Analysis of Double-Stranded RNA for Plant Virus Diagnosis

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Diagnosis of plant viruses can be difficult when one is dealing with unstable viruses, unusual strains, or viruses in woody plants. Observation of inoculated indicator plants, detection of virus-induced cellular inclusions, electron microscopy, test of the vector capability of certain insects, serology, and detection of viral nucleic acids are often used for virus diagnosis. The usefulness of each method depends on the virus and the host. A diagnostic method useful for one virus in a given host may not be reliable for other viruses or for the same virus in a different host. Diagnosis based on the use of indicator plants is not reliable because plant reactions and symptoms vary, depending on environmental conditions, plant cultivars, and virus strains. Other methods, such as electron microscopy, tests of vector capability, and detection of viral nucleic acids by means of radioactive probes, may not be practical for some laboratories. Serology is the most widely used method for virus diagnosis because serological tests are simple and practical. One drawback, however, is that in plants infected with more than one virus, a nontarget virus may pass undetected. Furthermore, some serological tests are strain-specific, which means that only certain strains of a given virus are detected. Therefore, it is usually necessary for the plant pathologist to utilize more than one method. The more methods employed, the greater the probability the diagnosis will be correct.

Morris and Dodds (9) developed a method for the isolation and analysis of double-stranded RNA (dsRNA) from virus-infected plants and fungi. This method detects dsRNAs that are produced in plants infected with RNA viruses. These dsRNAs, which are very resistant to enzymatic degradation, are not normally present in healthy plants. Detailed information regarding the applications of the dsRNA analysis to plant pathology has been published by Dodds and co-workers (3–5). The procedure has also been simplified (1,10). This simplification, together with improved equipment for nucleic acid analysis, has made the technique more practical and attractive to plant disease diagnosticians.

This paper considers the practicality of using analysis of viral-associated dsRNA as an alternative or complementary method for diagnosis of plant virus diseases. The advantages and disadvantages of this technique are presented, and a simplified version of the technique is described.

Double-stranded RNA in plants

Single-stranded RNA viruses compose approximately 90% of all known plant viruses. During their replication in plant cells, dsRNA is produced as an intermediate product (Fig. 1). This dsRNA is called the replicative form (RF) and is consistently present when a plant is infected with an ssRNA virus, regardless of the host. When researchers began to use dsRNA analysis for viral detection, however, it became clear that plants may also contain dsRNA of other kinds: the genome of a dsRNA virus (cryptic viruses and phytoreoviruses), the RF of a satellite RNA, and a dsRNA of unknown origin (cellular dsRNA) (8,13). Although the RF of an ssRNA plant virus is the most common type encountered, the other dsRNAs are important because they could be confused with that of an ssRNA virus. Nevertheless, the consistency with which dsRNAs can be extracted from most plant tissues infected with ssRNA viruses makes dsRNA analysis a practical method for virus diagnosis.

Purification and analysis of dsRNA from plant tissues

The dsRNA extraction procedure (Fig. 2) is basically as described by Morris and Dodds (9) and Jordan et al (8). Two cycles of cellulose chromatography are used to remove residual ssRNA, which may confuse the interpretation. The same procedure can be used to extract dsRNA from fungi or insects. The reagents are listed in Table 1. The procedure is as follows:

1. Grind 3.5 g of tissue in 6.0 ml of 1× STE buffer with a mortar and pestle or an equivalent tool. Transfer homogenate to a 50-ml centrifuge tube. Rinse the mortar and pestle with 2.0 ml of 1× STE and add this liquid to the homogenate. (If liquid nitrogen is used to grind the tissue, transfer the powder to the tube and add 8.0 ml of 1× STE.)
2. Add 1.0 ml of 10% SDS, 0.5 ml of bentonite (from a 2% aqueous suspension), and 9.0 ml of 1× STE-saturated phenol to the homogenate and shake it well for 30 min.
3. Centrifuge tubes at 8,000 *g* for 15 min. Withdraw 10.0 ml of the upper aqueous phase and place it in a 50-ml centrifuge tube. (If 10.0 ml is not available, adjust to 10.0 ml by adding 1× STE.)
4. Add 2.1 ml of 95% ethanol to each tube containing 10.0 ml of sample and mix well. (Samples can be stored overnight at 4 C.)
5. Weigh two 1.0-g portions of cellulose (Whatman CF-11 [Whatman, Clifton, NJ] or Cellex N-1 [Bio-Rad Laboratories, Richmond, CA]) per sample and place them in 50-ml tubes. Add 25 ml of 1× STE containing ethanol, 16.0% v/v.
6. Prepare two columns, using for each the barrel of a 20-ml plastic syringe plugged with a disk of Miracloth paper or glass wool. Mix the cellulose suspensions well, pour them into the columns, and allow the STE to drain through.
7. Add the sample (must be at room temperature) to one column and let it drain completely. Discard the liquid from the column. Flush the column with 40 ml of 1× STE containing ethanol, 16.0% v/v. Keep refilling the column until all the buffer is used. Let it drain completely, and discard the liquid.
8. Add 2.5 ml of 1× STE and let it drain completely. Add 10.0 ml of 1× STE, but this time collect 10.0 ml in 50-ml centrifuge tubes. Add 2.1 ml of 95% ethanol, then repeat step 7, using the second column. Go to step 9.
9. Add 2.5 ml of 1× STE and let it drain. Add 6.0 ml

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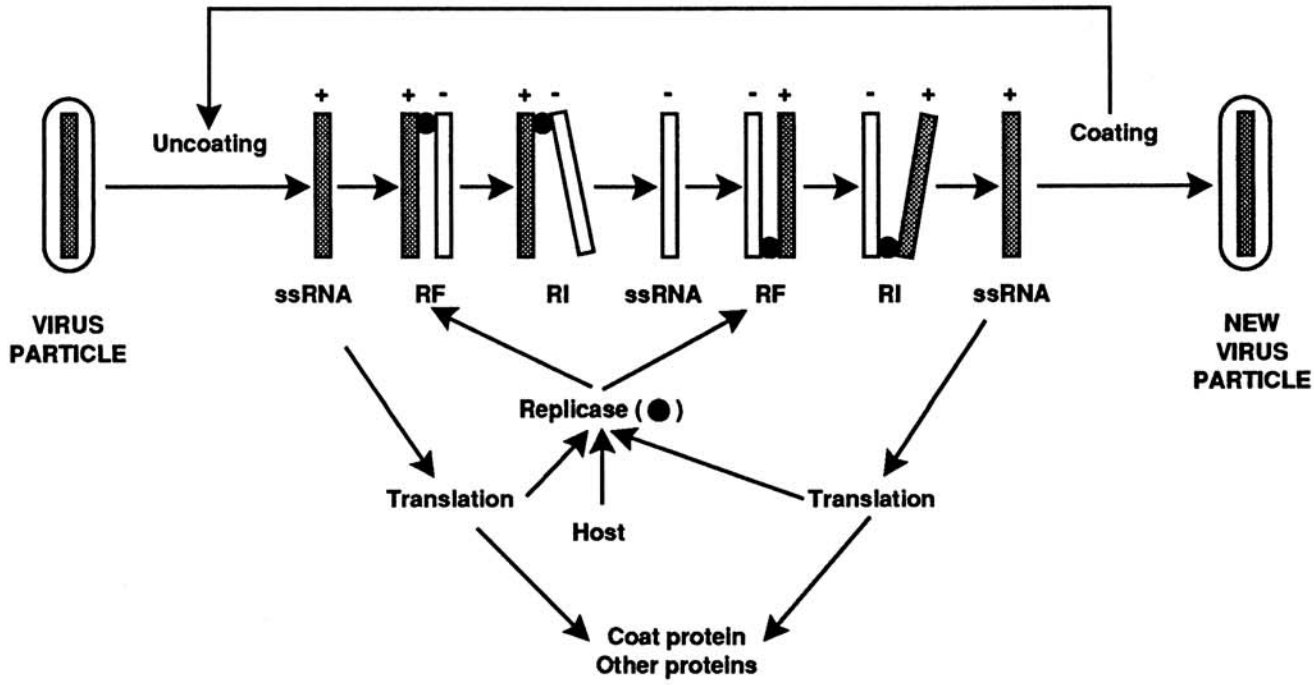

Fig. 1. Schematic representation of the proposed replication cycle of a typical single-stranded RNA (ssRNA) plant virus. Only the replication of the genomic RNA is illustrated. In some cases, however, replicative forms (RFs) of subgenomic RNAs are also produced. Double-stranded RNAs obtained from plants infected with ssRNA viruses are probably RFs. Replicative intermediate (RI) consists of dsRNA that is partially single-stranded and therefore more susceptible to RNase degradation.

of 1× STE and collect 6.0 ml in a 50-ml centrifuge tube. Add 0.5 ml of 3.0 M sodium acetate (pH 5.5) and 20.0 ml of 95% ethanol to each sample. Store for at least 2 hr at −20 C to precipitate the dsRNA.

10. Centrifuge samples at 8,000 *g* for 25 min. Pour off the ethanol and place the tubes upside down to drain for about 15 min. Add 200 μl of EG buffer to each tube and mix well to resuspend the dsRNA. Store samples (indefinitely) at −20 C.

Electrophoresis of dsRNA can be done in a variety of ways, but it is usually performed in 6% polyacrylamide gels or in 1.0–1.5% agarose gels. The appropriate volume of dsRNA extract to load on the gel varies according to the virus. Normally, 30–50 μl is needed. Electrophoresis parameters vary according to the size and type of gels. When a 6% polyacrylamide gel (1.5 mm × 7 cm × 8 cm) is used, electrophoresis can be performed at 100 V for 3 hr at room temperature. Gels are stained with ethidium bromide (50 $ng \cdot ml^{-1}$). The "stained" dsRNA fluoresces when exposed to ultraviolet radiation and can be photographed while fluorescing (Fig. 3A). Alternatively, acrylamide gels can be stained with silver as described by Blum et al (2) and photographed under visible light (Fig. 3B). Enzymatic treatment of the final sample is not needed because dsRNA obtained by means of the procedure described here is free from detectable host DNA and RNA.

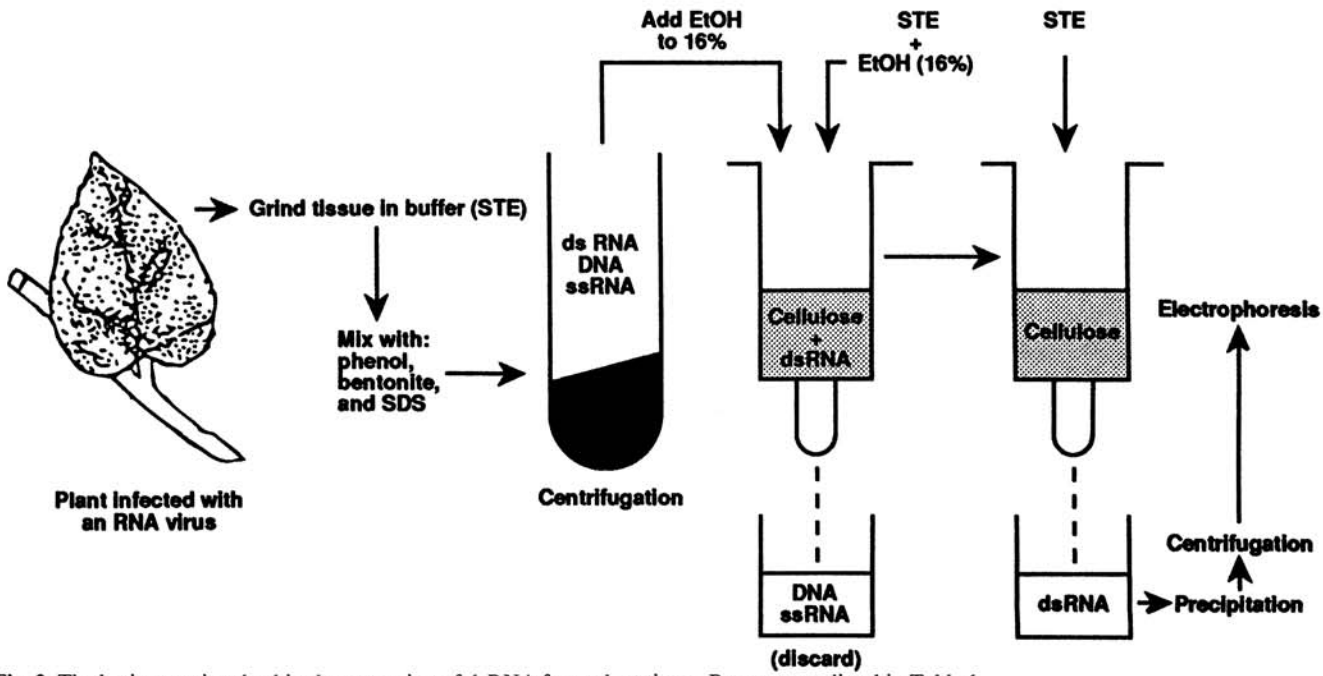

Fig. 2. The basic steps involved in the extraction of dsRNA from plant tissue. Reagents are listed in Table 1.

Evaluation of the results

One problem faced by the beginner using this technique is interpretation of dsRNA banding patterns. Different groups of plant viruses have characteristic dsRNA patterns (profiles) (5,6,13). Members within a group have similar dsRNA profiles. The uniqueness of a profile is based on the numbers and molecular weights of the dsRNA segments. A virus whose genome consists of one ssRNA should produce one major dsRNA of roughly twice the molecular weight of the ssRNA. In practice, however, other minor (less prominent) dsRNAs of lower molecular weight are obtained (4,10,13). This is illustrated by tobacco mosaic virus (TMV), tobacco necrosis virus, and potato virus X (Fig. 4). These less prominent dsRNAs are consistent and useful to differentiate viruses within a group (4,10,12,13). Some of these dsRNAs are the RFs of subgenomic RNAs derived from the genomic RNA (14); the origin of others is unknown. A virus with a genome consisting of three ssRNAs and a subgenomic ssRNA should yield a dsRNA profile with four bands. This is illustrated by alfalfa mosaic virus and cucumber mosaic virus (CMV) (Fig. 4).

The first step in interpreting the results of the dsRNA procedure is to determine, by means of molecular weight standards, the number and approximate size of the dsRNA bands. Some practical molecular weight standards are the dsRNAs extracted from plants infected with CMV and TMV. Once the number and molecular weight of the dsRNAs are known, one should consult the literature regarding described dsRNA profiles for members of different viral groups. Because information on dsRNA profiles is not available for all viral groups, one may have to look at information on ssRNA. Good sources of this information are the CMI/AAB descriptions of plant viruses, published by the Commonwealth Agricultural Bureaux (Kew, Surrey, England). Finally, findings should be confirmed by means of other detection techniques.

As pointed out before, there are other sources of dsRNA in plants. One of the most common types in some plants is cellular dsRNA, which is apparently nonviral (6,8,13). This

Table 1. Reagents used for extraction of dsRNA

Reagent	Amount
Extraction buffer (STE)	
(0.1 M NaCl, 0.05 M tris, 0.001 M EDTA)	
Stock solution (1.0 L, 10×)	
Tris-base	61.0 g
NaCl	58.0 g
$Na_2EDTA \cdot 2H_2O$ (ethylenediaminetetraacetic acid disodium salt)	3.7 g
Distilled H_2O	800.0 ml
Adjust pH to 6.8 with concentrated HCl, add distilled H_2O to a volume of 1,000 ml	
STE-ethanol (16%)	
10× STE	100.0 ml
95% Ethanol	174.0 ml
Distilled H_2O	726.0 ml
Electrophoresis buffer (E)	
(0.04 M tris, 0.02 M sodium acetate, 0.001 M EDTA)	
Stock solution (1.0 L, 20×)	
Tris-base	97.0 g
Sodium acetate·$3H_2O$	54.5 g
$Na_2EDTA \cdot 2H_2O$	7.4 g
Distilled H_2O	800.0 ml
Adjust pH to 7.8 with acetic acid, add distilled H_2O to a volume of 1,000 ml	
Polyacrylamide (40:1)	
30% Stock solution (100 ml)	
Acrylamide	30.0 g
Bisacrylamide	0.7 g
Materials for 6% polyacrylamide gel (10 ml)	
Distilled H_2O	3.5 ml
3× E	3.4 ml
30% Polyacrylamide stock solution	2.0 ml
1% Tetramethylethylenediamine (TEMED)	1.0 ml
10% Ammonium persulfate	0.1 ml
EG buffer	
20× E	5.0 ml
Glycerol	20.0 ml
Bromophenol blue	0.01 g
Distilled H_2O	75.0 ml

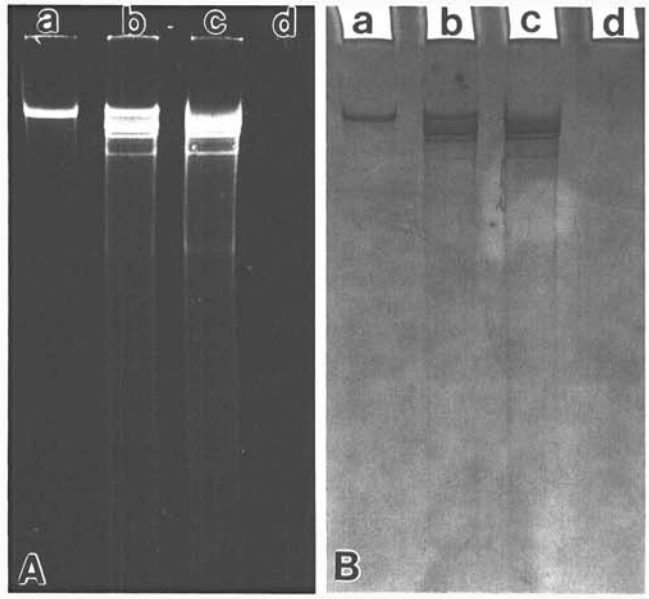

Fig. 3. Polyacrylamide gel electrophoresis of dsRNAs extracted from: (lanes a) healthy pepper (*Capsicum annuum* L. 'Yolo Wonder'), (lanes b) pepper cv. Yolo Wonder infected with potato virus X (PVX), (lanes c) tobacco (*Nicotiana tabacum* L. 'Turkish') infected with PVX, and (lanes d) healthy tobacco cv. Turkish. Gel stained with **(A)** ethidium bromide and **(B)** silver nitrate. Presence of cellular dsRNA in healthy pepper (lanes a) can lead to misinterpretations of results, which points out the importance of appropriate healthy controls. All plants used for dsRNA analysis illustrated in this figure and in Figures 4 and 5 were inoculated and grown in a greenhouse.

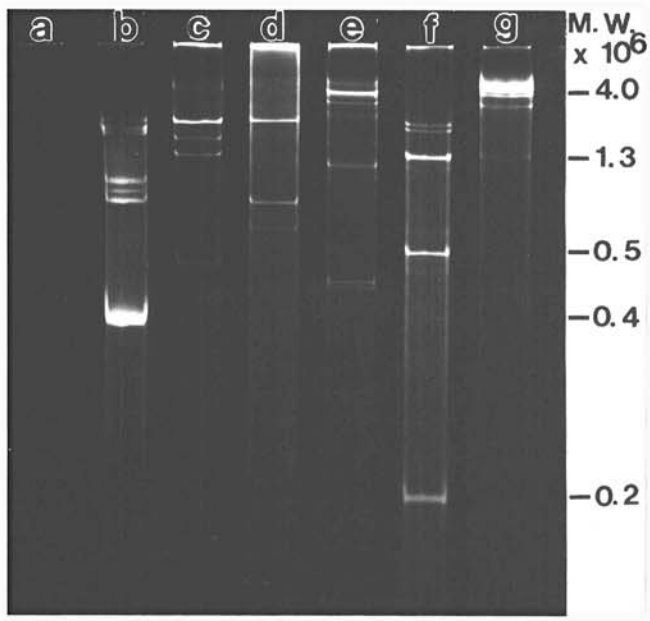

Fig. 4. Polyacrylamide gel electrophoresis of dsRNAs extracted from: (lane a) healthy tobacco cv. Turkish, (lane b) *Penicillium chrysogenum* Thom and *P. stoloniferum* Thom, and tobacco cv. Turkish infected with (lane c) alfalfa mosaic virus, (lane d) tobacco necrosis virus, (lane e) tobacco mosaic virus, (lane f) cucumber mosaic virus and its satellite RNA, and (lane g) potato virus X. Gel was run at 100 V for 2.5 hr.

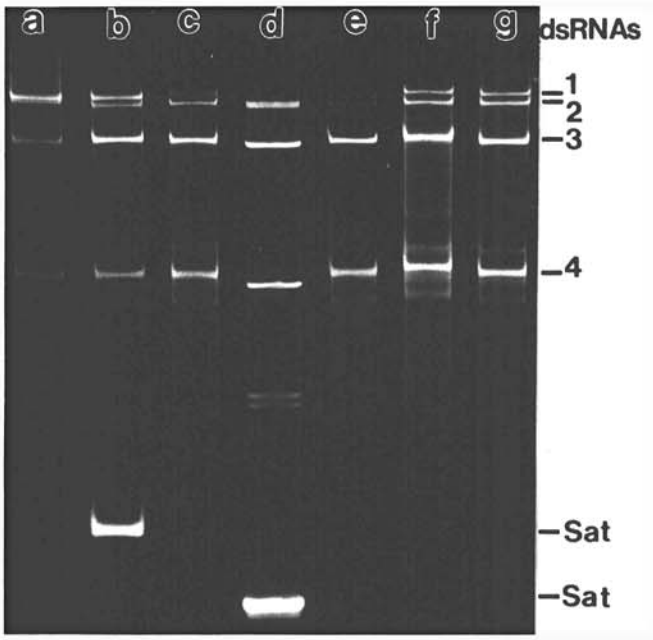

Fig. 5. Polyacrylamide gel electrophoresis of dsRNAs extracted from tobacco cv. Turkish infected with several strains and field isolates of cucumber mosaic virus (CMV): (lane a) CMV from *Apios americana* Medik., (lane b) CMV from *Nicotiana glauca* Graham containing also a satellite RNA (Sat) of CMV (prominent low molecular weight band), (lane c) CMV from cucumber, (lane d) S strain containing also a satellite RNA, (lane e) purslane strain, (lane f) Hall strain, and (lane g) CMV from malva. Gel was run at 100 V for 2.5 hr. Faint bands observed in lanes c, e, f, and g were consistently obtained with those CMV isolates, although their origin is uncertain.

dsRNA is similar in size to that of many ssRNA plant viruses. It is usually present in specific cultivars or plant species but is not detected in others. Figure 3 illustrates this problem. The use of appropriate controls (same plant species or same cultivar) is therefore recommended.

Advantages and disadvantages of the dsRNA analysis

This technique has several advantages over other methods for virus diagnosis. In plant virus diagnosis, interfering host components and instability of the virus or viral RNA are two of the problems encountered by diagnosticians when using traditional methods. The dsRNA technique overcomes these problems. The technique is simple and relatively inexpensive, and dsRNA is obtained regardless of the host or the RNA virus. Results are obtained in a relatively short time (8–12 hr). The technique detects mixed infections, which often go undetected with other methods and result in inadequate diagnoses. Unlike most other diagnostic techniques, dsRNA analysis is nonspecific. It can be used to distinguish not only different viruses but also strains of the same virus as well as satellite RNAs (12,13) (Fig. 5). The technique can also be used to purify dsRNAs of unstable viruses. The purified dsRNA could then be used as a reagent for inoculation, probe preparation, or molecular cloning, as reported by various researchers (3,7,11,12).

The dsRNA analysis has limitations. Only RNA viruses can be detected. Knowledge of the number and sizes of viral RNAs of the different viral groups is required. Some plants contain cryptic viruses and/or cellular dsRNAs that yield dsRNAs similar in size to those associated with ssRNA viruses. Certain viral groups, such as the luteoviruses and most potyviruses, yield very low quantities of dsRNA, making the method impractical for their routine diagnosis. Sometimes, field-collected samples yield low amounts of dsRNA and greenhouse inoculations are needed to improve dsRNA yields.

The need for rapid and reliable methods for diagnosing diseases and identifying pathogens is increasing as new technologies become available to researchers and diagnosticians. Despite its limitations, the described procedure could be used in most plant disease clinics as a primary screening technique, as a complement to other techniques, and as primary technique to diagnose such viruses as TMV, CMV, and citrus tristeza virus (3,10,12,13).

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