Double-Stranded RNA of the Diagnosis of Citrus and Avocado Viruses

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ABSTRACT. Until recently presence of homogeneous segments of double-stranded (ds)RNA, corresponding to twice the size of viral genomic ssRNA, in infected plants have been overlooked in plant virus diagnosis. Such molecules are not present in virus-free plants and their presence is an indicator for virus infection. The number and size of such genomic dsRNAs detected by gel electrophoresis are diagnostic parameters by which an unknown could be identified to one of the groups of plant viruses. Discrete patterns of additional minor dsRNAs which have sequence homology with the genomic dsRNAs, but are not full length, are also diagnostic.

A consistent pattern of genomic and other dsRNAs can be repeatedly isolated from different tissues and different hosts for a given virus. Methods for rapid isolation of dsRNA from small (1g or less) samples by cellulose chromatography and analysis by gel electrophoresis have been developed. Success is largely independent of the type of virus and host in contrast to virus purification and even serology. Hundreds of samples have been analyzed weekly. The method has detected citrus tristeza virus (CTV) and can distinguish strains of CTV, including seedling yellows (SY) strains. It has also detected three previously unsuspected virus-like agents in avocado. Results can be obtained from field collected samples.

The dsRNA, when melted, can be used as a source of (+) or (—) sense labelled probe to detect opposite sense RNA (e.g., viral genomic ssRNA) in plant extracts. This technique, and related techniques using c-DNA, are known as the dot-blot assay. It has many of the advantages associated with ELISA serology but can detect homology in all parts of the viral genome, not just that part which codes for the capsid protein.

The techniques described permit virus detection without purifying the virus. They are powerful tools and could be used to great effect in certification programs.

This paper was prepared on request to review past and present research on dsRNA based analysis of citrus and avocado viruses. The purpose of this paper is to inform virologists of new approaches to virus diagnosis and it has been written to convey ideas, more than techniques. Space does not allow for complete descriptions of methods and experiments but further information can be obtained from publications cited or by writing to the authors. The techniques described permit virus detection without knowing how to purify the virus being detected. They are powerful tools and could be used to great effect in certification programs such as the one described by Navarro et al. (16).

We are very fortunate to have suitable methods for indexing and diagnosing some viruses of citrus (8). The use of ELISA serology, now so common in plant virology, was brought to the attention of other virologists in part by its successful application to indexing of citrus tristeza virus (CTV). The tools we use are good ones, they are familiar, and will continue to give us years of service.

Serology does have two disadvantages as an ideal approach to virus diagnosis. The first is the need for an immunogen, which is usually a quite pure virus particle preparation. Years of research is frequently needed to develop a sufficient purification scheme. A classic example of this is CTV and we still continue to try to improve our schemes for this virus. The second is a generally overlooked shortcoming when serology is used to explore genetic diversity between strains of plant viruses. The variation that exists between the capsid proteins is all we can measure with antibodies. For a virus like CTV, which has a genome size of

6.3 x 10⁶ daltons (3, and Dodds, unpublished results), larger than any other plant virus, this fact is frustrating. We need new techniques that can detect differences in all parts of this large genome. Despite biological, and presumably genetic differences between seedling yellows (SY) and non-SY strains of CTV, we cannot distinguish between them with available antisera (1).

Techniques which detect and analyze genomes are not so familiar as serology, but are available, and do not require virus particle purification and do examine the entire genome. The isolation, analysis and use of viral replicative form (RF) double-stranded RNAs (ds RNAs) will be discussed in this report. Other techniques, including the synthesis of cDNA by reverse transcription from ssRNA and the cloning of viral genes from cDNA, are equally exciting.

The simple logic to exploit the presence of dsRNA in plants as a diagnostic tool is that homogeneous, high molecular weight dsRNA (greater than 0.1 x 10⁶ daltons) does not normally exist in most healthy plants (9, 13, 14). Therefore the presence of dsRNAs, which are the replicative templates from which viral genomes are made, is a "red flag" test for the presence of an RNA virus. Additional information of diagnostic value can be obtained by further analysis of any dsRNAs present as will be shown in the results section. The key features of the methods and results to be presented are:

1. The ease with which ds-RNAs can be isolated rapidly by simple methods from small amounts of tissue from multiple, field-collected samples using relatively simple laboratory equipment.

2. The use of the number,

size, intensity and complexity of dsRNAs detected by gel electrophoresis to aid in the diagnosis of a virus to group, member and even strain.

3. The use of dsRNAs to manufacture probes by various means which can rapidly detect virus nucleic acids in sap by dot-blot hybridization. This technique has many of the advantages of ELISA with the additional advantage of that the entire genome, not just the portion which codes for the capsid protein is analyzed.

4. The possibility of using purified dsRNA to generate an infectious ssRNA preparation free of troublesome inhibitors.

5. The diagnostic information, reagants and inoculum are obtained without needing to know how to purify the virus particle. A single method purifies dsRNA regardless of which virus, known or unknown, is causing dsRNA to accumulate in whichever host is being tested.

The examples to be discussed include strains of CTV and three newly discovered virus-like agents in avocado (*Persea americana* Miller), a tree-fruit crop commonly grown in citrus producing areas, including California.

MATERIALS AND METHODS

A unique feature of dsRNA is that it is adsorbed to cellulose powder in buffered 15-17% ethanol (6). Other nucleic acids, including ssRNA (ribosomal RNA, m-RNA, 7S, 5S, 4S RNA, and viroid RNA) and dsDNA are bound at higher ethanol concentrations. When nucleic acid solutions are passed through a solumn of cellulose powder or when cellulose powder is added to the solution, only dsRNA is adsorbed in the presence of 15-17% ethanol. Washing the powder (with dsRNA adsorbed) in buffered 15-17% ethanol removes unad-

sorbed nucleic acids. The dsRNA can be eluted from the washed powder with ethanol-free buffer. The eluted dsRNA can be concentrated by ethanol precipitation. A detailed scheme is described in a companion paper (4) and others have been reported elsewhere (2, 3, 11, 13).

The purified dsRNA can be detected by ELISA (17) or other (12) serological techniques and by hybridization techniques (7, 10, 15). It can be analyzed by gel electrophoresis (3, 4, 11, 13, 14) or electron microscopy (3). The dsRNA can be melted to single strands by boiling (5), or by treat-

ing it with such agents as formamide and methyl mercury (7, 10). These single strands will be of two polarities. The plus sense (+) population can hybridize with minus sense (—) molecules in plant sap (found in replicative structures), in media used to analyze nucleic acids (density gradients, gels and blots), or it can be used as an inoculum (5). The minus sense molecules can hybridize with (+) sense molecules in plant sap (every virion contains a (+) sense molecule and there are others in plant sap), or in fractions from a virus purification scheme. It may be necessary to release the
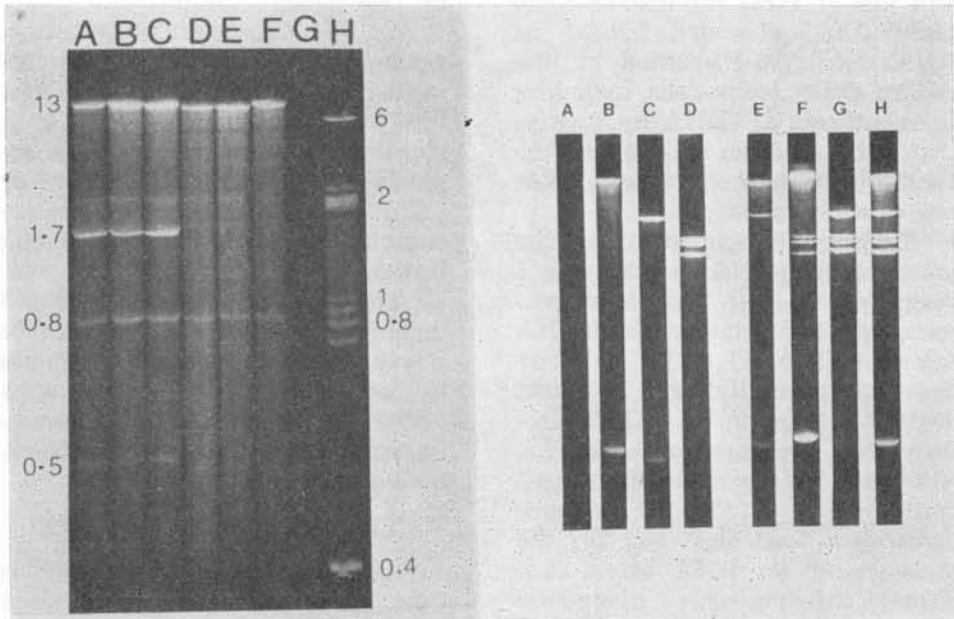

Fig. 1. Cylindrical (6 x 95 mm) polyacrylamide gels (5%) containing double-stranded (ds)RNAs isolated from individual avocado trees. Three distinct patterns were detected (B, C, or D) and these are believed to represent either genomic or replicative dsRNAs of three virus-like agents. Extracts from some individual trees (E-H) contained mixtures of the three dsRNA types. Extracts from some individuals (A) contained no dsRNA. Electrophoresis was for 10 hours at 5 mA/gel in 0.04 M Tris, 0.02 M sodium acetate, 1 mM EDTA, pH 7.8. Gels were stained in 10 ng/ml ethidium bromide and destained in water.

Fig. 2. Polyacrylamide gels (5%) containing double stranded (ds)RNAs isolated from citron infected with either a seedling yellows (A-C) or a non-seedling yellows (D-F) strain of citrus tristeza virus. A major (MW = 13.3 x 10⁶) and several minor diagnostic dsRNAs were present in extracts from plants infected with either strain. Two dsRNAs (MW = 1.7 x 10⁶ and 0.5 x 10⁶) were only present in detectable amounts in extracts from plants infected with the seedling yellows strain. No dsRNA was detected in extracts from uninoculated plants (G). Results from different sets of plants (A, B, or C; D, E, or F) were the same. Molecular weight markers are shown in channel H. Electrophoresis was as in figure 1.

(+) sense molecules from virions by treatment with detergents or other reagents which disassemble virus particles (7). Minus sense molecules can also hybridize with opposite sense molecules in density gradients, electrophoresis gels and nucleic acid blots.

In order to detect that hybridization has occurred, the probe [either (+) or (—) sense] has to be labelled, usually with a radioactive molecule such as 32p. This can be done by end-labelling of randomly fragmented RNA molecules (7, 10, 15) or by transcribing a complementary DNA copy of the RNA molecules using reverse transcriptase in the presence of labelled nucleotides.

An alternate source of (+) sense molecules is the genome isolated from purified virus particles. It is necessary to have a good purification scheme worked out when probe is made from this source.

RESULTS AND DISCUSSION

Gel analysis. Avocado viruses. The dsRNAs detected by polyacrylamide gel electrophoresis in extracts from 3.0 g of leaf tissue from 8 individual avocado trees is shown in fig. 1. No dsRNA was detected in one plant (fig. 1A). The other plants did contain dsRNA, and three patterns were resolved by gel electrophoresis. The patterns were detected alone (fig. 1 B-D) or in various combinations (fig. 1 E-H). These results are interpreted as evidence for three virus-like entities in avocado, which can occur in single or mixed infections. This example illustrates the value of dsRNA analysis for detection of previously unrecognized virus infections. These and other results on dsRNAs from avocado are reported in greater detail elsewhere (11).

Citrus tristeza virus. The dsRNAs detected by gel electrophoresis of samples prepared from bark of greenhouse grown citron seedlings infected with a non-seedling yellows isolate and a SY isolate of CTV is shown in fig. 2. Electrophoresis of the sample from the non-SY isolate separated the dsRNA molecules by size into a major RF (MW = 13.3 x 10^6, twice the size of the encapsidated ssRNA genome) and two other readily detected dsRNAs (MW = 1.9 and 0.8 x 10^6). Two additional dsRNAs (MW = 1.7 and 0.5 x 10^6) were characteristic for the SY isolate. Numerous other minor dsRNAs were also detected. Results for major and minor bands were the same for each of three sets of tissue analyzed for each isolate. No dsRNA was detected in extracts from healthy citron.

The dsRNAs of five different SY isolates and five different non-SY isolates were also analyzed by electrophoresis of extracts from greenhouse-grown sweet orange seedlings (fig. 3). The characteristic dsRNAs of CTV were detected in all samples. A dsRNA (MW = 0.5 x 10^6, see arrow) was detected in extracts from each of the five SY isolates which was not detected in extracts from each of the five non-SY isolates. Results for field trees are described elsewhere (4).

It can be concluded from these results that: 1) dsRNA was only present in infected plants; 2) a major replicative form (RF) dsRNA of expected number (one) and size (MW = 13.3 x 10^6) for CTV was detected; 3) numerous additional dsRNAs were detected (function presently unknown); 4) the electrophoretic pattern of dsRNAs was complex and consistent, and therefore diagnostic for CTV; and 5) at least one of the additional dsRNAs was specifically associated with SY iso-
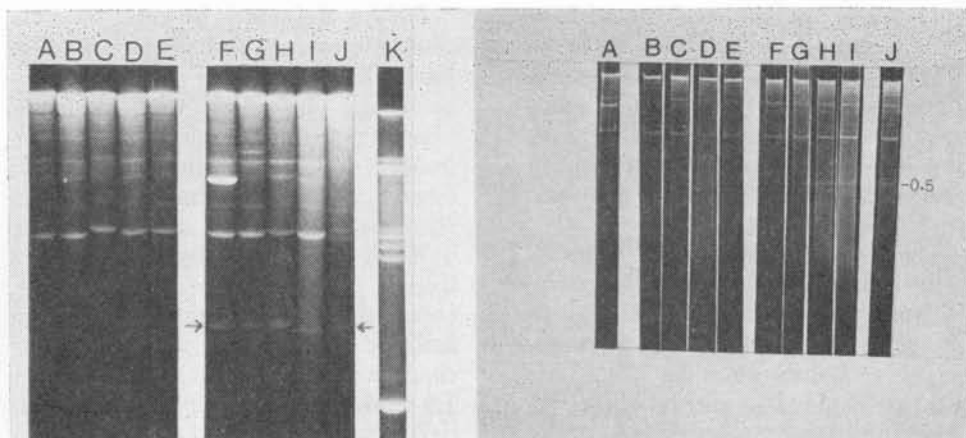
Fig. 3. Polyacrylamide gels (5%) containing double stranded (ds)RNAs isolated from sweet orange infected with five different non-seedling yellows isolates (A-E) and five different seedling yellows isolates (F-J) of citrus tristeza virus. Additional dsRNAs characterize seedling yellows isolates, one of which (arrowed, MW = 0.5×10^6) appears to be consistent. Molecular weight markers are shown in channel K (see also figure 2, channel H). Electrophoresis as in figure 1.

Fig. 4. Slab (9 cm x 8 cm x 0.8 mm) polyacrylamide gels (5%) containing double stranded (ds)RNA isolated from sweet orange infected initially with four different non-seedling yellows isolates of citrus tristeza virus (B-E and, in the same order, F-I). Plants were (F-I) or were not (B-E) re-inoculated by budding with a seedling yellows isolate (UCR isolate 12B). The expected dsRNAs for a non-seedling yellows isolate (A) and the 12B seedling yellows isolate (J) are shown for comparison. Symptoms (stunting and stem pitting) and the 0.5×10^6 dsRNA diagnostic for isolate 12B were detected in challenge inoculated plants. Electrophoresis was for 2 hours at 100 V.

lates, the first promising physical test for discriminating between isolates of CTV.

These results illustrate the use of dsRNA analysis for diagnosis of CTV, and for detection of strains of CTV. The ability to physically distinguish between SY and non-SY strains should prove useful in cross protection experiments. A preliminary result is illustrated in fig. 4. Expected dsRNAs were detected in extracts from plants inoculated with each of four non-SY isolates (fig. 4, B-E). Other plants which were initially inoculated with the same four isolates and were later reinoculated with a SY isolate (USR strain 12B) were also analyzed. These plants developed symptoms of strain 12B (severe stunting and severe stem-pitting) and the 0.5×10^6 dsRNA diagnostic for SY isolates was detected in extracts purified from them (fig. 4, F-I). The failure of
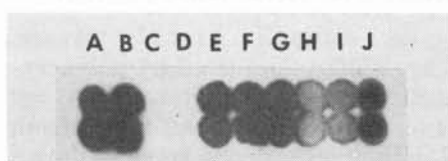

Fig. 5. Autoradiograph illustrating the results of the hybridization of 5'-^{32}P-end-labelled citrus tristeza virus (CTV) double stranded (ds)RNA probe to purified nucleic acids or extracts from bark of sweet orange seedlings which have been spotted to nitrocellulose paper. Before spotting, CTV single stranded (ss) and dsRNAs were treated for 10 minutes at room temperature in 10mM methyl mercury hydroxide (MMH) in 50mM borate buffer. Bark was extracted in 1.5% sodium dodecyl sulphate in borate buffer. Extract supernatants were treated with MMH before spotting. All samples were run in duplicate: (A) CTV ssRNA; (B) CTV dsRNA; (C, D) extracts from uninoculated plants; and (E-J) extracts from one of 6 different isolates of CTV. Spotted paper was incubated in 5'-end labelled CTV dsRNA probe, and hybridization of probe to specific dots was detected by autoradiography. Details of methods have been published (7, 10).

cross protection in this experiment was assayed by both biological (appearance of symptoms of the challenge strain) and physical (accumulation of a strain specific dsRNA) methods.

Hybridization analysis by dot blot assays. End-labelled (+) and (—) sense single-stranded RNA fragments derived from CTV dsRNA were used as probe to de-tect opposite sense molecules in samples of sap dotted onto nitro-cellulose paper. Hybridization only occurred with samples prepared from CTV infected plants (fig. 5).

ACKNOWLEDGMENTS

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