

## Identification of dsRNAs Associated with Soybean Dwarf Virus-Infected Soybean

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### ABSTRACT

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Double-stranded (ds) RNA was isolated from leaves of soybean cultivar Wayne infected with the dwarfing (D) or yellowing (Y) strain of soybean dwarf virus (SDV). Each strain produced two virus-specific dsRNAs. SDV-D dsRNAs were estimated to have molecular weights of 3.4 and  $1.9 \times 10^6$  Da. The corresponding SDV-Y species were larger, having estimated molecular weights of 3.6 and  $2.2 \times 10^6$  Da. Northern blot hybridization analyses showed the strains are related and that the two

dsRNAs correspond to viral genomic-length and 3' subgenomic-length species. The detection of SDV dsRNA by polyacrylamide gel electrophoresis (dsRNA profiles) was improved by combining  $S_1$  nuclease with DNase in a one-step treatment of nucleic acids isolated by CF-11 column chromatography to remove contaminating single-stranded RNA. The size-specificity of SDV-D and SDV-Y dsRNAs was shown to be a useful phenotypic marker for in vitro strain differentiation.

Soybean dwarf virus (SDV) is an aphid-transmitted luteovirus that can cause high soybean yield losses in Japan (23) and is capable of infecting more than 50 leguminous species (4,22). Although SDV has not been reported to occur in U.S. soybeans, SDV-like isolates have been identified in U.S. forage legumes and are considered a threat to agriculture (11,15).

Tamada (22) identified two strains of SDV, dwarfing (D) and yellowing (Y), based on distinct differences in the symptomatology of SDV-infected soybeans. The two strains have small differences in host range (4,22) and physiochemical properties (9,13). SDV-D is transmitted more efficiently than SDV-Y by the foxglove aphid, *Aulacorthum solani* (Kaltenbach) (3). Subterranean clover red leaf virus (SCRLV), previously reported as a distinct virus of forage legumes in Australia and New Zealand, is now considered a strain of SDV (12).

SDV-D and SDV-Y are closely related serologically; antiserum to each strain completely neutralizes the infectivity of the heterologous strain (13). This has been corroborated by our laboratory, which has demonstrated that SDV-D and SDV-Y antigens are indistinguishable when a polyclonal SDV-D ELISA system is used (10). Furthermore, monoclonal antibody technology has been applied to SDV; however, strain-specific antibodies have not been identified (2).

As an alternative to serological approaches, we have investigated the application of dsRNA analysis to the problem of SDV strain differentiation. Here we report that both strains of SDV produce two dsRNAs in virus-infected soybean. Northern blot hybridization analysis has confirmed that these dsRNAs are viral, corresponding to genomic-length and 3' subgenomic length species. These dsRNAs are size-specific to each strain and are useful phenotypic markers for strain differentiation using dsRNA gel profiles.

### MATERIALS AND METHODS

**Virus strains and purification.** SDV-D and SDV-Y were maintained in soybean (*Glycine max* (L.) Merr. 'Wayne') by serial

aphid transmissions by a Japanese population of the aphid vector, *A. solani*, in the containment facility at Frederick (3). Virus was purified by an enzyme extraction method as previously described (9). Leaf tissue for dsRNA analysis was harvested 20–22 days postinoculation and stored for less than 1 mo at  $-20$  C. Uninoculated soybean plants were used as controls.

**dsRNA isolation.** Double-stranded RNA was isolated by CF-11 column chromatography (7,16). Leaves (20 g frozen weight) were pulverized in liquid nitrogen, transferred to a Model 534 mini-chopper (Varco, Inc., Belleville, NJ), and ground for 45 sec to a fine powder. After transfer to a beaker, 40 ml of  $2\times$  STE (50 mM Tris, 100 mM NaCl, 1 mM EDTA, pH 6.8, 6 ml of 10% SDS, 50 mg of macaloid clay (19), and 50 ml of saturated phenol) were added. The resulting slurry was stirred for 30 min at room temperature and then centrifuged (4,700 g) for 30 min at 12 C. The aqueous phase was collected and stirred (15 min) at room temperature with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). The final aqueous phase was collected by centrifugation, diluted with  $2\times$  STE to a total volume of 60 ml, and then adjusted to 16% ethanol by adding 12 ml of 95% ethanol. Whatman CF-11 cellulose powder (5 g) was added to the mixture and stirred for 45 min at room temperature followed by standing for 45 min. The supernatant was decanted and the CF-11 bound dsRNA was transferred to a column (2.5  $\times$  10 cm) (Bio-Rad, Rockville, NY) followed by washing at room temperature with 350 ml of  $1\times$  STE containing 16% ethanol to elute single-stranded (ss) RNA and then with 36 ml of  $1\times$  STE to elute dsRNA. Eluted dsRNA was collected by isopropanol/sodium acetate precipitation (20), suspended in 400  $\mu$ l of water, and stored at  $-70$  C.

**Northern blot hybridization.** Samples of ssRNA and dsRNA were recovered by ethanol/sodium acetate precipitation using 5  $\mu$ g of tRNA carrier, washed with 70% ethanol ( $-20$  C), and processed for formamide/formaldehyde denaturation by the methods of Palukaitis et al (17). Denatured samples were electrophoresed in a 1.2% agarose gel containing 0.66 M formaldehyde followed by transfer to nitrocellulose with  $10\times$  SSC ( $1\times$  SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) (5). Isolation of ssRNA from purified virions, 3' poly(A) tailing of ssRNA, oligo(dT) priming of first strand cDNA synthesis, cDNA cloning techniques, and hybridization techniques were as previously

described (20). RNA size markers (Bethesda Research Laboratories [BRL], Gaithersburg, MD) were detected by hybridization to lambda DNA (International Biotechnologies, Inc., New Haven, CT). Hybridization probes were labeled with  $^{32}\text{P}$  by nick-translation (18).

**DNase treatment.** Thawed preparations from CF-11 chromatography were treated with DNase for 30 min at 37 C in reactions (500  $\mu\text{l}$ ) containing 30 mM sodium acetate, pH 6.0, 5 mM  $\text{MgCl}_2$ , and 100  $\mu\text{g}/\text{ml}$  RNase-free DNase I (19). Reactions were terminated by two extractions with equal volumes of phenol/chloroform/isoamyl alcohol (25:24:1). After addition of one-tenth volume 3.0 M sodium acetate, pH 5.5, and one volume isopropanol, the preparations were stored at  $-20\text{ C}$ .

**Combined DNase and  $S_1$  nuclease treatment.** Thawed preparations from CF-11 chromatography were incubated at 68 C for 3 min to heat denature ssRNA. After ice quenching for 2 min, preparations were treated with DNase and  $S_1$  nuclease for 30 min at 37 C in reactions (500  $\mu\text{l}$ ) containing 30 mM sodium acetate, pH 4.5, 300 mM NaCl, 5 mM  $\text{MgCl}_2$ , 2 mM zinc acetate, 100  $\mu\text{g}/\text{ml}$  RNase-free DNase I (19), and 1,000 units/ml  $S_1$  nuclease (Boehringer Mannheim, Indianapolis, IN). Reactions were terminated as described for the DNase treatment followed by addition of one volume of isopropanol and storage at  $-20\text{ C}$ .

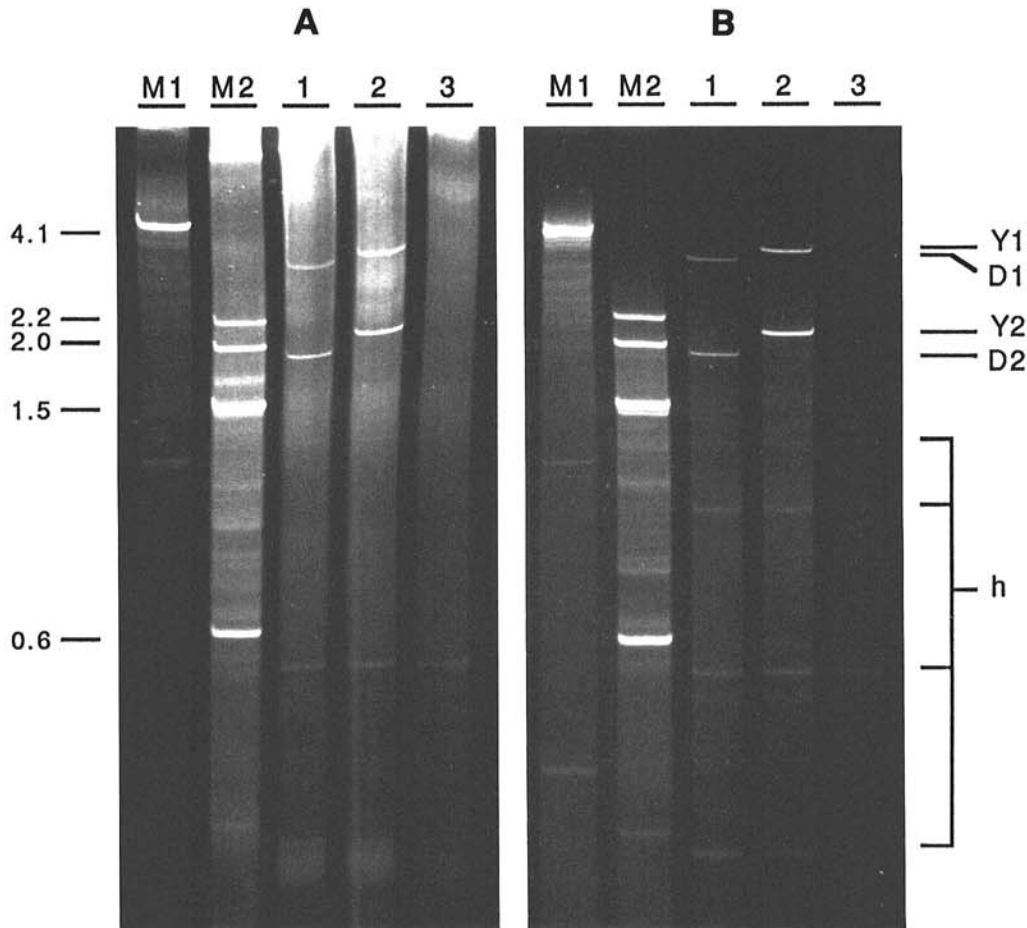
**Polyacrylamide gel electrophoresis (dsRNA profiles).** Samples were collected by centrifugation, washed with 70% ethanol ( $-20\text{ C}$ ), and suspended in water. After adjustment to TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) (14) containing 2.5% Ficoll Type 400 and 0.025% each of bromophenol blue and xylene

cyanole, samples were electrophoresed in vertical 5% polyacrylamide slab gels ( $180 \times 160 \times 15\text{ mm}$ ) using GelBond PAG support film (FMC BioProducts, Rockland, ME). After electrophoresis for 18 hr at 70 V using TAE buffer, gels were stained for 1 hr with 0.5  $\mu\text{g}/\text{ml}$  ethidium bromide (in TAE) followed by photography using transmitted UV illumination and Polaroid Type 57 film. The dsRNAs of tobacco mosaic virus (TMV) ( $4.1 \times 10^6\text{ Da}$ ) and brome mosaic virus (BMV) ( $2.2$ ,  $2.0$ ,  $1.5$ , and  $0.6 \times 10^6\text{ Da}$ ) were extracted from infected tissue as described above and used as size standards (8).

## RESULTS AND DISCUSSION

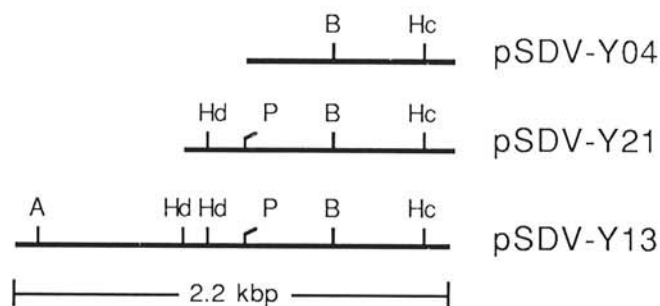
In repeated extractions, ssRNA was observed to contaminate preparations of dsRNA isolated by CF-11 chromatography and treated with DNase. We attempted lithium chloride fractionation (6) to remove this ssRNA but significant amounts remained (data not shown). We therefore supplemented the DNase treatment of dsRNA preparations to include enzyme activity for ssRNA by combining  $S_1$  nuclease (14) with DNase in a one-step treatment. The activities of DNase and  $S_1$  nuclease in this reaction were confirmed using lambda DNA, BRL ssRNA size markers, and total nucleic acid (DNA and RNA) from healthy soybean leaves (data not shown).

Figure 1 shows the gel profiles of dsRNA preparations treated with DNase alone (Fig. 1A) or DNase combined with  $S_1$  nuclease (Fig. 1B). The combined use of DNase and  $S_1$  nuclease improved the detection of dsRNA species by removing contaminating ssRNA (Fig. 1B). The dsRNA profiles of preparations from

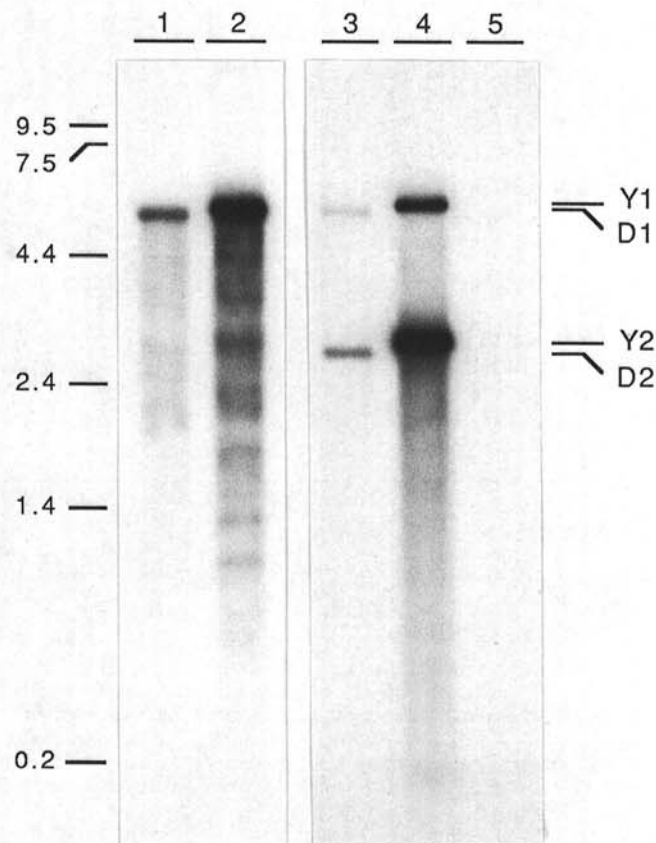


**Fig. 1.** Polyacrylamide gel electrophoresis of the dsRNAs associated with the dwarfing (D) and yellowing (Y) strains of soybean dwarf virus (SDV). **A**, Treatment of samples with DNase. **B**, Treatment of samples with DNase and  $S_1$  nuclease. Lanes M1 and M2 contain markers, TMV dsRNA ( $4.1 \times 10^6\text{ Da}$ ) and BMV dsRNAs (2.2, 2.0, 1.5, and  $0.6 \times 10^6\text{ Da}$ ), respectively (shown on the left), from 5 g-equivalents of virus-infected leaves. Lanes 1, 2, and 3 are dsRNAs extracted from 20 g-equivalents of SDV-D-infected, SDV-Y-infected, and healthy soybean cultivar Wayne leaves, respectively. The location of SDV-specific dsRNAs are shown on the right, abbreviated as follows: SDV-D (D1 and D2, lane 1) and SDV-Y (Y1 and Y2, lane 2). The positions of faint, ethidium bromide-stained, dsRNAs observed in healthy controls (lane 3) are marked on the right by the letter h. These same dsRNAs stained more intensely in virus-infected plants (lanes 1 and 2).

soybean plants infected with either SDV-D or SDV-Y showed that each produced two virus-specific dsRNAs (Fig. 1B, lanes 1 and 2). At least four additional, smaller-sized dsRNAs were observed; however, these dsRNAs were identical in both strains and present in healthy controls (Fig. 1B, lane 3 "h"). The dsRNAs identified in healthy controls stained less intensely with ethidium bromide relative to corresponding species from virus-infected plants (Fig. 1B). This suggests that these endogenous healthy plant



**Fig. 2.** Physical maps of SDV-Y 3' cDNA clones. Clones were produced from SDV-Y genomic ssRNA isolated from virions and 3' poly (A)-tailed prior to cDNA synthesis using oligo(dT) as a primer. Restriction enzyme sites are: *AccI* (A), *BamHI* (B), *HincII* (Hc), *HindIII* (Hd), and *PstI* (P). Clones are bordered by oligo(G)-oligo(C) tails and *PstI* sites (not shown).



**Fig. 3.** Northern blot hybridization of the RNAs associated with the dwarfing (D) and yellowing (Y) strains of soybean dwarf virus (SDV). The hybridization was conducted using on 3' SDV-Y cDNA clone, pSDV-Y13 (see Fig. 2). Lanes 1 and 2, 0.5  $\mu$ g of genomic ssRNA from virions of SDV-D and SDV-Y, respectively. Lanes 3, 4, and 5, denatured dsRNAs from 10 g-equivalents of SDV-D-infected, SDV-Y-infected, and healthy soybean cultivar Wayne leaves, respectively (treated with DNase and  $S_1$  nuclease). The location of denatured SDV dsRNAs are shown on the right, abbreviated as follows: SDV-D genomic RNA (D1, lanes 1 and 3), SDV-D subgenomic RNA (D2, lane 3), SDV-Y genomic RNA (Y1, lanes 2 and 4), and SDV-Y subgenomic RNA (Y2, lane 4). The position of BRL ssRNA size markers ranging from 0.24 to 9.5 kb are shown on the left.

dsRNAs may increase in concentration as a result of virus infection. Based on the migration of TMV and BMV dsRNA size markers, SDV-D dsRNAs were estimated to have molecular weights of 3.4 and  $1.9 \times 10^6$  Da. Similar estimates for SDV-Y dsRNAs were 3.6 and  $2.2 \times 10^6$  Da. The double-stranded nature of these virus-specific dsRNAs was confirmed by sensitivity to RNase A in low salt and resistance in high salt (1) (data not shown).

Confirmation that the two dsRNAs associated with each strain were SDV-related was obtained by northern blot hybridization analysis. The hybridization probe used for this analysis was selected from cDNA cloned to SDV-Y virion (genomic) ssRNA which was 3' poly(A)-tailed prior to cDNA synthesis. Restriction enzyme maps of three cDNAs are shown in Figure 2. Conservation of the *BamHI* and *HincII* map positions in these cDNAs indicates that variable lengths from the 3' terminus of SDV-Y genomic ssRNA were cloned. The largest clone, pSDV-Y13, was selected for use as a 3' hybridization probe. Hybridization of this probe to denatured dsRNA and genomic ssRNA from both viral strains (Fig. 3) demonstrated sequence relatedness and is consistent with reports that SDV-D and SDV-Y are closely related serologically (2,10,13). This probe did not hybridize to denatured dsRNA from healthy plants (Fig. 3, lane 5). The larger species of denatured dsRNA from each strain comigrated with the corresponding genomic ssRNA isolated from virions, indicating that this species is a double-stranded form of the genomic ssRNA. This genomic-length RNA had denatured length estimates of 5.8 and 6.1 kilobases (kb) for SDV-D and SDV-Y, respectively. The second, subgenomic-length, species had denatured estimates of 2.9 and 3.1 kb for SDV-D and SDV-Y, respectively. Based on the standard conversion that 1 kb of ssRNA approximates  $3.4 \times 10^5$  Da, these molecular weight estimates are in reasonable agreement with the values predicted for denaturation of the native dsRNAs (Fig. 1).

Polyacrylamide gel profiles of virus-specific dsRNA have been reported for two luteoviruses, barley yellow dwarf virus (BYDV) (8), and beet western yellows virus (BWYV) (6). The dsRNA profiles of two possible members of the luteovirus group, including strawberry mild yellow edge virus (SMYEV), also have been described (1,21). The number of virus-specific dsRNAs identified in the aforementioned studies ranged from two to five species, depending on the strain. As we have observed for SDV (Fig. 1), investigators studying BWYV (6) and SMYEV (21) also have reported dsRNA in healthy plant controls. The two dsRNAs associated with SDV infections are similar in molecular weight to the major dsRNAs reported for strains of BYDV (8) and BWYV (6). Applying the dsRNA nomenclature system of Gildow et al (8) to compare the genomic-length (ds-1) and subgenomic-length (ds-2) dsRNAs of SDV, BYDV (8), and BWYV (6), greater molecular weight variability occurs with luteovirus ds-2 species ( $1.4-2.2 \times 10^6$  Da) than with luteovirus ds-1 species ( $3.4-3.8 \times 10^6$  Da).

The use of polyacrylamide gel electrophoresis to distinguish two strains of SDV based on size-specific dsRNAs and the availability of cloned SDV cDNA should facilitate investigations into the etiology and epidemiology of SDV-like viruses reported to occur in U.S. forage legumes (11,15).

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