VIRUS AND VIROID DETECTION

Indexing of Citrus Tristeza Virus Double-Stranded RNA in Field Trees

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ABSTRACT. Samples of bark and buds were collected from commercial groves and experimental plantings of sweet orange, and indexed by the Mexican lime test and the dsRNA test. Infected trees (approximately 50) were detected equally well by each index. Three dsRNAs (MW = 13.3 x 10^6, 1.9 x 10^6, and 0.9 x 10^6) were consistently detected by electrophoresis of 10 μ liter samples (equivalent to 0.25 g of bark tissue) on 6% polyacrylamide gels in a mini (9 cm x 8 cm x 0.8 mm) vertical gel apparatus. Starting quantity of peeled bark was approximately 1.0 g which can be removed from a single 10-20 cm long twig. Positive results were obtained from green twigs, which need not be new flush growth.

A companion paper (1) in these proceedings describes the potential of using the presence and properties of virus-specific double-stranded (ds) RNAs for detection and diagnosis of citrus and avocado viruses. Previous results have demonstrated that a major (MW = 13.3 x 10^6) and numerous other (MW = 13.0 x 10^6 to 0.5 x 10^6) double-stranded RNAs can be isolated from citrus tristeza virus (CTV) infected tissue extracts by selective binding to cellulose powder (2). The overall pattern of major and minor dsRNAs resolved by polyacrylamide gel electrophoresis was diagnostic for CTV. Bark samples gave the best results from infected greenhouse grown seedings of several *Citrus* spp. One important question is whether such molecules can be isolated with ease from samples from commercial, field-grown trees. This was possible for avocado viruses (3) and the objective of this study was to test recovery of dsRNA from sweet orange infected with CTV.

MATERIALS AND METHODS

The sweet orange plants chosen for sampling were from a 15-year-old commercial block at Redlands, Riverside County, California, and from experimental trees at the UCR Citrus Experiment Station. Trees tested for dsRNA had previously been indexed on Duncan grapefruit, Eureka lemon, and Mexican lime. Those at Redlands had all shown the seedling yellows reaction in grapefruit and lemon. All field trees tested CTV-positive on Mexican lime. Twig samples with green bark were taken from growth that was 1 or 2 years old.

The dsRNA was isolated from bark peeled from stems by a rapid extraction method similar to methods previously described (4, 5, 6). Bark was peeled from single 10-20 cm long twigs and 0.5-1.0 g of tissue was pulverized to a fine powder in a mortar in the presence of liquid nitrogen. The powder was transferred to a beaker (5.0 ml) to which was added 1.3 ml of double strength TSE buffer [0.05 M tris-hydroxy methylaminomethane, 0.1 M NaCl, 0.001 M ethylene-diaminetretraacetic acid (EDTA), pH 7.0] containing 3% sodium dodecyl sulfate (SDS) and 0.5% 2-mercaptoethanol and

0.7 ml of TSE buffer-saturated phenol. The tissue was mixed well with the solution and transferred to a glass tube to which 0.7 ml of a 24:1 mixture of chloroform and isopropyl alcohol was added. The contents were mixed well and allowed to stand for 20 minutes with occasional mixing. The tube was then centrifuged (5,000 g for 15 minutes) and the aqueous phase (1.0 ml) was transferred to a plastic tube (microfuge) containing 50 mg of cellulose powder (Whatman CF-11). Ethanol was then added to a final concentration of 16.5% and the contents of the tube were shaken for 15-20 minutes. The cellulose powder was collected by centrifugation for 5 minutes in a Beckman Microfuge B in a cold box (4 C). The cellulose was washed three times with 1.0 ml aliquots of 16.5% ethanol in TSE buffer. Following these washes, 100 μ liters of TSE buffer was added to each tube for a final wash. The washed CF-11 cellulose was resuspended in 500 μ liters of TSE buffer, centrifuged and the supernatant was transferred to a 1.5 ml microfuge tube. The dsRNA contained in this supernatant was precipitated by the addition of 25 μ liters of 3.0 M sodium acetate pH 5.5 and 1.1 ml of 95% ethanol. The solution was stored at -20° until samples were prepared for electrophoresis.

The dsRNA was collected by centrifugation and the pellet was resuspended in 20 μl of electrophoresis buffer (0.04 M Tris, 0.02 M sodium acetate, 0.01 M EDTA, pH 7.8) containing glycerol (5%). An aliquot (10 μ liters) was analyzed by electrophoresis on 6% polyacrylamide gels in a mini (9 cm x 8 cm x 0.8 mm) vertical gel apparatus. Electrophoresis was for 4 hours at 100 V in electrophoresis buffer. Electrophoresed gels were stained in ethidium bromide (100 ng/ml) for 10 minutes in electrophoresis buffer and photographed on a UV light box.

RESULTS AND DISCUSSION

Three dsRNAs (MW = 13.3 x 10^6, 1.9 x 10^6 and 0.9 x 10^6) were consistently detected in gels used to analyze extracts from approximately 50 trees previously diagnosed positive from indexing on Mexican lime seedlings. An additional dsRNA (MW = 0.5 x 10^6) was consistently detected in gels used to analyze extracts from 15 of these trees, all of which were from the grove at Redlands (figure 1). These trees were all indexed

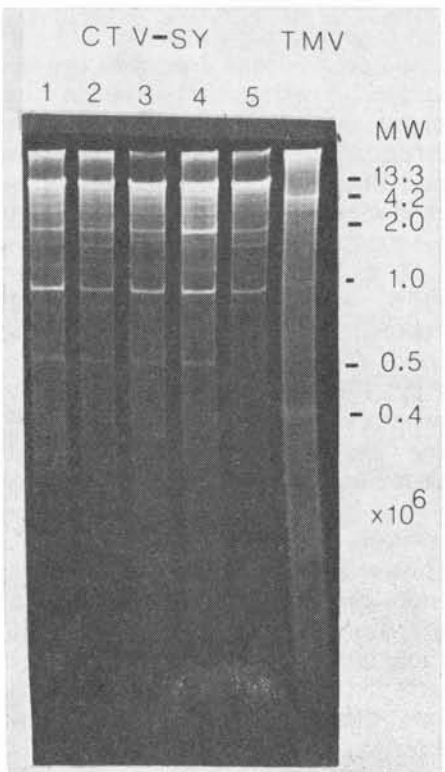

Fig. 1. A polyacrylamide gel, stained with ethidium bromide, on which the dsRNA isolated from 0.25 g of bark was analyzed. The samples were from 5 different sweet orange trees infected with seedling yellows type strains of CTV. The dsRNA from tobacco plants infected with tobacco mosaic virus (TMV) is shown at the extreme right. Electrophoresis was for 2.0 hours at 100 V/gel.

positively for seedling yellows strains of CTV in prior indexing on grapefruit and lemon seedlings. Samples have been taken over several months (September-March), from all sides of individual trees and from shoots on the outside or from within the tree canopy. None of these variations affected the recovery of the dsRNAs diagnostic for CTV. Detection was possible with extracts recovered from as little as 0.05 g of starting tissue. Resolution of minor dsRNA bands other than the four previously mentioned required more tissue, but never more than 0.5 g. The use of "mini" gels greatly increased sensitivity. The complexity of the pattern represented by the minor dsRNA bands was variable between isolates, and different patterns were detected in adjacent trees. Whether the complexity of the dsRNA patterns can be correlated with biological properties of the virus strains is under investigation.

The results presented offer some promise for the future development of nucleic acid-based diagnosis for citrus viruses, using small samples collected from field trees. Sufficient dsRNA was recovered from 1.0 g of tissue or less to be resolved on polyacrylamide gels. Such quantities are also sufficient to be detected by dot-blot hybridization techniques which, therefore, should be developed as an alternative to ELISA for indexing of citrus viruses. The methods used in this report have been presented in some detail in order to encourage others to adapt these approaches and a set of 35 mm color slides has been prepared to illustrate the steps involved. A discussion of the advantages of diagnosis based on detection and analysis of dsRNAs is contained in a companion paper (1).

ACKNOWLEDGMENTS

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