Extraction of Double-Stranded RNA from Plant Tissues Without the Use of Organic Solvents

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ABSTRACT

DePaulo, J. J., and Powell, C. A. 1995. Extraction of double-stranded RNA from plant tissues without the use of organic solvents. Plant Dis. 79:246-248.

Double-stranded RNAs (dsRNAs) are commonly isolated from plants and insects with toxic organic solvents such as phenol and chloroform that denature and remove proteins from aqueous solutions containing nucleic acids. This procedure was modified by replacing the toxic solvents with sodium dodecyl sulfate and potassium acetate (SDS/KOAc). Samples were homogenized in liquid nitrogen mixed with buffer and SDS at 65 C and then with KOAc at 0 C. A precipitate, consisting of SDS, KOAc, and proteins, was removed by centrifugation. The supernatant containing nucleic acids was collected and subjected to standard CF-11 cellulose chromatography to separate dsRNAs from other nucleic acids. The SDS/KOAc method produced comparable yields and purity of dsRNA products compared to the phenol/chloroform method and is considered a convenient, effective alternative for isolating dsRNA from plant tissues.

Double-stranded RNAs (dsRNAs) are commonly found in plant tissues infected with single-stranded RNA (ssRNA) viruses. The presence of dsRNAs in plant tissues is frequently used to diagnose and characterize viral infections (3,8).

Florida Agricultural Experiment Station Journal Series R-03119.

Accepted for publication 24 October 1994.

Double-stranded RNA is isolated from virus-infected and noninfected (control) plant tissue, electrophoresed on agarose or acrylamide gels, and the results are compared with the dsRNA banding patterns of known viruses. In addition, other less common dsRNAs found in plant tissue may represent the genome of reoviruses or cryptic viruses (4). Other dsRNAs of unknown origin also have been reported (5,11,12).

Double-stranded RNA is usually isolated from plant and insect tissues using (11) phenol, chloroform, and sodium dodecyl sulfate (SDS). These reagents solubilize proteins separating them from nucleic acids that remain in the aqueous phase. This method requires safety precautions, such as the use of fume hoods, and is expensive due to the cost of organic solvents and their disposal. In addition, disposal of these organic solvents creates environmental concerns. The combination of potassium acetate and SDS (SDS/KOAc) is less toxic and more easily and cheaply disposable than organic solvents and can be used as an alternative to remove proteins from nucleic acids in tissue homogenates (2). The following investigation compares and analyzes dsRNAs prepared by the phenol/chloroform and SDS/KOAc procedures.

MATERIALS AND METHODS

Plant samples. Samples processed for dsRNAs include phloem tissue (leaf midribs or bark from new flush) from *Citrus excelsa* Webster infected with citrus tristeza virus (CTV), Florida isolate T36; tobacco (*Nicotiana tabacum* L. 'Samsun') leaves infected with the type strain of tobacco mosaic virus (TMV); barley (*Hordeum vulgare* L. 'Barsoy')

leaves that contained a high molecular weight dsRNA (12); and tomato (*Lycopersicon esculentum* Mill. 'Sunny') leaves infested with sweetpotato whitefly (*Bemisia tabaci* Genn.) nymphs (11). Noninoculated plants of *C. excelsa*, Provar soybean, Sunny tomato, and Samsun tobacco were used as dsRNA negative controls. Samples were obtained from collections at the University of Florida, Agricultural Research and Education Center in Fort Pierce.

SDS/KOAc extraction of dsRNA. The procedure of Dellaporta et al (2) that uses KOAc for the isolation of plant DNA was utilized for the initial separation of nucleic acids from proteins and polysaccharides. Double-stranded RNA was separated from ssRNA and DNA as described by Morris and Dodds (8) with some modifications. Tissue samples, six each in 10 replications, were ground to a fine powder in liquid nitrogen with a mortar and pestle. The ground tissue was transferred to 30-ml Oak Ridge tubes (Nalgene Labware Division, Nalge/Sybron Corporation, Rochester, NY) and mixed with 20 ml of extraction buffer (100 mM Tris-HCl, 50 mM EDTA, pH 8.0, 500 mM NaCl, 10 mM β-mercaptoethanol) and 15 mg of bentonite. Samples were incubated with 1.0 ml of 20% SDS at 65 C for 20 min and then with 5.0 ml of 5 M KOAc at 0 C for 20 min. Centrifugation at 10 K for 15 min was used to remove the protein and polysaccharide precipitate. The nucleic acid supernatant was passed through one layer of Miracloth (Calbiochem-Behring, La Jolla, CA), and the volume was adjusted with 95% ethanol to obtain a final alcohol concentration of 15% (v/v).

Double-stranded RNA was purified from the supernatant by cellulose chromatography. The solution was mixed into a column containing 1.0 g of CF-11 powder (Whatman, Hillsboro, OR). After a 10 min incubation at room temperature, the column was washed with 200 ml of STE buffer (100 mM NaCl, 50 mM Tris-HCl, 1 mM EDTA, pH 7.0) containing 15% ethanol. Double-stranded RNA was eluted from the column with 14 ml of STE buffer, precipitated with 0.1 volume of 3 M sodium acetate, pH 6.0, and 3.0 volumes of 95% ethanol, and stored at −70 C for 1 hr. Precipitates were pelleted by centrifugation at 10 K for 20 min., washed with 70% ethanol, air-dried, and resuspended in 100 μl of distilled water (dH_2O). Samples were transferred to Eppendorf tubes and treated with deoxyribonuclease I (DNase I [Sigma Chemical Co., St. Louis]) as described below. Fifteen microliters of each sample was used for electrophoresis.

Phenol/chloroform extraction of dsRNA. Double-stranded RNAs were initially isolated using phenol/chloroform as described by Morris and Dodds (8) and purified using CF-11 as modified by Jordan et al (6). Six grams of tissue, 10 replications, was used in each sample for each extraction. The dsRNA products were resuspended in 100 μl of dH_2O, treated with DNase I as described below, and 15 μl was used for electrophoresis.

Nuclease treatment and gel analysis. DNase I and ribonuclease A (RNase A [Sigma]) treatments were conducted on dsRNA samples to confirm dsRNA structure. The DNAse I (final concentration = 10 μg/ml) treatments lasted 20 min at 30 C in the presence of 0.03 M $MgCl_2$ (8). The RNase A (final concentration = 10 ng/ml) treatments were conducted in high salt (0.3 M NaCl) (4) and in no salt (dH_2O) concentrations at 37 C for 30 min. Double-stranded RNA was analyzed by electrophoresis on 0.8% agarose gels (6.5 × 10 × 0.5 cm) containing ethidium bromide at 100 pg/ml for 1 hr at a constant voltage of 75 V. The dsRNAs were viewed with a UV-light transilluminator (300 nm) and photographed through a Cokin A 0.001 yellow filter with TMAX 400, 35-mm film (Kodak, Rochester, NY).

RESULTS

Comparison of methods. The recovery and resolution of dsRNAs was similar for both methods when equal amounts of tissues were extracted with SDS/KOAc or phenol/chloroform, DNase I treated, and electrophoresed in agarose gels (Fig. 1). The dsRNA from each tissue was located as a single band at approximately 20 kb for CTV (3), at 13.0 kb for barley (12), and at 6.5 kb for TMV (10). A consistent dsRNA band at 6.7 kb (11) and variable dsRNA bands at 6.1 (11), 4.6, and 4.2 kb (1) were detected in extracts from sweetpotato whitefly nymphs (Fig. 2). No dsRNAs were

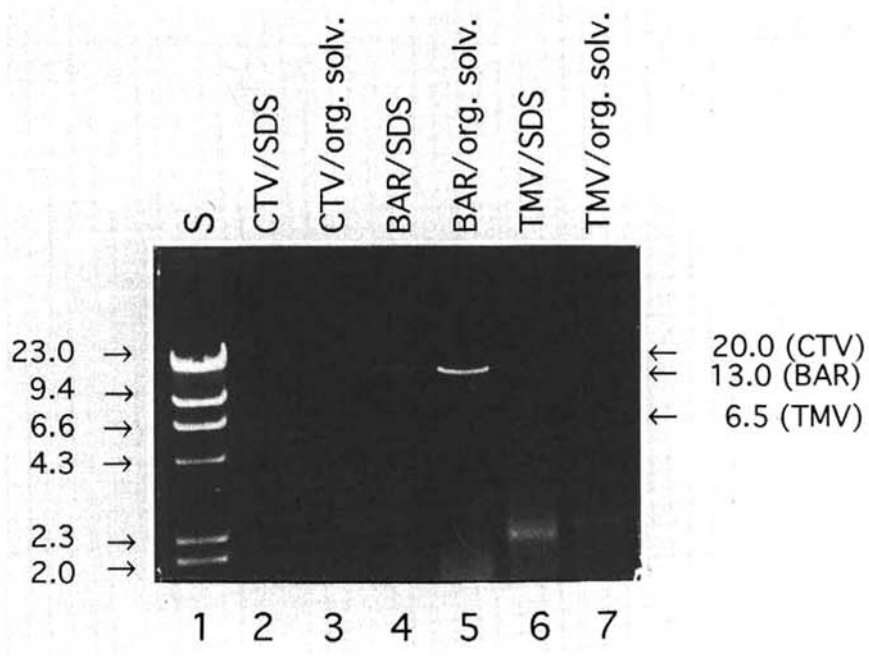

Fig. 1. Agarose gels (0.8%) showing a comparison of potassium acetate/sodium dodecyl sulfate (KOAc/SDS) and phenol/chloroform (organic solvents) dsRNA extractions. All samples were subjected to DNAse I treatment before electrophoresing. Lane 1, lambda phage DNA digested with *Hin*dIII; lane 2, SDS/KOAc extraction of citrus tristeza virus (CTV)-infected sweet orange; lane 3, phenol/chloroform extraction of CTV-infected sweet orange; lane 4, SDS/KOAc extraction of Barsoy barley (BAR); lane 5, phenol/chloroform extraction of BAR; lane 6, SDS/KOAc extraction of tobacco mosaic virus (TMV)-infected tobacco; and lane 7, phenol/chloroform extraction of TMV-infected tobacco.

Fig. 2. Nuclease treatments to confirm dsRNA structure of isolates prepared with sodium dodecyl sulfate/potassium acetate (SDS/KOAc) from Sunny tomato infested with sweetpotato whitefly nymphs. A DNA band was found at approximately 20 kb. Double-stranded RNAs were found at 6.7, 6.1, and 4.2 kb (1,11). Lane 1, lambda phage digested with *Hin*dIII; lane 2, no nuclease treatment; lane 3, DNase I treatment; lane 4, RNAse A treatment in 0.3 M NaCl (high salt); and lane 5, RNase A treatment in dH_2O (low salt).

detected in noninfected citrus or tobacco tissue.

Verification of dsRNA. Double-stranded RNA extractions were conducted with SDS/KOAc and phenol/chloroform on dsRNA-containing and healthy control tissue. Without pretreatment with DNase I, a band migrating at approximately 20.0 kb that was sensitive to DNase I was found in each sample except in healthy and CTV-infected citrus. Only the 20-kb DNase I-sensitive band was detected in healthy control tissues. Samples prepared by both methods were subjected to ribonuclease treatments in high (dsRNA was not RNase susceptible) and low (dsRNA was RNase susceptible) salt to confirm the double-stranded structure of DNase I-resistant bands. As expected, when samples were subjected to RNase A treatment in low salt concentrations, all RNA bands were degraded (Fig. 2). Degradation was not observed when these samples were treated with RNase A at high salt concentrations, confirming their dsRNA nature (4).

DISCUSSION

The SDS/KOAc method for dsRNA extraction is fast, convenient, and sensitive enough for diagnostic purposes. The SDS/KOAc method is as efficient as the phenol/chloroform method with respect to the tissues used in this study. The major advantage of this method is that it eliminates the need for organic solvents. Nucleic acid extraction with phenol requires numerous safety precautions, including the use of fume hoods and protective clothing such as gloves, coats, and goggles. In addition, the disposal of organic solvent waste is an environmental concern and increases the costs of conducting research. These problems are reduced when handling and disposing of SDS/KOAc because the reagents are less toxic, nonvolatile, and miscible in water.

Analysis of dsRNAs is commonly carried out by electrophoresis in polyacrylamide gels that have a high resolving power but that are more difficult to prepare and handle than agarose gels (9). We believe agarose is more suitable for routine or preliminary analysis, while polyacrylamide should be used for detailed characterization of dsRNAs present in a sample. Although exact molecular weights cannot be determined from agarose gels and minor dsRNAs may be overlooked, characteristic band separation and resolution of major dsRNAs can be accomplished in about 1 hr. The same determinations with polyacrylamide tube gels can take up to 12 hr (6) and with polyacrylamide slab gels can take up to 3 hr (7). When purifying dsRNA with SDS/KOAc and agarose gels for analyses, reliable diagnostic results for major dsRNAs can be obtained in 4 hr. The dsRNAs prepared by SDS/KOAc can be used in other applications, such as polymerase chain reaction assays and dot blot hybridizations.

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