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Development of a probe-based blotting technique for the detection of Tobacco streak virus

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Summary. – Digoxigenin (DIG)-labeled DNA probe was developed for a sensitive and rapid detection of the Tobacco streak virus (TSV) isolates in India by dot-blot and tissue print hybridization techniques. DIG-labeled DNA probe complementary to the coat protein (CP) region of TSV sunflower isolate was designed and used to detect the TSV presence at field levels. Dot-blot hybridization was used to check a large number of TSV isolates with a single probe. In addition, a sensitivity of the technique was examined with the different sample extraction methods. Another technique, the tissue blot hybridization offered a simple, reliable procedure and did not require a sample processing. Thus, both non-radioactively labeled probe techniques could facilitate the sample screening during TSV outbreaks and offer an advantage in quarantine services.

Keywords: Tobacco streak virus isolates; DIG-labeled DNA probe; dot-blot hybridization; tissue print hybridization technique; diagnosis

Introduction

Tobacco streak virus (the genus *Ilarvirus*, the family *Bromoviridae*) contains a single stranded, tripartite RNA genome. The complete RNA 3 consists of 2,205 nts and encodes a movement protein (MP) required for cell-to-cell movement (Cornelissen *et al.,* 1984). The viral CP is encoded by a subgenomic RNA designated RNA 4 that is collinear with the 3'-end of RNA 3. Since TSV has a broad host range, it emerged as a major threat to many economically important agricultural and horticultural crop species like sunflower, pumpkin, gherkin, marigold, and globe amaranth in tropical and subtropical countries. Comparative studies of TSV diagnostic methods indicated that RT-PCR techniques are very sensitive, but expensive and time-consuming. ELISA techniques are more economical and better suited for the screening of large numbers of samples, but are limited by the supply and quality of specific antibodies as well as by the method of sampling. Hybridization techniques are used to detect several plant viruses and are more sensitive and specific than ELISA (Hahm *et al.*, 1993). Because of the limited variability of CP region of TSV isolates all over the India, a corresponding DIG-labeled probe was developed for the detection of TSV isolates.

This study reports the probe applicability in dot-blot hybridization and tissue print hybridization techniques that were used for diagnostics of TSV infection.

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Abbreviations: CP = coat protein; DIG-labeled DNA = digoxigenin-labeled DNA; TSV = Tobacco streak virus

Materials and Methods

Collection of plant material and total RNA extraction. The suspected TSV-infected sunflower, gherkin, pumpkin, marigold, and globe amaranth plants showed characteristic symptoms of chlorosis and necrosis. The plants were collected in Andhra Pradesh, India. Total RNA was isolated from virus-infected as well as from healthy plants using trizol method (Sigma) following manufacturer's instructions.

Primer design from TSV sunflower isolate. Primer pair was designed for CP+3'- UTR regions of TSV using Oligo version 5 software using reported sequence data of TSV RNA 3 (Cornelissen *et al.,* 1984).

cDNA synthesis and PCR amplification of *CP+3'-UTR regions.* First strand cDNA synthesis was carried out in a 20 µl reaction containing 2 µg of total RNAs of infected and healthy leaf samples, 10 pmol of TSV specific reverse primers, 4 µl of 5x reaction buffer, 2 mmol/l dNTPs, 20 units of ribonuclease inhibitor (Fermentas) and 200 units of Revert Aid Moloney murine leukemia virus (MuLV) reverse transcriptase (Fermentas). The reaction was performed according to the manufacturer's instructions. The PCR was performed in 50 µl volume containing 1 µl of cDNA, 10 pmol upstream (ATG AAT AAT TTG ATC CAA RGT CCA) and 10 pmol of downstream primers (GCA TCT GGT ATA AAG GAG GCA T), Taq buffer, 2 mmol/l dNTPs, 1.75 mmol/l $MgCl_2$, 1.25 units Taq (Fermentas). The reaction mixture was heated at 94°C for 10 mins followed by 40 cycles of amplification including denaturation at 94°C for 30 secs, annealing at 58°C for 1 min, extension at 72°C for 1.5 min and final extension at 72°C for 30 mins, in a thermal cycler (Corbette Research). The amplification was confirmed by 1% agarose gel electrophoresis and the gel was extracted using DNA gel extraction kit (QIAquick).

Cloning, nucleotide sequencing and sequence comparisons. The PCR product was cloned into the pGEM-T Easy vector (Promega) and transformed into $CaCl_2$ competent *Escherichia coli* strain of DH5α cells. Recombinant plasmids were isolated by alkaline lysis (Qiagen) and the positive clones were subjected to restriction enzyme digestion with *ECO*RI (New England Biolabs). The positive clones identified by restriction enzyme analysis were sequenced in both orientations by using T7 and SP6 forward and reverse primers (MWG, Sequencing Department, Bangalore). Comparison of the nucleotide sequences of the positive clones was performed by using Blast V.2.1.2 with those data available in the GenBank (Altschul *et al*., 1990, 1997). The sequence analysis and calculation of identity and similarity were carried out using BioEdit V.5.0.9 for all the nucleotide and deduced amino acid sequences (Hall, 1999). The phylogenetic relationships were established using Mega 4.1 beta software (www.software.net) by bootstrap test with 100 replicates and 11 random odd seed number, where Parietaria mottle virus (PMoV) served as an outgroup.

Probe design and samples preparation for dot-blot hybridization. The DIG-labeled DNA probe was designed by using DIG High prime DNA labeling and detection starter kit I (Roche Applied Sciences) with a mixture of sunflower PCR product and DIG-High prime mix kept for overnight incubation. Total RNAs were extracted from sunflower, gherkin, pumpkin, marigold, and globe amaranth leaf tissues collected from fields (100 mg) by Trizol method (Sigma) according to the manufacturer's instructions. Partially purified preparations of healthy and TSV-infected sunflower leaves (100 mg/0.1ml) were prepared using PBS and the filtrate was extracted with chloroform. Crude extracts of healthy and TSV-infected leaves (100 mg/0.1ml) were prepared by maceration in cold TNE buffer with 1% 2-mercaptoethanol (10 mmol/l Tris, 1 mmol/l EDTA, 100 mmol/l NaCl) and the centrifuged supernatants were used for blotting. To determine the broad spectrum detection of TSV isolates, total RNA samples were denatured in RNA denaturation buffer (Fermentas) at 70°C for 5 mins and chilled on ice. RNA samples (1 µl) of the TSV isolates and healthy samples of corresponding isolates (blank) were spotted onto positively charged nylon membrane (Schleicher & Schuell). To determine the sensitivity limit of this assay, total RNA (112 ng to 0.00112 ng), 10^{-1} to 10^{-6} dilutions of partially purified and crude extracts were prepared in 10 x saline-sodium citrate (SSC) buffer, denatured at 70°C for 5 mins, and spotted onto positively charged nylon membrane. For tissue print hybridization the fresh leaf samples of sunflower, pumpkin, marigold, globe amaranth were cut with sharp razor blade and immediately pressed onto a nylon membrane to form a visible print.

Hybridization. The probe was denatured at 100°C for 10 mins and then chilled on ice for 5 mins. Pre-hybridization and post hybridization of dot-blots were performed following the manufacturer's instructions (Roche Applied Sciences) for nucleic acid blots. Hybridization was carried out overnight at 50°C. The membranes were washed twice for 5 mins in 2 x SSC and 0.1% SDS at 15–25°C and twice for 15 mins in 0.5 x SSC and 0.1% SDS at 65–68°C under constant agitation. The hybridized probes were treated with anti-DIG-AP Fab fragments and made visible for colorimetric detection with nitroblue tetrazolium/bromo-chloro-indolyl phosphate (NBT/BCIP).

Results and Discussion

The positively amplified RT-PCR amplicon of sunflower TSV isolate was further used in the dot-blot and tissue print hybridization assays. Positive reaction was observed with all tested TSV isolates obtained from sunflower, gherkin, pumpkin, marigold, and globe amaranth. No hybridization signal was observed with the healthy samples of their respective hosts in dot-blot (Fig. 1). Total RNA prepared from TSV-infected sunflower and its ten-fold serial dilutions were applied onto the nylon membrane. The color intensity decreased with increased RNA dilutions. The sensitivity limits were proved up to 0.00112 ng of total RNA. The intensity of the hybridization signal correlated with the virus concentration in leaf samples. In order to simplify the extraction procedure, partially purified and crude extracts from the same infected sample were also applied on the membrane and the end point dilutions were detected as 10^{-5} in partially purified, and 10^{-5} in crude extract preparations. No signals were observed in the healthy plants control (Fig. 2). In tissue print hybridization, the print made by the leaf inoculum of sunflower, pumpkin, marigold, and globe amaranth evidently reacted with the probe and was observed as a purple color print on the membrane after keeping in the dark (Fig. 3). No hybridization print was observed with the healthy leaf samples of their respective hosts.

Ilarviruses are "unpromising subjects for purification and the raising of good antisera" (Francki *et al*., 1985). Dot-blot hybridization with DIG-labeled probes was used for the plant viruses other than tospoviruses (James *et al*., 1999). The principal advantage of this assay is that it does not use any virus specific antibodies that are difficult to produce and are in short supply, especially in less developed countries or regions. The major disadvantage is a relative complicated preparation

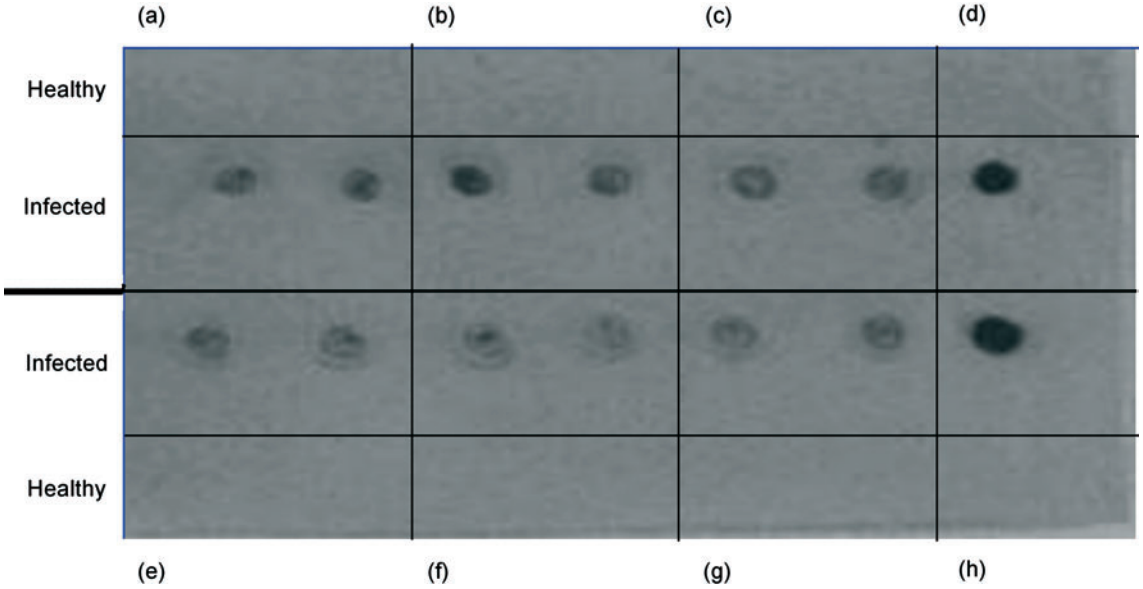

Fig. 1

Dot-blot hybridization of TSV sunflower isolate probed with total RNA of other TSV isolates in duplicates

Healthy – TSV-infected sunflower (a), gherkin (b), pumpkin (c, g), marigold (e), globe amaranth (f), positive control – PCR amplicon of sunflower (d, h).

of the nucleic acid samples. The dot-blot hybridization with the CP-based probes proposed in this study is a sensitive and wide-ranging method for the TSV isolates detection that is suitable for use in quarantine services. Here, the probe with CP region prepared from sunflower PCR amplicon was hybridized with the TSV field isolates, because of its high conservation with little variability. Hybridization technique using the nylon membrane starting with crude total RNA of 112 ng with 10 fold dilution ending with 0.00112 ng from the infected and healthy leaf samples showed decreasing sensitivity and the hybridization signal was detected up to 0.0112 ng/µl. The hybridization signals started from 1000 µg, 100 µg, 10 µg, 1 µg, and end at 0.1 µg from partially purified samples. The positive signals on the nylon membrane were observed from 1000 µg, 100 µg, 10 µg, 1µg, 0.1 µg, and the titer of the signal was 0.1 µg with crude extract preparations. No hybridization signals were detected from the healthy leaf samples. It was concluded that there was a decrease in the sensitivity levels from total nucleic acid to the partially purified and crude extract preparations.

In this work, the tissue print hybridization was another probe-based technique, which is similar to the dot-blot hy-

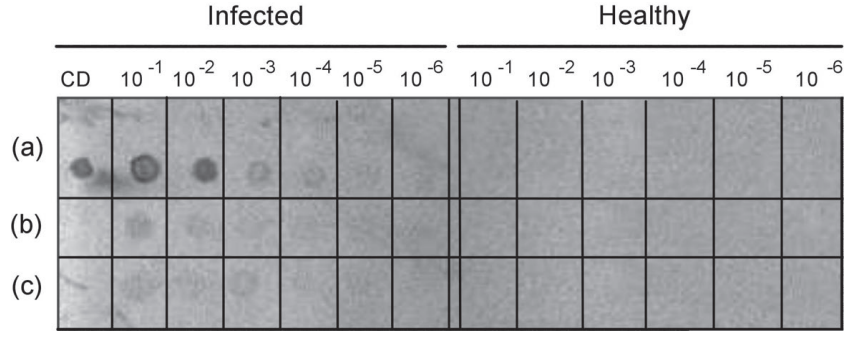

Fig. 2

Sensitivity of dot-blot hybridization with probe of TSV sunflower isolate

Total RNA extracts of infected sunflower CD (crude) and healthy sunflower – dilutions 10^{-1} to 10^{-6} ng (row a); partially purified TSV from the infected sunflower and healthy sunflower – dilutions 10^{-1} to 10^{-6} (row b); crude extract of TSV-infected sunflower and healthy sunflower – dilutions 10^{-1} to 10^{-6}(row c).

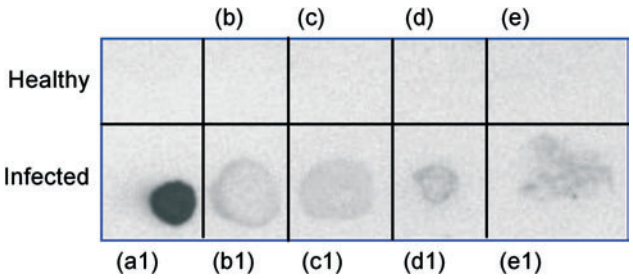

Fig. 3

Tissue print hybridization with TSV sunflower isolate

Positive control of PCR amplicon (a1), healthy – infected sunflower (b, b1), healthy – infected pumpkin (c, c1), healthy – infected marigold (d, d1), healthy – infected globe amaranth (e, e1).

bridization that does not involve a disruption of tissues for the extraction of nucleic acids. The freshly cut tissue surface was pressed directly on the membrane (Hsu and Lawson, 1991). Positive hybridization signals were detected in the infected leaf samples and no signals were detected in the healthy leaf samples. This rapid hybridization technique simplified all the extraction procedures described above.

In conclusion, the high sensitivity and reliability of molecular hybridization assays were used to understand the distribution and frequencies of virus species in the different regions and on various crops. This information increases the understanding of TSV epidemiology over larger scale surveys and proves useful in the introduction of effective control measures.

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