricultural Research Center, Giza, and Egypt by our group and has worked very well. This method offers several advantages. Many samples can be tested and the species-specific probes are effective in distinguishing between TYLCV and TYLCSarV, where as the general probe hybridizes effectively with these two viruses. Similar methods have been developed by Potter et al. (2003) for detection of bean-infecting begomoviruses.

References

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