Dot Blot Detection of Tomato Spotted Wilt Virus RNA in Plant and Thrips Tissues by cDNA Clones

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ABSTRACT

Rice, D. J., German, T. L., Mau, R. F. L., and Fujimoto, F. M. 1990. Dot blot detection of tomato spotted wilt virus RNA in plant and thrips tissues by cDNA clones. Plant Dis. 74:274-276.

A cDNA library was constructed that contained sequences complementary to tomato spotted wilt virus (TSWV) RNA. Two plasmids, pTSWV7 and pTSWV80, containing inserts of 460 and 870 bp, respectively, were characterized for specificity to TSWV RNA and were selected to develop a diagnostic dot blot assay. ^{32}P-labeled hybridization probes from these clones can detect TSWV in as little as 16 ng of total RNA from tobacco, 80 ng from tomato and lettuce, and 400 ng from chrysanthemum and pepper. TSWV is detected by these hybridization probes in dot blots of nucleic acids extracted by simple methods using 40–50 mg of leaf tissue and in all the major developmental stages of individual thrips.

Tomato spotted wilt virus (TSWV) has a worldwide distribution (1,9) and an extremely broad host range that includes 192 dicotyledonous species in 33 families and eight monocotyledonous species in five families (1,3,5,13). It is a serious problem in temperate and subtropical regions throughout the world, causing economic losses in important vegetable, legume, and ornamental crops. TSWV has a unique relationship with its only known vectors, several species of thrips. The virus is acquired during feeding of the larval stages and is transmitted by adult insects throughout their life span (18). The ubiquitous occurrence of vectors, susceptible crop plants, and reservoir species makes the control of epidemics caused by TSWV very difficult. Rapid, accurate, and inexpensive diagnostic procedures are an important feature of any management strategy for viruses. The only such assay currently available for TSWV is the enzyme-linked immunosorbent assay (ELISA) described by Gonsalves and Trujillo (11). In a recent review article describing a multidisciplinary approach to control of TSWV (2), Cho et al reported a nucleic acid dot blot procedure for detecting TSWV. In this paper, we report the development and implementation of the dot blot procedure and describe how it can be used to complement and extend diagnostic data obtained with ELISA or indicator plants. A preliminary report of this work has been published (10).

Journal Series Paper 3355 of the Hawaii Institute of Tropical Agriculture and Human Resources, Honolulu.

Accepted for publication 27 September 1989 (submitted for electronic processing).

MATERIALS AND METHODS

Virus isolate and identification. An isolate of TSWV from lettuce (*Lactuca sativa* L.) grown in the Kula district on Maui, Hawaii, was provided by John Cho. The isolate was serially maintained in the systemic host *Nicotiana benthamiana* Domin, on the local lesion host *N. glutinosa* L., and in the maintenance host *Emilia sonchifolia* (L.) DC. TSWV symptom expression was checked on these plants and on *Petunia* × *hybrida* Hort. Vilm. TSWV was detected by the double-antibody sandwich (DAS) ELISA, as previously reported (2).

Thrips. Healthy laboratory *Frankliniella occidentalis* (Pergande) were reared on bean pods (*Phaseolus vulgaris* L. 'Green Crop') and handled as previously described (4). Sap-inoculated burdock (*Arctium lappa* L.) was used as the acquisition host for thrips when TSWV symptoms (veinclearing) began to appear. Leaves without signs of TSWV were removed. Viruliferous thrips were produced by transferring second instar larvae from healthy bean pods to a caged acquisition host and allowing continuous access feeding—2 days for second instar larvae, 6 days for prepupae, and 8 days for adults. Viruliferous adult thrips were transferred to healthy bean pods and allowed to feed for 7–10 days before harvesting. Thrips transfers were facilitated by lightly anesthetizing thrips with CO_2.

Putative ribonucleoprotein (RNP) purification. We followed the initial TSWV purification steps described by Gonsalves and Trujillo (11), except that the supernatant from the first differential centrifugation, which is usually discarded, was used for RNP isolation. The RNP in buffer A (0.1 M potassium phosphate, 0.01 M sodium sulfite, 0.01 M cysteine hydrochloride, pH 7.2) was pelleted in polycarbonate bottles for 70 min at 204,000 *g* (maximum) in a Spinco Type 60 Ti rotor. A paint brush was used to resuspend the pellet in a volume of buffer B (0.01 M sodium sulfite, 0.01 M cysteine hydrochloride, pH 7.2) numerically equal (in milliliters) to the starting tissue weight (in grams). The suspension was gently agitated on a shaker for at least 2 hr at 4 C and then clarified by centrifugation at 8,000 *g* for 15 min. The supernatant was applied to linear 10–40% sucrose-density gradients in 0.01 M sodium sulfite at 150,000 *g* (maximum) for 190 min in a Spinco SW-28 rotor. RNP in sucrose gradient fractions was diluted at least 4:1 in buffer A, then pelleted at 204,000 *g* for 2.5 hr. RNP was resuspended either in buffer B for infectivity assays or in another appropriate buffer for further analysis.

RNA isolation. RNA was isolated from TSWV RNP pellets and thrips by the acid guanidinium thiocyanate-phenol-chloroform (AGPC) extraction method described by Chomczynski and Sacchi (6). Thrips were frozen in liquid nitrogen and homogenized in 0.1 ml of extraction buffer (4.2 M guanidinium thiocyanate, 0.025 M sodium citrate [pH 7.0], 0.5% sodium sarcosyl, and 0.1 M 2-mercaptoethanol) in a microcentrifuge tube fitted with a plastic pestle. RNA was quantified from its UV spectrum assuming that 1 A_{260} unit equals 40 μg/ml of single-stranded RNA. Absorbance ratios (260/280) for AGPC-isolated RNA were usually 2.2.

Nucleic acids for leaf disk dot blot assays were extracted from 40–50 mg of leaf tissue (two to four leaf circles obtained with a No. 4 cork borer) by homogenizing the tissue for 30 sec in 125 μl of extraction buffer (0.2 M K_2HPO_4, 10 mM sodium diethyldithiocarbamate, and 0.1% [w/v] Triton X-100) (15) in a 1.6-ml microfuge tube with a plastic pestle. Then, 125 μl of buffer-equilibrated phenol (14) was added and the tubes were vortexed vigorously for 3–5 sec. A mixture of chloroform and isoamyl alcohol (24:1, 125 μl) was added, and the vortexing was repeated. The phases were separated by microcentrifugation for 1

min, and 75 μl of the clear upper phase was transferred to a new Eppendorf tube containing 175 μl of 6.15 M formaldehyde in 10× SSC (14). Samples were stored on ice until all had been processed, then heated at 65 C for 15 min, and finally cooled on ice until blotted.

Nucleic acid for total RNA blots was extracted from 0.2–0.5 g of leaf tissue that was frozen in liquid nitrogen and ground to a powder with a mortar and pestle. Then, 3 ml of extraction buffer (0.1 M TRIS-HCl, 0.001 M EDTA, 0.15 M NaCl, and 0.1% sodium sarcosyl, pH 8.0) was added to the leaf powder, mixed, and allowed to thaw before transfer to a 30-ml centrifuge tube. The mixture was phenol-chloroform extracted and the nucleic acids were ethanol precipitated (14). The resulting pellet was washed with 70% ethanol, dried under vacuum, and dissolved in 0.05 M TRIS-HCl, pH 8.0. Magnesium chloride was added to a final concentration of 5 mM, and the solution was incubated at 37 C for 30 min with 50 units per milliliter of RNase-free DNase. The solution was phenol-chloroform extracted and RNA was ethanol precipitated.

cDNA clones to TSWV RNA. The cloning, screening, and characterization of a library of cDNA clones to TSWV will be published elsewhere by Rice and German. A brief description of the cloning and screening of two clones, pTSWV7 and pTSWV80, follows.

The RNP purification protocol was followed for TSWV-infected leaves of *N. benthamiana*. Template RNA was extracted from pooled regions of sucrose gradients that tested positive for TSWV by ELISA and where TSWV infectivity was found. The protocol for cDNA synthesis by Polites and Marotti (16) was followed to construct a cDNA library in pBR322. First-strand synthesis was initiated with random primers (14). Blunt-ended, double-stranded cDNA was deoxycytidine-tailed using terminal deoxynucleotidyl transferase in a potassium cacodylate buffer with freshly prepared $MnCl_2$ and annealed directly to a commercial Pst I cut, oligo deoxyguanosine-tailed pBR322 (16). We established cDNA libraries with the hybrid plasmids in both RR-1 and HB101 *Escherichia coli* by following the transformation protocol of Hanahan (12). Putative TSWV clones were screened by differential colony hybridization. Differential ^{32}P-cDNA hybridization probes were reverse-transcribed using the same type of template RNA preparation used for library construction and from its corresponding healthy control RNA. Colonies were selected that gave a positive signal with the probe derived from RNA containing TSWV sequences but not with the probe derived from its corresponding control. Selected plasmids were digested with Pst I to release the cloned insert DNA from the plasmid, and the digest was analyzed by agarose-gel electrophoresis. Two clones, plasmids pTSWV7 and pTSWV80, which contained inserts of 460 and 870 bp, respectively, were further screened by dot blot hybridization for specificity to TSWV RNA. Neither insert hybridized to blots derived from healthy or mock-inoculated plants or plants infected with tobacco mosaic virus. Both inserts, however, hybridized to the RNA template used to construct and screen the library and to any dot blot derived from TSWV-infected plants. Southern blot analysis demonstrated that inserts 7 and 80 each represent unique sequences of the TSWV genome. Northern blot analysis revealed that inserts 7 and 80 hybridize to the tripartite RNA genome of TSWV.

Preparation of probes for dot blot experiments. Plasmids pTSWV7 and pTSWV80 were purified by the procedure of Sadhu and Gedamu (17), except that bacterial selection was done with 12.5 μg/ml of tetracycline and plasmid amplification was done with 170 μg/ml of chloramphenicol. Restriction enzyme digests were performed according to Maniatis et al (14). Then, 6.7 μg of the Pst I digest fragments was separated by electrophoresis on four adjacent 6.0 × 1.0 mm lanes of a minisubmarine horizontal gel apparatus using 4% NuSieve GTG agarose gels in 40 mM TRIS-acetate and 1 mM EDTA buffer (14). Electrophoresis was performed at 8.4 V/cm of gel until the bromophenol blue marker dye had migrated about 7 cm. The gel was stained for 30 min in 1 L of 0.2 μg/ml of ethidium bromide in water. The desired band was excised and radiolabeled with ^{32}P in the melted gel slice using the random primer extension technique of Feinberg and Vogelstein (7,8). The hybridization probe was used without further purification.

Blotting and hybridization protocols were selected from Wahl et al (19) according to the nucleic acid and type of membrane material. The prehybridization solution was 50% (v/v) formamide, 5× SSPE, 5× Denhardt's, 0.2% (w/v) SDS, 200 μg/ml of yeast tRNA, and 200 μg/ml of phenol-extracted, low molecular weight salmon sperm DNA. Hybridization buffer was prehybridization buffer containing probe. Hybridized blots were washed with a final stringency of 0.1× SSPE/0.2% SDS at 60 C for 1 hr.

RESULTS

Total RNA was extracted from a variety of TSWV-infected plant leaves and their corresponding healthy controls. The RNA was quantified, and fivefold serial dilutions of each sample were blotted onto nitrocellulose. Blots were hybridized simultaneously with ^{32}P-labeled probes from Pst I inserts of plasmids pTSWV7 and pTSWV80. The resulting autoradiogram is shown in Figure 1. This dot blot shows complete specificity for TSWV. It detects TSWV sequences in as little as 16 ng of total RNA in tobacco, whereas it requires 80 ng of RNA in tomato and lettuce and 400 ng of RNA in chrysanthemum and pepper.

Figure 2 shows a similar dot blot detection of TSWV by the same probes when total nucleic acid is extracted and blotted from 40–50 mg of leaf disks or when RNA is extracted by AGPC and blotted from five pooled adult thrips (*F. occidentalis*). TSWV can be detected in as little as 0.3 mg of infected *N. benthamiana* leaves, and the blot shows the same sensitivity pattern seen in Figure 1 for infected leaf tissue. Since five thrips were pooled and fivefold serial dilutions were performed on this blot, the hybridization assay appears to be sensitive enough to detect less than an individual infected thrip.

In a single blind test, groups of larval, prepupal, and adult thrips were allowed

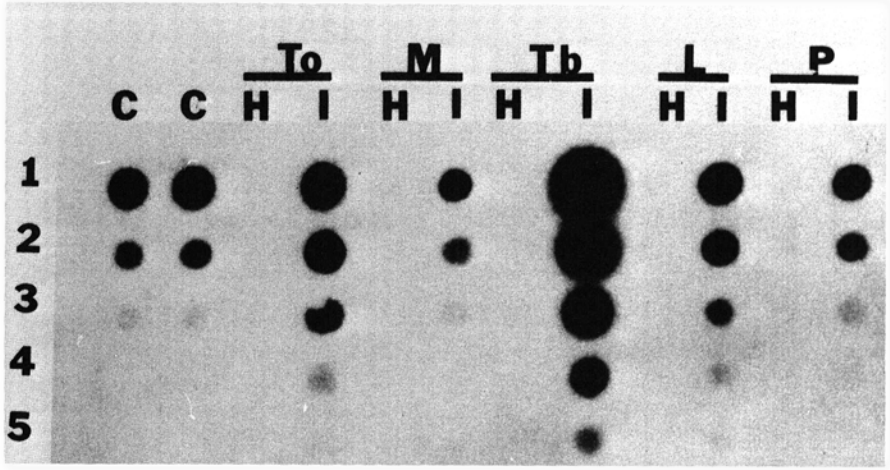

Fig. 1. Dot blot hybridization starting with equal masses of RNA. Serial fivefold dilutions of total RNA (10 μg, 2 μg, 400 ng, 80 ng, and 16 ng) were spotted in rows 1–5. Samples are healthy (H) or infected (I) tomato (To), chrysanthemum (M), tobacco (Tb), lettuce (L), and pepper (P). Columns marked C are control fivefold dilutions (3.2 ng, 640 pg, 130 pg, 26 pg, and 5 pg) of pBR322 containing insert used to make the probes.

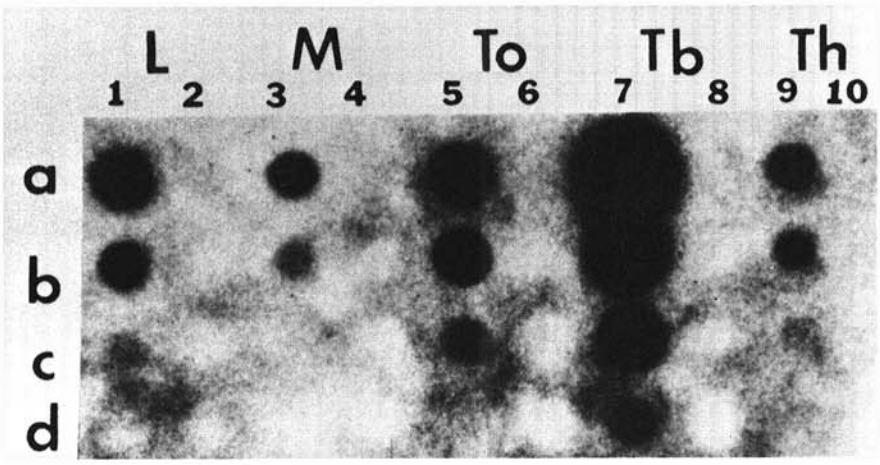

Fig. 2. Dot blot hybridization starting with equal masses (40–50 mg) of plant leaf disk tissue or with five thrips. Rows a–d are fivefold serial dilutions of starting material. Letters over columns indicate lettuce (L), chrysanthemum (M), tomato (To), tobacco (Tb), and thrips (Th). Infected samples are in odd-numbered lanes and uninfected samples, in even-numbered lanes.

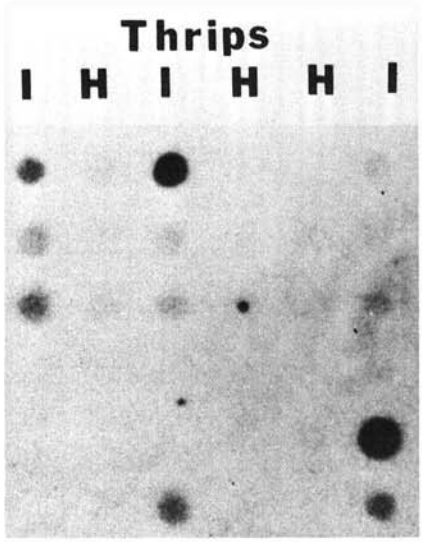

Fig. 3. RNA dot blot detecting individual TSWV-infected thrips at three major developmental stages in life cycle of thrips. Larval thrips were fed on TSWV-infected burdock leaves (I) or healthy bean pods (H) and harvested at adult, prepupal, and second instar larval stages. RNA in pairs of infected and healthy columns (right to left): individual second instar larvae, individual prepupae, individual adults.

access to either healthy or TSWV-infected plants and individually blotted using six replicates of each developmental stage. Some of the individual thrips from each developmental stage that were fed from TSWV-infected burdock tested positive for the presence of TSWV, whereas all thrips fed on healthy control plants tested negative (Fig. 3). Individual thrips showed great variability in the quantity of TSWV detected.

DISCUSSION

cDNA clones constituting 1,330 bp of the TSWV genome, radiolabeled with ^{32}P, hybridize specifically to a wide variety of plant and thrips tissues infected with TSWV. Two independent cloned inserts were chosen to increase the sensitivity of the assay and broaden the range of TSWV strains that can be detected. Clearly, enough TSWV RNA is present in 40–50 mg of plant leaves to unequivocally test plants for TSWV. The sensitivity of this assay was equivalent to the double-antibody sandwich ELISA (2). The assay is especially useful for detecting low titers of TSWV in hosts such as pepper that give high background readings with ELISA but not with the hybridization dot blot test (Fig. 1).

TSWV could be detected in individual thrips allowed continuous access feeding on TSWV-infected plants during all stages of the thrips life cycle. As in the report of the ELISA assay that detects TSWV in individual thrips larvae and adults (4), only a percentage of the thrips in our experiment tested positive for TSWV. In the thrips-rearing procedure presented here, about 40–50% of second instar larvae tested positive to varying degrees for TSWV by ELISA. The data presented in Figure 3 appear to correlate well with that result, suggesting that variability in sensitivity seen for individual thrips and different types of plant tissue is due to varying titers of TSWV.

Our results show that nucleic acid dot blot detection of TSWV will extend our capability for diagnosing viral infections in a variety of circumstances. These tools will make it possible to develop the epidemiological data needed for management practices that will address the complex problems associated with this disease. This library of clones will also facilitate the elucidation of the genomic structure, organization, and replication strategy of TSWV and possibly clarify its taxonomic position.

ACKNOWLEDGMENTS

We thank Sam Sun for critical reading of the manuscript and for helpful suggestions throughout the course of this research, and Wayne Borth for proofreading the manuscript. We also thank John Cho and Diana Custer for providing virus isolates and performing ELISA studies, and Randall Hamasaki and Westley Otani for providing thrips. This study was supported by grant 86-CRSR-2-2829 from the USDA/CSRS Special Grants Program in Tropical and Subtropical Agriculture.

LITERATURE CITED

1. Best, R. J. 1968. Tomato spotted wilt virus. Pages 65-146 in: Advances in Virus Research. Vol. 13. K. M. Smith and M. A. Lauffer, eds. Academic Press, New York.
2. Cho, J. J., Mau, R. F. L., German, T. L., Hartmann, R. W., Yudin, L. S., Gonsalves, D., and Provvidenti, R. 1989. A multidisciplinary approach to management of tomato spotted wilt virus in Hawaii. Plant Dis. 73:375-383.
3. Cho, J. J., Mau, R. F. L., Gonsalves, D., and Mitchell, W. C. 1986. Reservoir weed hosts of tomato spotted wilt virus. Plant Dis. 70:1014-1017.
4. Cho, J. J., Mau, R. F. L., Hamasaki, R. T., and Gonsalves, D. 1988. Detection of tomato spotted wilt virus in individual thrips by enzyme-linked immunosorbent assay. Phytopathology 78:1348-1352.
5. Cho, J. J., Mau, R. F. L., Mitchell, W. C., Gonsalves, D., and Yudin, L. S. 1987. Host list of plants susceptible to tomato spotted wilt virus. Res. Ext. Ser. 078 Hawaii Inst. Trop. Agric. Hum. Resour. 10 pp.
6. Chomczynski, P., and Sacchi, N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162:156-159.
7. Feinberg, A. P., and Vogelstein, B. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. 132:6-13.
8. Feinberg, A. P., and Vogelstein, B. 1984. Addendum: A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. 137:266-267.
9. Francki, R. I. B., and Hatta, T. 1981. Tomato spotted wilt virus. Pages 491-512 in: Handbook of Plant Virus Infections and Comparative Diagnosis. E. Kurstak, ed. Elsevier, New York.
10. German, T. L., and Rice, D. 1988. Dot blot detection of tomato spotted wilt virus using cloned cDNA probes. (Abstr.) Phytopathology 78:1599.
11. Gonsalves, D., and Trujillo, E. E. 1986. Tomato spotted wilt virus in papaya and detection of the virus by ELISA. Plant Dis. 70:501-506.
12. Hanahan, D. 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. 166:557-580.
13. Iwaki, M., Honda, Y., Hanada, K., Tochihara, H., Yonaha, T., Hokama, K., and Yokoyama, T. 1984. Silver mottle disease of watermelon caused by tomato spotted wilt virus. Plant Dis. 68:1006-1008.
14. Maniatis, T., Fritsch, E. F., and Sambrook, J. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
15. Owens, R. A., and Diener, T. O. 1981. Sensitive and rapid diagnosis of potato spindle tuber viroid disease by nucleic acid hybridization. Science 213:670-672.
16. Polites, H. G., and Marotti, K. R. 1986. A stepwise protocol for cDNA synthesis. BioTechniques 4:514-520.
17. Sadhu, C., and Gedamu, L. 1988. A procedure for the preparation of RNA-free plasmid DNA. BioTechniques 6:20-21.
18. Sakimura, K. 1962. The present status of thrips-borne viruses. Pages 33-40 in: Biological Transmission of Disease Agents. K. Maramorosch, ed. Academic Press, New York.
19. Wahl, G. M., Ong, E., Meinkoth, J., Franco, R., and Barinago, M. 1987. Transfer and immobilization of nucleic acids to Schleicher and Schuell solid supports. Publ. 700. Schleicher and Schuell, Keene, NH. 34 pp.