Subterranean Clover Distortion: A New Viruslike Disease with Unusual Characteristics

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ABSTRACT

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A new mechanically transmissible viruslike disease, subterranean clover distortion, was found in subterranean clover (*Trifolium subterraneum*) in a pasture in New South Wales. The pathogen has a wide experimental host range. Symptoms varied from thickening and distortion of young leaves, reddening of older leaves, and production of axillary shoots on subterranean clover to chlorosis of leaves, flower malformation, flower abortion, and stem necrosis on other hosts. Symptomless shoots often developed on infected plants. No local lesion host was identified. A disease-specific double-stranded RNA was isolated from tissues of infected subterranean clover, phlox, alfalfa, and broad bean plants with typical symptoms but not from tissues of healthy control plants or parts of inoculated plants that failed to show symptoms. The dsRNA was estimated to be 11-13 kbp (M_r 7.3-8.6 × 10^6). Three size classes of quasi-spherical particles (85-90, 66-70, and 50-55 nm in diameter) were detected in partially purified preparations. Electron microscopy indicated that the 85- to 90-nm and 66- to 70-nm particles possessed double membrane envelopes and single-walled membrane envelopes, respectively.

Subterranean clover (*Trifolium subterraneum* L.) is the most important legume in improved pastures in the southern states of Australia. It is an annual with seed germination in autumn, flower production in spring, and seed set in early summer. It is the host of three persistent aphid-transmitted virus diseases with red pigmented leaves as part of the disease syndrome: subterranean clover red leaf (1), which is closely related serologically to soybean dwarf, and beet western yellows (19), both of which are incited by luteoviruses (1,10), and subterranean clover stunt, which is associated with a DNA-containing viruslike particle (6).

In 1981, a plant with a mechanically transmissible disease was found among subterranean clover plants with red leaves collected on the Monaro Tablelands, New South Wales (isolate 1). A second isolate of the disease (isolate 2) was later obtained from greenhouse-grown alfalfa (*Medicago sativa* L. 'Hunter River'). Previously described as the "new disease" (16), it is now designated subterranean clover distortion (SCD) disease.

This paper reports symptoms and host range of the disease and the attempts to identify the pathogen.

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MATERIALS AND METHODS

Plant maintenance. Plants were grown in an aphid-proof greenhouse fumigated at 2-wk intervals and with temperatures ranging from 16 to 30 C. The two isolates were maintained on subterranean clover, phlox (*Phlox drummondii* Hook.), and alfalfa. Unless stated otherwise, all experimental work was done using isolate 1.

Mechanical inoculation. Leaf samples were ground in 0.1 M phosphate (pH 7.0 or 7.4) containing Celite and rubbed on leaves of test plants sprayed with Celite. In more recent experiments, 0.1% sodium sulfite was added to the phosphate buffer.

Chemotherapy with antibiotics. Rolitetracycline (Reverin) at 0.35 mg/ml or benzylpenicillin (sodium salt) at 0.5 mg/ml was applied at weekly intervals for six successive weeks each to four phlox and four subterranean clover plants with symptoms of SCD disease. The antibiotics were applied to the soil at 60-100 ml per 10-cm-diameter pot. Plants were maintained for 84 days after the first treatment. At the time of the first application of antibiotics, plants were trimmed and pots were placed in plastic saucers. Controls were two infected plants of each species treated with distilled water.

Nucleic acid extraction. Nucleic acids were extracted from plant tissues frozen in liquid nitrogen or from gradient fractions by the phenol-sodium dodecyl sulfate (SDS) method. Fractionation of nucleic acids into LiCl-soluble and LiCl-insoluble fractions was as described previously (5).

Enzymatic analysis of nucleic acids. Digestion of nucleic acids with pancreatic ribonuclease A (RNase A) in 2× SSC or 0.01× SSC (1× SSC = 0.15 M NaCl + 0.015 M sodium citrate, pH 7.0) and deoxyribonuclease I (DNase I) (Promega Biotech RQI DNase) was done as described previously (5).

Gel electrophoresis of proteins and nucleic acids. Proteins were analyzed in discontinuous SDS-polyacrylamide gels (21) and silver-stained (Bio-Rad). Nucleic acids were electrophoresed in either agarose gels in TBE buffer (89 mM Tris, 89 mM boric acid, and 2 mM EDTA, pH 8.3) or in polyacrylamide gels containing 7 M urea in TBE buffer (5). Nucleic acid bands were detected by ethidium bromide or silver stain (polyacrylamide gels only).

Purification of viruslike particles (VLPs). Two methods were used to purify VLPs from infected plants. The first was based on the method described by Gould et al (14). Briefly, infected plants showing typical symptoms were harvested 4, 6, or 10 mo postinoculation, pulverized in liquid nitrogen, and homogenized in 2 vol of 0.1 M phosphate buffer (pH 7.4) containing 0.5% thioglycolic acid. The homogenates were clarified in the absence of organic solvent and concentrated by polyethylene glycol precipitation. The precipitate was resuspended in phosphate buffer at a rate of 40 ml per 100 g of tissue. After differential centrifugation, the pellets were resuspended in 10 mM phosphate buffer with or without the addition of 10 mM EDTA or 10 mM EDTA + 1% Triton X-100, at a rate of 2 ml per 100 g of tissue. The extracts were then centrifuged through 10-40% sucrose density gradients at 40,000 rpm for 100 min in a Beckman SW41 rotor. Nine 1.5-ml fractions and one pellet were collected from each gradient. Material in each fraction was sedimented by high-speed centrifugation, resuspended in 150-μl buffer, and analyzed by electron microscopy, gel electrophoresis, and nucleic acid hybridization.

The second method was that used to purify tomato spotted wilt virus (TSWV) (27). Gradient fractions obtained from both the supernatant and the pellet after low-speed centrifugation of the extract were examined for TSWV-like particles by electron microscopy. The fractions were also analyzed by gel electrophoresis

and nucleic acid hybridization studies and subjected to enzyme-linked immunosorbent assay (ELISA) using TSWV antiserum.

Tests for presence of circular, viroid-like RNA. Both a one-dimensional and a two-dimensional gel electrophoresis system were used to determine if circular RNAs were present in infected tissues. Subterranean clover mottle virus, which contains a circular, satellite RNA (12), was used as a control. For one-dimensional gel electrophoresis, up to 2 μg of the LiCl-soluble and LiCl-insoluble nucleic acids from infected phlox, clover, and broad bean (*Vicia faba* L.) were electrophoresed in a 3% urea-polyacrylamide gel. Two-dimensional gel electrophoresis was done as described by Schumacher et al (25). In the first dimension, up to 50 μg of total nucleic acids was electrophoresed under native conditions in 4% polyacrylamide gel. In the second dimension, electrophoresis was in a 5% polyacrylamide gel containing 7 M urea and 25% formamide at room temperature. Gels were silver-stained.

Rapid detection of dsRNA in infected tissues. Approximately 8 μg of the LiCl-soluble nucleic acid was digested with 2 units of DNase I (Promega Biotech), then with either another 2 units of DNase I or 10 μg/ml of RNase A in 2× SSC or 10 μg/ml of RNase A in 0.01× SSC. After digestion, all reactions were stopped by addition of EDTA to a final concentration of 10 mM, followed by digestion of earlier added enzymes with 100 μg/ml of proteinase K (22). The nucleic acids were extracted with phenol-chloroform and concentrated by ethanol precipitation for gel electrophoresis. The molecular weight of the dsRNA was estimated using linear dsDNA fragments derived from lambda DNA cut with *Hin*dIII and *Eco*RI restriction enzymes (Bresatec).

Purification of dsRNA from infected tissues. dsRNA was extracted from healthy and SCD-infected tissues using a method described previously (20). Samples were then electrophoresed on 1% agarose gel, and nucleic acids were detected by staining with ethidium bromide.

Cloning of dsRNA from SCD-infected tissues and nucleic acid hybridization assays. SCD-specific DNA clones were obtained by cloning complementary DNAs (cDNAs) of the purified dsRNA (13). For first strand synthesis, 2 μl of purified dsRNA (approximately 0.5 μg) was boiled in water for 1 min in the presence of 1 μl of formamide, cooled in ice, and then random primed using avian myeloblastosis virus reverse transcriptase in the presence of hexamer primers (Bresatec) at 42 C for 90 min (22). The cDNA product was boiled, cooled in ice, and rehybridized at 65 C for 1 hr before being subjected to second strand synthesis using the Klenow fragment DNA polymerase (22). The double-stranded cDNA was treated with S1 nuclease and subsequently cloned into pUC8 by blunt-end ligation into the Sma I site, followed by transformation into *Escherichia coli* using the Hanahan method (15). SCD-specific clones were identified by hybridization of the radiolabeled nick-translated clones against purified nucleic acids from healthy and SCD-infected alfalfa using dot blot hybridization assay (22).

ELISA. An ELISA system for detection of TSWV was developed as described by Clark and Adams (7) from an antiserum specific for TSWV. Both the TSWV isolate and antiserum were gifts from R. I. B. Francki of the Waite Agricultural Research Institute.

Electron microscopy. SCD-infected tissue from phlox, subterranean clover, and tomato was fixed in 25 mM sodium phosphate buffer (pH 7.1) for 2 hr at 22 C, postfixed in 2% OsO_4, dehydrated through a graded series of ethanol, and embedded in Spurr's epoxy resin. Thin sections were stained with lead or uranyl salts and examined at either 60 or 80 kV (JOEL JEM 100S). Crude sap (using the leaf dip method) and density gradient fractions of partially purified preparations of SCD-infected and control phlox and subterranean clover were negatively stained with 2% ammonium molybdate (pH 6.5) and examined at 60 kV. The diameters of the negatively stained quasi-spherical particles observed in the gradient fractions were measured from prints, and magnification was checked against a diffraction grating replica.

RESULTS

Symptoms and host range. The first definitive symptoms of isolate 1 in subterranean clover (cv. Mt. Barker) were young, thick, irregularly shaped leaflets (Fig. 1). Symptoms on older plants included distorted leaves, short internodes, proliferating axillary shoots, apical necrosis, and reddened older leaves. Shoots of alfalfa plants inoculated with isolate 1 became chlorotic (Fig. 2) and, as with subterranean clover, showed reddening of older leaves and proliferation of axillary shoots. Symptoms developed slowly and erratically, with latent periods ranging from about 6 wk to 6 mo. Frequently, inoculated plants failed to develop symptoms.

The greenhouse-grown alfalfa plants from which isolate 2 was obtained (Fig. 3A) had been cut back at monthly intervals; this may have increased the severity of symptoms and account for the observed high incidence of the disease in that greenhouse. Plants were chlorotic and stunted, with numerous thin, upright shoots and with small, slightly cupped and rounded leaves, some with a yellow-green mottle. Eventually, plants became necrotic and died. Plants inoculated with this isolate were maintained in the same way as those inoculated with isolate 1, but in a separate greenhouse. Symptoms on phlox, subterranean clover, and alfalfa inoculated with isolate 2 were similar to those of plants inoculated with isolate 1, though perhaps more severe.

Phlox was the most useful host plant for diagnosis of SCD disease (Fig. 3B–D) and therefore was used most commonly for maintenance of the pathogen. The earliest time for definite symptoms to develop after inoculation of seedlings was 5–6 wk, when flowers were sometimes distorted (Fig. 3D), or petals were sometimes streaked with pigment or small but otherwise normal. A few weeks after inoculation, leaf veins were sometimes swollen. Some leaves showed white or yellow necrotic speckles or white tips (Fig. 3C). Leaves of older plants were often small and thickened, internodes were short, and shoots were stunted, giving the plants a bushy appearance. From time to time, plants apparently recovered and developed relatively normal appearing shoots and flowers. This "recovery" phenomenon also occurred sometimes in species other than *Phlox*.

No local lesion host was identified in any species tested, but a variety of systemic symptoms (Table 1) was observed in plants of six different families: Apocynaceae (*Vinca alba* L.); Asteraceae (*Chrysanthemum* × *morifolium* Ramat. 'Bonnie Jean', *Gynura aurantiaca* (Blume) DC., *Senecio cruentus* (Masson ex L'Hér.) DC., *Cineraria* spp.); Fabaceae (*Arachis hypogaea* L. 'Bunchy Top', *Medicago sativa* L. 'Hunter River', *Trifolium repens* L. 'New Zealand White', *T. subterraneum* L. 'Mt. Barker', *Vicia faba* L. 'Dwarf'); Polemoniaceae (*Phlox drummondii* Hook. 'Dwarf'); Solanaceae (*Lycopersicon esculentum* Mill. 'Rutgers' and 'White Cloud', *Nicotiana clevelandii* Gray, *N. glutinosa* L., *Petunia* × *hybrida* Hort. Vilm.-Andr.); and Tropaeolaceae (*Tropaeolum majus* L.). In some tests, only a few inoculated plants developed symptoms, presumably because of low levels of the pathogen in the inoculum or of unfavorable environmental conditions, or both.

No symptoms were observed in plants of the following: *Gomphrena globosa* L. (Amaranthaceae); *Helianthus annuus* L. (Asteraceae); *Chenopodium amaranticolor* Coste & Reyn. and *C. quinoa* Willd. (Chenopodiaceae); *Cucumis sativus* L. (Cucurbitaceae); *Phaseolus vulgaris* L. 'Pinto' (Fabaceae); *Antirrhinum majus* L. (Scrophulariaceae); and *Datura stramonium* L. and *Nicotiana tabacum* L. 'Samsun' (Solanaceae).

Treatment of SCD-infected plants with antibiotics. Neither tetracycline nor penicillin inhibited symptoms of SCD disease in subterranean clover or phlox. The experiment was discarded 84 days after the first treatment, at which time the two infected plants of subterranean clover that were treated with antibiotics

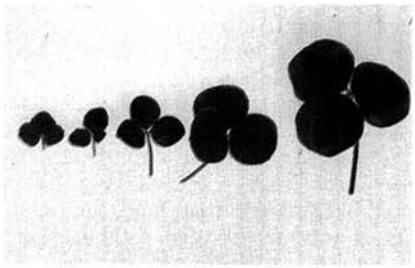

Fig. 1. Leaves of *Trifolium subterraneum* infected with isolate 1 of subterranean clover distortion disease. (Left to right) First two leaves are small, with distorted leaflets, slight rugosity, and veinclearing; next two leaves are older and show red-brown pigmentation on the margins; and leaf at right shows necrotic margin.

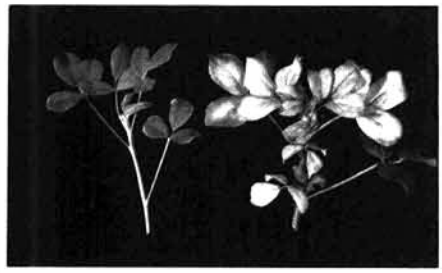

Fig. 2. (Left) Shoot from a healthy alfalfa plant and (right) plant inoculated with subterranean clover distortion disease, with general chlorosis and reddening of an older leaf.

Fig. 3. (A) Alfalfa plant (cv. Hunter River) naturally infected in a greenhouse with subterranean clover distortion (SCD) disease. Symptoms are similar to those of the source plant of isolate 2, including spindly stems, short internodes, and small, rounded leaves that may roll upward or downward. **(B–D)** Phlox infected with isolate 1 of SCD disease: **(B)** Two stunted plants showing partial recovery, with younger flowers less distorted than older proliferating ones; **(C)** white-tipped and speckled leaves on stunted shoot; and **(D)** small distorted flowers from infected plant shown in B (right) compared with flowers from an uninoculated control plant.

were dead. Infected control plants of subterranean clover and phlox that were sprayed with distilled water continued to produce symptoms. Symptoms that developed in healthy control plants of subterranean clover presumably resulted from cross-contamination. This was unexpected, as care had been taken to avoid accidental mechanical transmission. A search in the greenhouse for possible virus vectors such as aphids or thrips proved negative.

Absence of low molecular weight circular, viroidlike RNAs. Attempts to detect circular RNA in infected alfalfa, phlox, subterranean clover, and broad bean plants were unsuccessful. No disease-specific RNA species with molecular weights characteristic of viroids (8) or satellite RNAs (11) were detected by use of either a high-resolution one-dimensional urea-polyacrylamide gel capable of detecting viroidlike satellite RNA in total nucleic acid preparations (5) or a sensitive two-dimensional gel electrophoresis technique (25). These methods readily detected satellite RNA of subterranean clover mottle virus (12).

Detection of a high molecular weight dsRNA. A single major species of dsRNA was consistently detected in nucleic acid preparations from infected plants, but not from healthy control plants, of alfalfa and phlox (isolates 1 and 2), subterranean clover (isolate 2), and broad bean (isolate 1) showing typical symptoms. Figure 4 shows the dsRNA band identified by the rapid dsRNA detection method and confirmed by its resistance to DNase I and RNase A in 2× SSC but not to RNase A in 0.01× SSC. Some diffused staining material in the infected lanes that was resistant to both nucleases was probably polysaccharide. The dsRNA comigrated with the chromosomal DNA in this gel system and was detected only after digestion of the DNA and the ssRNAs. By use of this differential digestion method, approximately 10 ng of dsRNA could be detected in 20–40 mg of selected phlox or alfalfa leaf tissues. The same dsRNA was obtained from infected tissues at various times after infection but not before the onset of symptoms. The yield of dsRNA was higher from tissues with severe stunting symptoms than from tissues with milder symptoms.

The approximate molecular weight of the dsRNA from infected alfalfa (isolates 1 and 2) was obtained using CF-11 cellulose column purified preparations. The dsRNA from both isolates was estimated to be between 11 and 13 kbp (relative molecular weights of $7.3–8.6 \times 10^6$) (Fig. 5A). On storage, the dsRNA degraded to produce five minor, smaller molecular weight RNA bands, with sizes ranging from 1.1 to 3.5 kbp (Fig. 5B). Similar minor bands were also detected in dsRNA prepared from shoots of deteriorating older plants that had been

infected for more than 18 mo. These results suggest that the minor RNA bands were derived from degradation of the high molecular weight RNA band at specific sites, possibly single-stranded.

Detection of disease-specific nucleic acids in infected tissues by nucleic acid hybridization. The sizes of the inserts in the cDNA clones of the SCD-specific dsRNA ranged from approximately 100 to 650 bp. The specificity of the clones was demonstrated by hybridization of the radiolabeled clones on dot blots of LiCl-insoluble nucleic acids prepared from healthy plants and from infected alfalfa plants showing either mild yellowing or a severe necrotic symptom (Fig. 6). This result also demonstrated the presence of single-stranded disease-specific nucleic acids related to the dsRNA in SCD-infected plants but not in healthy plants.

Detection and properties of spherical VLPs in infected plant extracts. No VLPs were detected in sap from freshly ground infected tissue of phlox or subterranean clover by the leaf dip method or in thin sections of fixed and embedded infected leaves of phlox, subterranean clover, and tomato. However, large, quasi-spherical VLPs (50–90 nm in diameter) were identified in partially purified extracts from both infected phlox (isolate 1) harvested 4 and 10 mo postinoculation and subterranean clover (isolate 2) harvested 6 mo postinoculation using the first method described previously but not the second method. Extracts from the same plants had previously been found to contain disease-specific dsRNA. No such VLPs were detected in corresponding extracts from healthy control plants.

When the partially purified extracts from diseased plants obtained by the first method were purified by sucrose density gradient centrifugation, no disease-specific UV-absorbing peak was obtained. However, an examination of the resulting gradient fractions by electron microscopy showed the presence of VLPs (Fig. 7). These particles were distributed in three size classes: 85- to 90-nm diameter (mainly in fractions 7 and 8, corresponding to 30–40% sucrose), 66- to 70-nm diameter, and 50- to 55-nm diameter (mainly in fractions 5 and 6, corresponding to 20–30% sucrose), all with either thick or thin walls. Some 85- to 90-nm particles appeared to have membrane envelopes. VLPs were not detected in comparable gradient fractions from uninfected control plants. Although relatively few, VLPs were consistently present in duplicate preparations from several different hosts. Some 30-nm-diameter particles were detected in gradient fractions 3 and 4 (corresponding to 10–20% sucrose) from both healthy and infected phlox (Fig. 7, Table 2) but not in subterranean clover.

When phosphate extracts from infected plants were resuspended in the presence of 10 mM EDTA (phlox only) before sucrose density centrifugation, the numbers of 66- to 70-nm and 85- to 90-nm particles in the gradient fractions diminished (Table 2), and no 30-nm particles could be detected in extracts from healthy phlox plants. When phosphate extracts of phlox and subterranean clover were resuspended in the presence of 10 mM EDTA plus 1% Triton X-100, no 85- to 90-nm particles and only a few 50- to 55-nm and 66- to 70-nm particles remained (Table 2). These results suggest that the VLPs associated with SCD are indeed enveloped and require divalent cations for stability.

Comparison of SCD with TSW. Sap from both TSWV-infected *N. glutinosa* and SCD-infected phlox was inoculated at the same time to 10 plants of each of eight species. Characteristic symptoms of TSW developed in the known hosts (2,18) *N. tabacum, N. clevelandii, N. glutinosa, L. esculentum*, and *T. majus* within 3–14 days and in phlox within 30 days. No symptoms of TSWV developed in subterranean clover cv. Mt. Barker, even though cv. Wenigup is a known host of TSWV (17), or in alfalfa, which is not listed as a host (2). Extracts of these TSWV-inoculated subterranean clover and alfalfa plants gave no detectable serological reaction when tested for TSWV by ELISA. In contrast, sap from SCD-infected plants produced symptoms on phlox, subterranean clover, and alfalfa, but only after 3–4 mo. After 6 mo, no clearly definitive symptoms of SCD had been observed on the three *Nicotiana* species or on tomato and

Table 1. Symptoms of subterranean clover distortion disease

Plant part Symptoms	Hosts
Leaves	
Reduced size	*Phlox drummondii, Vinca alba*
Distortion and asymmetry	*Cineraria* spp., *Gynura aurantiaca, Lycopersicon esculentum*
Veinclearing, mottle, or both	*Arachis hypogaea*
Vein enlargement, necrosis, or both	*G. aurantiaca, L. esculentum*
White or yellow speckles	*P. drummondii, Trifolium repens*
Occasional ring spots	*Petunia × hybrida*
Yellow, red, or purple pigment	*L. esculentum, Medicago sativa, Trifolium subterraneum*
Shoots	
Apical or stem necrosis, or both	*Nicotiana clevelandii, N. glutinosa, Vicia faba*
Proliferation	*M. sativa, P. drummondii*
Flowers	
Color break	*P. drummondii*
Distortion, abortion, or both	*L. esculentum, P. drummondii*
Reduced size	*P. drummondii, Vinca alba*

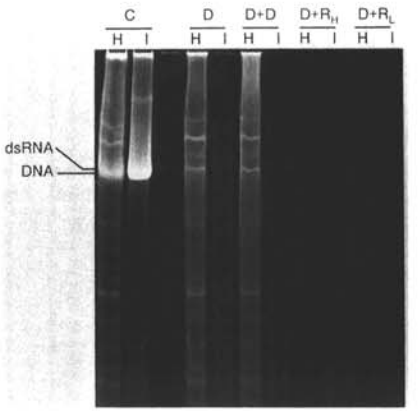

Fig. 4. Detection of disease-specific dsRNA in phlox infected with subterranean clover distortion disease. First, 20-μg aliquots of nucleic acids from 40 mg of fresh healthy (H) and infected (I) tissues were fractionated in 2 M LiCl, then the LiCl-soluble fractions were electrophoresed in a 3% polyacrylamide gel containing 6 M urea after digestion with enzymes. C = Control, D = DNase I digested, D+D = two consecutive digestions with DNase (to ensure complete digestion of DNA), $D+R_H$ = RNase A digestion in 2× SSC after DNase treatment, and $D+R_L$ = RNase digestion in 0.01× SSC after DNase treatment.

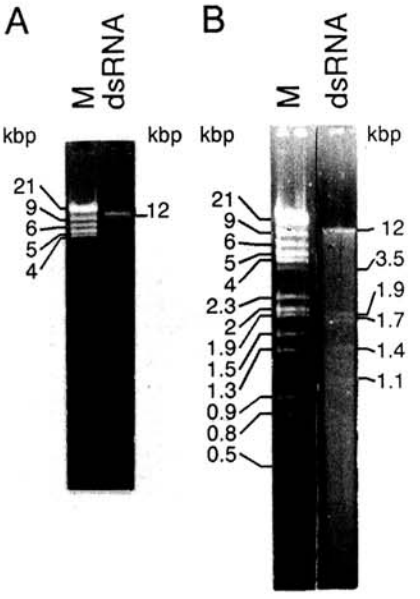

Fig. 5. Agarose gel electrophoresis of dsRNA isolated from alfalfa infected with subterranean clover distortion disease and specific for the disease. **(A)** Aliquot of dsRNA purified by CF-11 cellulose (dsRNA lane) was electrophoresed in 1% agarose gel. Molecular size of the dsRNA, estimated by dsDNA markers (M), was based on electrophoresis of five samples of dsRNA in three separate gels. **(B)** Gel profile of the dsRNA preparation after storage at −20 C for 1 mo. Molecular sizes of the minor bands range from 1.1 to 3.5 kbp.

nasturtium, even though in earlier tests *N. clevelandii*, tomato, and nasturtium were found to be hosts of the disease. Presumably, this difference in results relates to the inconsistency of development of symptoms in inoculated plants. Symptoms of TSW and SCD on phlox were distinct. After inoculation with TSWV, systemically infected leaves showed diffuse spots 3–5 mm in diameter and subsequently became chlorotic, but flowers showed no symptoms. After inoculation with SCD, petals were sometimes streaked or reduced in size or flowers were distorted. Leaf veins were sometimes swollen, and some leaves showed white or yellow necrotic speckles or white tips. Older plants often had a bushy appearance. The results indicate differences between the SCD and TSW diseases in respect to symptoms and host range.

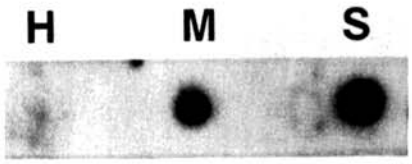

Fig. 6. Dot blot hybridization of nucleic acids specific for subterranean clover distortion disease. A clone (380 bp) specific for the disease was nick-translated and used as a probe. The probe was hybridized to 1-μl aliquots of LiCl-insoluble fractions of nucleic acids prepared from healthy alfalfa (H) and infected alfalfa showing either a mild (M) or a severe (S) symptom and spotted on nitrocellulose membranes.

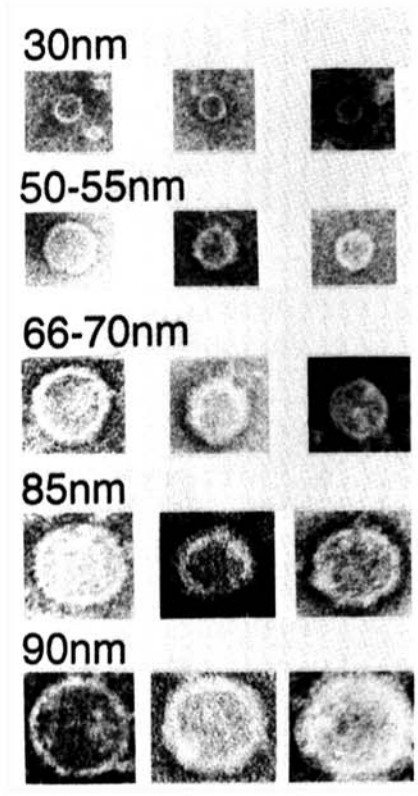

Fig. 7. Diameters of spherical viruslike particles associated with plants with subterranean clover distortion disease. Partially purified preparations of the particles were obtained from infected phlox and subterranean clover plants by sucrose density gradient centrifugation. The 30-nm particles were found in both healthy and infected phlox plants but not in subterranean clover plants.

Additional tests were made to examine possible relationships between the SCD and TSW diseases. When extracts from SCD- and TSW-infected plants were examined by means of ELISA, using antiserum to TSWV, no cross-reaction was observed. Also, a virus purification procedure known to produce large numbers of particles of TSWV (27) failed to produce similar particles with SCD-infected tissue when the gradient fractions were analyzed by electron microscopy. Gel electrophoresis, nucleic acid hybridization, and ELISA using TSWV antiserum also failed to indicate the presence of SCD VLPs. Moreover, electron microscopy of thin sections of SCD-infected tissues provided no evidence of the presence of TSWV-like particles, possibly because of the paucity of VLPs.

DISCUSSION

Symptoms of SCD disease in a range of hosts suggest that the pathogen could be a mycoplasmalike or a rickettsialike organism, a viroid, or a virus. Three lines of evidence provide no indication that the pathogen is a mycoplasmalike or a rickettsialike organism (24,28): 1) Such organisms were not detected in sap extracts or thin sections examined by electron microscopy, 2) no evidence was obtained that progress of the disease in phlox and subterranean clover was inhibited by treatment of inoculated plants with penicillin or tetracycline, and 3) mechanical transmission is unknown for these organisms. Similarly, the possibility that the disease is caused by either a viroid or a virus-associated circular satellite RNA is incompatible with the size and relatively large amounts of dsRNA in the absence of detectable circular RNA (4,5).

The presence of large amounts of dsRNA suggests that the pathogen is a dsRNA virus or that the dsRNA is the replicative form of an ssRNA virus (9).

Table 2. Numbers and diameters of quasi-spherical viruslike particles (VLPs) in sucrose density gradient fractions of extracts from phlox and subterranean clover infected with subterranean clover distortion disease[a]

Host	Particle diameter (nm)	Phosphate buffer[b]					
		Alone		+EDTA		+EDTA+TX	
		H	I	H	I	H	I
Phlox	30	8	6	0	2	0	1
	50–55	0	4	0	10	0	4
	66–70	0	14	0	8	0	6
	85–90	0	28	0	12	0	0
	Sum (≥50)	0	46	0	30	0	10
Subterranean clover	30	0	0	...[c]	...	0	0
	50–55	0	10	...	...	0	3
	66–70	0	22	...	...	0	1
	85–90	0	30	...	...	0	0
	Sum (≥50)	0	62	...	...	0	4

[a] Particle counts were obtained by electron microscopy of two grids per treatment.
[b] Healthy (H) and infected (I) plants of phlox were harvested 4 mo, and those of subterranean clover 6 mo, postinoculation. (In phlox extracted with phosphate buffer 10 mo postinoculation, the numbers of VLPs 50–55, 66–70, and 85–90 nm in diameter were 38, 32, and 26, respectively.) VLP preparations were resuspended in 10 mM phosphate buffer (pH 7.4), phosphate buffer + 10 mM EDTA (+EDTA), or phosphate buffer + 10 mM EDTA + 1% Triton X-100 (+EDTA+TX).
[c] Not done.

Table 3. Characteristics of subterranean clover distortion (SCD) and tomato spotted wilt (TSW) diseases

Characteristic	SCD	TSW
Genome	Single species dsRNA, 11–13 kbp	Segmented ssRNA, 2.7, 1.7, and 1.1 kb
Particle morphology	Quasi-spherical, 85–90 nm in diameter, enveloped	Quasi-spherical, 85 nm in diameter, enveloped
Particle detection	Few, in partially purified extracts only	Many, in thin sections, by leaf dip, in partially purified extracts
Transmission	Mechanical, vector transmission not examined	Mechanical, thrips
Latent period	30–180 days	2–14 days in some diagnostic hosts
Experimental host range	Wide	Wide
Symptoms	Variable, systemic only	Variable, local or systemic or both

If the pathogen is a dsRNA virus, it differs from any known group of dsRNA viruses, e.g., the Reoviridae, Cystoviridae, Birnaviridae, or the mycoviruses and cryptic viruses (3,23). Likewise, if it is an ssRNA virus, the pathogen differs from any known group of plant ssRNA viruses (23). Because large quasi-spherical VLPs, as well as dsRNA, were detected in infected tissues at various times after inoculation, we suggest that the particles were those of the pathogen inciting SCD disease and that the dsRNA was a form of the nucleic acid of the pathogen.

The composition of the VLPs is unknown. Insufficient material was available for determination of spectrophotometric and sedimentation characteristics, and no virus-specific protein or nucleic acid bands could be detected when gradient fractions were analyzed by gel electrophoresis. For the same reason, no signals were detected when nucleic acids prepared from these gradient fractions were subjected to dot blot hybridization using the cloned SCD-specific DNA probes, and relationships between different classes of VLPs could not be elucidated. The observed differences in particle size may represent different states of decomposition of the large, double membrane particles.

Several characteristics of SCD and TSW suggest that the two diseases could be related (Table 3). For example, infected tissues of both diseases contain large enveloped VLPs and both diseases infect a wide host range that includes subterranean clover (17). Further, inconsistencies of transmission and symptom expression observed with SCD have been reported for TSW (2). For example, Selman and Milne (26) reported cyclical changes in virus multiplication in TSW-infected plants. Failure in the present work to obtain a cross-reaction between extracts of the two diseases with ELISA, to purify VLPs by means of a method known to be suitable for purification of TSW, and to observe VLPs by electron microscopy in thin sections could be due to major strain differences between the isolates examined, to low levels of VLPs in SCD-infected tissues, or to both.

The pathogen inciting SCD disease is distinct from that inciting TSW because infected plants contain unsegmented dsRNA of high molecular weight and small numbers of VLPs in sap extracts. Furthermore, no TSWV local lesion host produced local lesions when inoculated with SCD, and both the symptoms on key diagnostic hosts and the long latent period were uncharacteristic of plants infected with TSWV.

These characteristics of SCD differ from those of any known plant pathogen. Therefore, we suggest that SCD represents a new group of RNA viruses. Further work will be required for this to be established conclusively.

Difficulties in characterizing the pathogen of SCD disease relate to the variable and slow development of symptoms and the small numbers of VLPs in infected tissues. Work would be facilitated if a host plant were found in which the pathogen of SCD increased to high concentrations and the latent period was short.

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