Techniques

Cloned cDNA Probes for the Detection of Tomato Spotted Wilt Virus

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ABSTRACT

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As a first step toward the development of a detection test for tomato spotted wilt virus (TSWV) based on nucleic acid hybridization, cDNA clones have been obtained and used to probe viral RNA from purified virions and infected plants. DNA reverse transcribed from TSWV unfractionated RNA was ligated to linearized pUC13 plasmid and cloned in *Escherichia coli*. Cloned inserts ranged from 0.13 to 1.0 kb and represented TSWV RNA2 and RNA3 sequences. Two clones—0.73 and 1.03 kb long—were labeled with ^{32}P by nick translation and used as probes in dot blot and Northern blot hybridization assays. The dot blot assay allowed detection of as little as 2 ng of RNA from purified virions as well as TSWV RNA present in total RNA extractions from 80 mg of infected leaves of *Nicotiana rustica* and in crude saps from 8 mg of the same plants. After a slight modification in the homogenization procedure, TSWV RNA was detected in crude extracts from 3 mg of infected tomato plants, by the dot blot assay. Viral RNA was also unambiguously detected in total RNA preparations from 5 g of infected tomato leaves by Northern blot hybridization.

Additional keyword: diagnosis.

Tomato spotted wilt virus (TSWV), a possible member of the Bunyaviridae family (8), has enveloped spherical particles with an average diameter of about 85 nm and a genome consisting of three ssRNA segments with approximate molecular weights of 2.7×10^6, 1.7×10^6, and 1.1×10^6 (15,17). This virus has a very wide host range and can cause serious diseases in important crops (2).

The serological detection of TSWV in infected plants exhibits some degree of cross reactivity with healthy plants (14), although Gonsalves and Trujillo succeeded in eliminating cross reactivity

by adsorbing the antiserum with healthy antigens (3).

Nucleic acid hybridization is also useful for virus detection in crop plants, reservoirs, and vectors. It has proved to be a sensitive and specific diagnostic method for several plant viruses and viroids (1,9,10,13,16). Although serological detection is at present widely used and has significantly contributed to the control of important viral diseases, nucleic acid hybridization techniques are rapidly evolving and offer several potential advantages: 1) there is no need for repeated virus purification for antibody production, as clones can be maintained for as long as one desires; 2) multiprobes can be easily constructed to check for several viruses and viroids simultaneously; 3) the probe detects the same molecular entity from which it was derived while sera may fail to detect proteins or protein domains which show antigenic variability in crude saps, fractionated extracts, and purified virions (12) or else in plants and vectors; this may well hold true for enveloped, vector transmited viruses, like TSWV; 4) and viral strains with identical proteins but differing in regulatory genome regions that may produce significant differences in pathogenicity or vector transmissibility cannot be discriminated by antisera while differentiation by appropriate probes is possible.

In addition, the increasing use of nonradioactive labeling of nucleic acids will probably make hybridization methods as safe and reproducible as the widely used immunological tests.

As a first step in our attempt to develop a method for the detection of TSWV we have obtained cDNA clones and used them to probe viral RNA from purified virions and infected plants.

MATERIALS AND METHODS

Virus purification and RNA extraction. TSWV M-316 was isolated and characterized as TSWV by O. Gracia in Mendoza, Argentina, by electron microscopy, inoculation on diagnostic species, and stability in sap (6).

The virus was purified by the procedure described by Tas et al (14) from systemically infected leaves of *Nicotiana rustica*. Batches of 100 g of leaves, either fresh or kept frozen at −70 C, were homogeneized in 0.01 M Tris-HCl, 0.01 M sodium sulfite, and 0.1% cysteine hydrochloride, pH 8.0 buffer. The homogenate was passed through cheesecloth and centrifuged for 10 min at 10,000 *g*. The pellet was reextracted by suspension in 2 volumes of resuspension buffer (0.01 M Tris-HCl, 0.01 M sodium sulfite, 0.01 M glycine, and 0.1% cysteine, pH 7.9) for 1 hr and clarified by centrifugation at 10,000 *g* for 30 min.

Supernatants were combined and centrifuged for 30 min at 25,000 rpm in a Spinco type #30 rotor to concentrate the virus. The pellets were resuspended in 20 ml of resuspension buffer and incubated with 0.3 ml of antiserum against healthy leaf material (titer 1:32) for 1 hr. The precipitate was removed by low-speed centrifugation and the supernatant was layered on 3–30% (w/v) sucrose gradient in resuspension buffer and centrifuged at 17,000 rpm for 80 min in a Spinco SW25 rotor. The virus bands were collected and concentrated by centrifugation as indicated above. The presence of TSWV in the selected gradient bands was confirmed by infectivity assay on detached leaves of *Petunia hybrida* cultivar Pink Beauty (11). The temperature was kept at 4 C throughout the purification procedure.

RNA was purified from the selected gradient bands as described by van den Hurk et al (15). Virions were disrupted in 0.01 M Tris-HCl, 0.1 M NaCl, 1 mM Na_2EDTA, 2% SDS, pH 7.6 buffer, stirred for 10 min at room temperature. After repeated phenol extractions, the aqueous phase was adjusted to 0.3 M sodium acetate (pH 5.5) and the nucleic acid was precipitated with 2.5 volumes of ethanol at −20 C overnight. After centrifugation the RNA was resuspended in water and tested for purity in denaturing agarose gels containing methyl-mercuric hydroxide (7).

cDNA synthesis and molecular cloning. Complementary DNA synthesis was initiated on the TSWV RNA (mixture of the three segments) with random primers (prepared according to Maniatis et al [7]), AMV reverse transcriptase (Promega Biotec, Madison, WI), and DNA polymerase I (New England Biolabs, Beverly, MA) using procedures described by Gubler and Hoffman (4). ^{32}PdATP (New England Nuclear, Boston, MA; or prepared in this laboratory) was used for labeling.

The products of first and second strand syntheses were electrophoresed on 1 and 2% alkaline agarose gels, as were molecular size standards. The synthesis was followed by blunt end repair of the cDNAs with T4 DNA polymerase (Pharmacia-PL, Piscataway, NJ). Excess labeled dATP was eliminated by ethanol precipitation of the cDNAs.

An *Eco*RI cohesive termini addition was performed on the cDNAs so they could be inserted in the *Eco*RI site of the pUC13 plasmid (18,20). This step required *Eco*RI methylation (New England Biolabs, Beverly, MA) followed by addition of phosphorylated *Eco*RI linkers (New England Biolabs, Beverly, MA) with T4 DNA ligase (New England Biolabs, Beverly, MA). Sepharose 4B chromatography was employed to remove the excess *Eco*RI linkers and size fractionate the ds cDNAs. Aliquots of the radioactive fractions were electrophoresed on agarose gels as were labeled standards. The size-fractionated *Eco*RI linkered cDNAs were ligated with *Eco*RI-cut, dephosphorylated pUC13 plasmid cut with *Eco*RI (Pharmacia-PL, Piscataway, NJ).

The ligation mixtures were used to transform competent *E. coli* TG1 and DH5α (5). Recombinant clones with β-galactosidase negative and ampicillin resistant phenotypes were selected. The presence and size of inserts was determined by plasmid isolation followed by *Eco*RI digestion and electrophoresis on 1% agarose gel. Cloned inserts corresponding to viral sequences were assigned to one of the three TSWV RNA segments by hybridization in Northern blots (19).

Electrophoresis and blotting of RNA in agarose-methylmercuric hydroxide gels. Agarose/CH_3HgOH gels were prepared as described by Maniatis et al (7). RNA and molecular size marker samples were denatured with methylmercuric hydroxide at a final concentration of 12.5 mM in 50 mM boric acid, 5 mM sodium borate, 10 mM sodium sulfate, pH 8.2 buffer. After electrophoresis, gels were renatured and stained in 14 mM β-mercaptoethanol and 1 μg/ml of ethidium bromide, respectively, and then transferred onto Gene screen membrane (New England Nuclear, Boston, MA) for hybridization with cDNA probes.

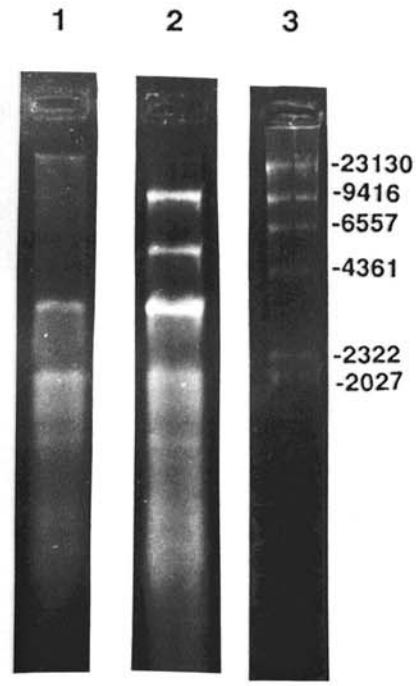

Fig. 1. Tomato spotted wilt virus (TSWV)-RNA fragments in a 1% agarose-methylmercuric hydroxide denaturing gel electrophoresis stained with ethidium bromide. RNA from healthy (1) and TSWV M316 infected (2) *Nicotiana rustica* leaves subjected to the same virus purification and RNA extraction procedures (see Materials and Methods). (3) *Hin*dIII digested lambda DNA. Molecular sizes (kb) are indicated on the right margin.

Radioactive labeling and hybridization conditions. TSWV cDNA clones were labeled by nick translation (7) with α^{32}PdATP or α^{32}PdCTP. Unincorporated nucleotides were removed either by ethanol/ammonium acetate precipitation or by passage through a 1-ml Sephadex G-25 column.

Prehybridization was carried out overnight in sealed polyethylene bags at 42 C in 50% formamide (Sigma Chemical Company, St. Louis, MO), 2× Denhardt's solution (1× Denhardt is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin, all from Sigma Chemical Company), 5× SSC (1× SSC is 0.15 M NaCl, 15 mM sodium citrate, pH 7), 1% SDS, and 200 μg/ml of denatured yeast RNA (Gallard-Schlesinger Chemical Mfg. Corp., Carle Place, NY). For hybridization the solution was changed to 50% formamide, 1× Denhardt's solution, 5× SSC, 1% SDS, 0.2 mg/ml tRNA (yeast), and denatured labeled probe and carried out at 42 C for 24–48 hr. Membranes were then washed twice, 5 min each, in 2× SSC buffer at room temperature; twice, 15 min each, in 2× SSC plus 1% SDS at 65 C and twice, 15 min each, in 0.1× SSC at room temperature. Autoradiography of the membranes was at −80 C with intensifying screen for at least a day (Kodak X-OMAT-K-Film).

Sample preparation for Northern and dot blotting. Three types of samples of *N. rustica* were dot spotted onto membranes: 1) RNA from purified virions; 2) total RNA extracted from infected and uninfected leaves; leaves were ground in liquid nitrogen and the RNA was extracted with 2 ml of 2× SSC (containing 1% SDS and 1 mg of bentonite) and 2.5 ml of phenol per gram of tissue; RNA was concentrated from the aqueous phase by ethanol/sodium acetate precipitation; and 3) crude extracts of infected and uninfected plants, simply squeezed from leaves and used immediately.

Samples for *L. esculentum* were: 1) RNA isolated from leaves, as for *N. rustica*; 2) crude extracts obtained by homogenizing leaves in mortar with 8 M guanidinium isothiocyanate, 20 mM MOPS (3-[*N*-morpholino]propanesulfonic acid), 20 mM EDTA, 50 mM β-mercaptoethanol, pH 7 (1 ml/0.5 g of leaves), incubated at 0 C for 1 hr, sedimented for 10 min in microfuge and diluted to 2 M guanidinium isothiocynate with 20 mM MOPS, 20 mM EDTA, pH 7. Aliquots of the different samples were either electrophoresed in agarose gels and blotted onto membranes or directly spotted onto membranes previously treated with 20× SSC (19); blots were dried and baked at 80 C for 2 hr.

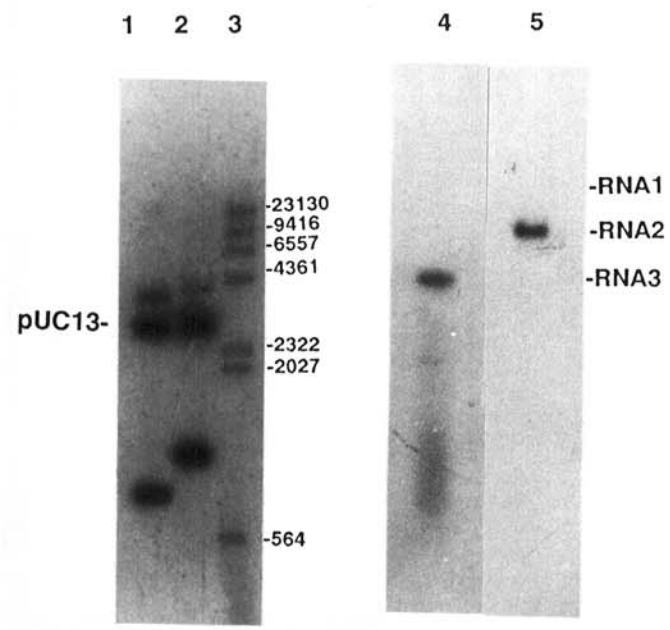

Fig. 2. Size determination and characterization of tomato spotted wilt virus (TSWV) inserts in clones $_2$TSWV1 and $_3$TSWV14. **A,** Autoradiograph of a 1% agarose gel loaded with: (1) $_2$TSWV1 clone; (2) $_3$TSWV14 clone; (3) ^{32}P-labeled *Hin*dIII digest of lambda DNA. The clones were digested with *Eco*RI and labeled with ^{32}P by fill-in with ^{32}PdATP and DNA polymerase I (Klenow fragment). Sizes (kb) of markers and linearized pUC13 plasmid are indicated. **B,** Northern blots of TSWV RNAs probed with: (4) $_3$TSWV14 clone and (5) $_2$TSWV1 clone labeled with ^{32}PdATP.

RESULTS

cDNA synthesis and cloning. RNA preparations from plants of *N. rustica* infected with TSWV were analyzed by denaturing agarose gel electrophoresis, as shown in Figure 1. The high molecular weight bands in lane 2 are 9,000, 4,900, and 3,100 nucleotides in length, in agreement with the sizes reported by van den Hurk et al (15), Verkleij et al (17), and Milne and Francki (8) for the three TSWV genomic RNAs. Some contamination comes from ribosomal and other lower molecular weight RNAs of plant origin (Fig. 1, lane 1). This TSWV-enriched unfractionated RNA preparation was used as template for random primed reverse transcription, rendering a population of ds-cDNA molecules up to 2,000 bp long. This population was size fractionated into molecules shorter than 500 bp and those ranging between 500 and 2,000 bp in length. Ligation to the *Eco*RI site of linearized pUC13 dephosphorylated plasmid was performed separately with both fractions. *E. coli* TG1 competent cells were transformed with recombinant plasmids bearing short cDNA inserts while *E. coli* DH5α was used for transformation with those carrying inserts longer than 500 bp. Three clones containing inserts of 130, 140, and 185 bp and another three clones containing inserts of 780, 785, and 1,030 bp corresponding to the TSWV RNA3 were isolated (Fig. 2A). One clone containing an insert of 730 bp corresponding to the TSWV RNA2 was also identified. The assignment of the cloned sequences to any of the viral RNAs was done by using each of them as a probe on Northern blots of an RNA extracted from sucrose gradient purified virions. Figure 2B shows the results of such an analysis for two clones containing sequences of TSWV RNA2 and RNA3, respectively. The viral nature of these clones was confirmed by their inability to detect (by Northern blot hybridization) complementary sequences in RNA obtained from healthy plants of *N. rustica* (not shown) and by negative results in dot blot hybridization with materials obtained from uninfected plants of *N. rustica*, as will be shown later; they also failed to detect—by Northern blot hybridization—complementary sequences in RNA from healthy tomato plants.

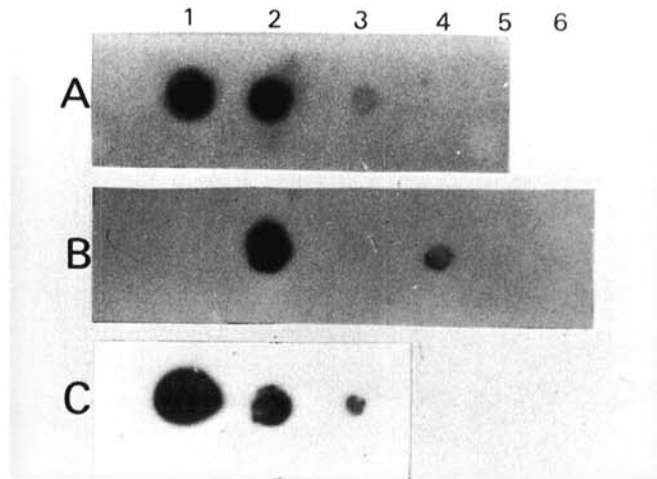

Fig. 3. Detection of tomato spotted wilt virus (TSWV)-RNA by dot blot hybridization. **A,** 200 (1), 20 (2), and 2 ng (3) of RNA extracted from sucrose gradient purified virus, hybridized with ^{32}P-$_3$TSWV14 probe; 200 ng (4) of RNA extracted from equivalent zones of a sucrose gradient layered with material from healthy *Nicotiana rustica*; **B,** RNA extracted from 7.5 (1, 2), 0.4 (3, 4), and 0.08 g (5, 6) of uninfected (1, 3, 5) and TSWV-infected (2, 4, 6) *N. rustica* leaves, hybridized with ^{32}P-($_3$TSWV14 + $_2$TSWV1) probe; (C) crude sap samples from 400 (1), 80 (2), and 8 mg (3) of infected *N. rustica* leaves, hybridized with ^{32}P-($_3$TSWV14 + $_2$TSWV1) probe.

Detection of TSWV in *N. rustica* and *L. esculentum* by dot blot and Northern blot hybridization. The availability of TSWV cloned sequences made it possible to start working out a procedure based on nucleic acid hybridization for detection of the virus in diseased plants. As a first step toward this purpose, clones were used to probe viral RNA in samples of increasing complexity, ranging from RNA extracted from purified virus to crude plant sap. The following results were obtained:

1) As little as 2 ng of RNA from sucrose gradient purified virions could be detected with any of the selected clones labeled by nick translation in a dot blot assay (Fig. 3A); no background signal was observed with 200 ng of RNA from equivalent fractions of a sucrose gradient loaded with healthy material.

2) As shown in Figure 3B, TSWV RNA was clearly detected in dotted RNA extracted from 0.4 g of TSWV infected leaves of *N. rustica* and a weak signal was visible even with a sample from 0.08 g; no background signal was seen in dotted healthy preparations from up to 7.5 g of leaves.

Fig. 4. Tomato spotted wilt virus (TSWV) detection in *Lycopersicon esculentum* by Northern blot hybridization. Electrophoresed and blotted RNA extracts from 5 g of leaves of infected (1) and healthy (2) *L. esculentum* (cv. Roma) and of infected (3) and healthy (4) *L. esculentum* (cv. Platense) were hybridized with ^{32}P-($_3$TSWV14 + $_2$TSWV1) probe.

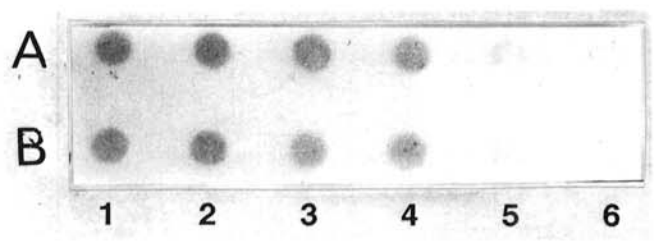

Fig. 5. Tomato spotted wilt virus (TSWV) detection in *Lycopersicon esculentum* by dot blot hybridization. Crude sap samples obtained by the guanidinium isothiocyanate procedure from 6 (A) and 3 mg (B) of infected (1, 2) and uninfected (5, 6) *L. esculentum* (cv. Platense) and from infected (3, 4) leaves of a comercial tomato hybrid.

3) TSWV sequences were detected in dotted crude sap from as little as 8 mg of infected leaves of *N. rustica* (Fig. 3C). No background signal from healthy material was observed.

4) Strong hybridization signals were obtained for dotted total RNA and crude sap from tomato leaves prepared by the procedures used for samples of *N. rustica*, so that discrimination between uninfected and infected tomato plants was ambiguous. This background was never observed with material from *N. rustica*. To overcome this difficulty a different method was employed to obtain tomato crude saps.

5) TSWV sequences were unambiguously detected in total RNA preparations from 5 g of tomato leaves electrophoresed in agarose gel, blotted, and probed with viral clones. Figure 4 shows the results obtained by this Northern blotting hybridization assay for two cultivars of *L. esculentum*. Samples from either cultivar infected with TSWV hybridized strongly to ($_3$TSWV14 + $_2$TSWV1) probe (Fig. 4, lanes 1 and 3), while uninfected controls produced hardly visible signals (Fig. 4, lanes 2 and 4).

6) TSWV sequences were also detected in crude tomato extracts by dot blot hybridization provided that the extracts were prepared by homogenization in MOPS buffer containing 8 M guanidinium isothiocyanate (Fig. 5). Samples from 3 mg of infected leaves of two tomato cultivars gave clear hybridization signals with very faint background for the healthy counterparts.

DISCUSSION

Clones representing sequences of tomato spotted wilt virus genomic RNAs 2 and 3 have been obtained by random primed cDNA synthesis and subsequent cloning in pUC13 plasmid. Two of these clones have been used to detect the presence of viral RNAs in infected plants. The results presented in Figures 3, 4, and 5 showed that cloned TSWV cDNA can be used in hybridization assays on membranes to detect TSWV in plants. As little as 2 ng of viral RNA could be detected by dot blot assay (Fig. 3A). Detection of the virus in *N. rustica* infected leaves was at least 10 times more sensitive for crude sap extracts than for total RNA extractions in the same assay (Fig. 3B and C). This difference may be due to significant losses of viral RNA during isolation and purification from infected tissue. The dot blot hybridization assay—performed under identical conditions—was of no value for tomato samples. Both, healthy total RNA and crude saps bound labeled probe. We found no similar behavior with samples of *N. rustica* processed in the same way. Our assumption is that tomato leaves contain somewhat different or more abundant polysaccharides which unspecifically bind DNA. Whatever the real cause, the problem was overcome in two ways: 1) electrophoresis of tomato total RNA preparations eliminated the unspecific binding of the probe (Fig. 4); the smeared image in lanes 1 and 3 of Figure 4 is probably due to degradation of RNA during its isolation and purification and/or to adsorption of RNA to mobility-heterogeneous components (i.e., polysaccharides); 2) crude tomato extracts obtained by homogenization of the tissue with MOPS buffer containing 8 M guanidinium isothioyanate gave unambiguous results when spotted onto membranes and subsequently probed with TSWV clones.

Although detection by Northern blotting was quite convincing, it is more laborious, expensive, and requires more equipment than the dot blot method employing crude sap. In addition, even when the Northern blotting method is routinely employed in research laboratories its use would be difficult in low complexity units—as may be the case in agronomical or horticultural experimental stations. The results in Figure 5 show that a simple and fast dot blot procedure could be used for TSWV detection in tomato. Attempts will be made to apply this assay to other crop plants, weeds, and vectors.

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