Properties and Cytopathology of Diodia Vein Chlorosis Virus—A New Whitefly-Transmitted Virus

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ABSTRACT

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A virus causing chlorosis and vein-clearing symptoms in *Diodia virginiana* from Arkansas was identified. The virus, designated as *Diodia* vein chlorosis virus (DVCV), was transmitted by the bandedwinged whitefly, *Trialeurodes abutilonea*, and by grafting but not by sap inoculation. *T. abutilonea* has not been reported to be a vector of plant viruses. Attempts to purify the virus resulted in low yields of fragmented flexuous rods approximately 12 nm in diameter. *D. virginiana* is the only known host plant for this virus. Ultrastructural studies of diseased leaves revealed the consistent presence of cytopathic effects characteristic of closterovirus infections. These include the occurrence of flexuous, rod-shaped viruslike particles, approximately 12 nm in diameter, in the cytoplasm of phloem parenchyma cells and sieve elements associated with membranous vesicles containing fibrils. In addition, greatly proliferated tubular membranes in the cytoplasm and unique double membrane-bound bodies within the vacuoles, which have not been reported to be associated with other closteroviruses, also were found. Double-stranded RNA isolated from DVCV-infected *Diodia* and subjected to polyacrylamide gel electrophoresis consisted of three bands with estimated M_r of 4.6, 4.3, and 1.9×10^6, which were similar in nature to those exhibited by the closterovirus group. We conclude that the agent causing the symptoms in *D. virginiana* is probably a closterovirus.

Additional keywords: virus-induced inclusions.

Virginia buttonweed, *Diodia virginiana* L., is a common low ground weed occurring throughout the southeastern United States. A previously undescribed disease of *D. virginiana* with viruslike symptoms of vein clearing and chlorosis (Fig. 1) is widely distributed in Arkansas and Louisiana. Some of the diseased plants were colonized by the abutilonea or bandedwinged whitefly, *Trialeurodes abutilonea* (Haldeman), which was suspected to be the vector of the agent causing the disease. This whitefly has not been demonstrated previously to be a vector of plant viruses.

Preliminary ultrastructural examination of leaves of *D. virginiana* with symptoms revealed long, flexuous, rod-shaped viruslike particles and membranous vesicles containing fibrils, both of which are characteristic cytopathic structures associated with plants infected by viruses of the closterovirus group (13,19). This paper describes studies of the transmission of the disease agent and the ultrastructure and double-stranded RNA (dsRNA) analysis of *D. virginiana* with vein-clearing and chlorosis symptoms. The results suggest that the disease is caused by a closterovirus.

MATERIALS AND METHODS

Virus source and whitefly maintenance. Plants of *D. virginiana* exhibiting disease symptoms were collected from their low ground habitat and maintained in the greenhouse. Symptomless plants also were collected and propagated from stem cuttings or seed.

T. abutilonea were collected from cotton growing at the Agricultural Experiment Station, University of Arkansas, Fayetteville. Colonies were reared and maintained in a greenhouse at 18–32 C on the Chinese lantern plant, *Physalis alkekengi* L., in muslin-covered cages (2). Whiteflies were tested periodically for contamination by transmission tests on healthy *Diodia* plants.

Transmission and host-range studies. Approximately 50 nonviruliferous whiteflies in leaf cages (3) were given a 24-hr acquisition access period on diseased *Diodia* plants and were transferred to five to 10 healthy plants for a 24-hr inoculation access period. Further tests for transmissibility of the disease-causing agent were performed using grafts of diseased *Diodia* plants onto healthy *Diodia* seedlings. Numerous attempts at mechanical transmission consisted of macerating diseased *Diodia* plants in 2% nicotine 0.01 M PO_4 buffer, pH 7.0, or in 0.1 M phosphate buffer (Na_2HPO_4-KH_2PO_4), pH 7.2, containing 50 mM Na_2SO_3, and rubbing the leaf extract onto symptomless Carborundum-dusted *Diodia* seedlings.

Virus purification. Three previously described purification methods for closteroviruses were used in attempts to purify the disease-causing agent. Leaves and stem tips were collected from infected *Diodia* plants exhibiting typical vein-clearing symptoms and processed by the methods described for citrus tristeza virus (18), a closterovirus from *Nandina* (1), and lettuce infectious yellows virus (LIYV) (8).

Purification and electrophoresis of dsRNA. Double-stranded RNA was extracted from freshly harvested leaves of *D. virginiana* exhibiting vein chlorosis symptoms using a modification of the method described by Morris and Dodds (20). Tissue (7 g) was harvested and ground with a mortar and pestle using liquid nitrogen. The powder was suspended in 14 ml of STE buffer (0.1 M NaCl, 0.05 M tris, 1 mM ethylenediaminetetraacetic acid [EDTA], pH 7.0), 18 ml of STE-saturated phenol, 2 ml of 10% sodium dodecyl sulfate (SDS), and 16 mg of fractionated bentonite (11). The mixture was shaken every 3–5 min for 30 min at room temperature. The emulsion was broken by centrifugation for 5 min at 3,000 *g* followed by 10 min at 8,000 *g*. The supernatant was removed, adjusted to 16% ethanol, and subjected to two cycles of fractionation through 2.5 g (dry weight) of a Whatman CF-11 cellulose column (22). The dsRNA suspension was applied to the column and washed with 100 ml of STE buffer adjusted to 16% ethanol. Double-stranded RNA was collected from the cellulose columns by elution with 15 ml of STE buffer. The sample was concentrated by the addition of 2.5 v of 95% ethanol and 0.05 v of 3.0 M sodium acetate, pH 5.5, and was stored overnight at −20 C. After centrifugation for 30 min at 12,000 *g*, pellets were washed in 70% ethanol, dried, and resuspended in 100 µl of electrophoresis buffer (40 mM tris, 10 mM sodium acetate, 1 mM EDTA, pH 7.8).

Electrophoresis of dsRNA was performed on a 6% acrylamide and bis-acrylamide gel (40:1) using a vertical mini-slab apparatus (6.5 cm × 9.5 cm × 0.8 mm) (Idea Scientific, Minneapolis, MN) for 4 hr at 100 V. Gels were stained with ethidium bromide (20 µg/ml) for 15 min, briefly destained in double-distilled water,

and visualized on an ultraviolet-light transilluminator at 302 nm. Gels were photographed with Wratten 23A and 9 filters using Polaroid type 665 positive/negative film. Reference dsRNA from tobacco mosaic virus (TMV) (22), tobacco necrosis virus (TNV) (4), and cucumber mosaic virus (CMV) (15) with known relative masses (M_r) were used as standard markers. Double-stranded RNA associated with beet yellows virus (BYV), an aphid-transmitted closterovirus (7), and LIYV (8), a virus transmitted by the whitefly *Bemisia tabaci* (Genn.) and believed to be a member of the closterovirus group, also were included in these experiments.

Serology. Antisera to BYV (kindly supplied by R. Reed, University of California, Davis) and to LIYV prepared as previously described (8) were used in SDS immunodiffusion tests (21). Positive control antigens were supplied by J. E. Duffus (U.S. Department of Agriculture-Agriculture Research Service, Salinas, CA).

Electron microscopy. Leaf samples, 1–2 mm square, from symptomless tissue and tissue showing typical vein-clearing and chlorosis symptoms were prepared for transmission electron microscopy as described previously (16). Sections cut with a diamond knife were double stained with 2% aqueous uranyl acetate followed by lead citrate.

RESULTS

Transmission studies and host range. *Diodia* plants began to show characteristic symptoms (Fig. 1) beginning with vein clearing on the newer leaves about 2 wk after exposure to whiteflies that had been given an acquisition access feeding on affected plants. *Diodia* plants exposed to whiteflies that had been fed on symptomless *Diodia* plants failed to produce symptoms.

Typical vein-clearing symptoms appeared on all *Diodia* seedlings within 1 mo after grafting. All attempts to transmit this presumed virus by mechanical means to healthy *Diodia* plants failed.

The following plants exposed to viruliferous whiteflies were not susceptible to the disease agent: Chenopodiaceae—*Beta vulgaris* L., *Chenopodium murale* L., *C. quinoa* Willd., *Spinacia oleracea* L.; Compositae—*Lactuca sativa* L.; Cucurbitaceae—*Cucurbita maxima* Duchesne, *Cucumis sativus* L.; Malvaceae—*Malva parviflora* L.; Solanaceae—*Datura stramonium* L., *Nicotiana tabacum* L. 'Kentucky 16'; Leguminosae—*Phaseolus vulgaris* L. 'Black Valentine.'

Virus purification. Of the three methods applied to tissue infected with *Diodia* vein chlorosis virus (DVCV), only the preparation obtained by the citrus tristeza purification technique (18) contained flexuous, rod-shaped particles approximately 12 nm in diameter. The virus yield, however, was so low that further characterization studies were not pursued.

Analysis of dsRNA. The dsRNA isolated from DVCV-infected plants of *D. virginiana* was lower in concentration than the dsRNA obtained from the same amount of tissue from plants infected with TMV, TNV, and CMV. Double-stranded RNA from the DVCV-infected plants consisted of two minor bands with estimated M_r of 4.6 and 4.3×10^6 and a more intensely fluorescing major band of about 1.9×10^6 M_r (Fig. 2). No bands were detected in healthy controls. The well-characterized closterovirus BYV produced a single major band with a value of 5.6×10^6 M_r and four smaller bands, which are consistent with those found by Dodds and Bar-Joseph for this virus (5). Two bands were detected from the LIYV transmitted by *B. tabaci*. The band with the slowest mobility was approximately 4.5×10^6 M_r. This is very close to the same M_r of the two slower migrating minor bands of DVCV. The second band of approximately 2.6×10^6 M_r was considerably different in its mobility compared with the major band of DVCV.

The double-stranded nature of the RNA was determined by incubation of the bands in the gels with ribonuclease (RNase) A at 4 μg/ml in distilled water or in 0.45 M NaCl (10). The RNA was incubated for 30 min at 20 C. Double-stranded RNA was digested by RNase in water but was not affected by RNase in NaCl.

Serology. Only the homologous antisera and antigens formed precipitin bands in SDS immunodiffusion tests. That is, LIYV reacted only with its antiserum, and BYV reacted only with its antiserum. No reaction occurred with DVCV antigen.

Electron microscopy. Ultrastructural studies of the diseased leaves of *D. viginiana* revealed a consistent presence of flexuous, rod-shaped viruslike particles and associated cytopathic changes characteristic of plants infected by most closteroviruses (Figs. 3–5), which were absent in symptomless leaves. These particles, which were assumed to be virus particles and which will be referred to as such hereafter, were approximately 12 nm in diameter with

Fig. 1. Leaves of Virginia buttonweed, *Diodia virginiana*, from a healthy plant (left) and an infected plant with typical vein-clearing and chlorotic symptoms (right).

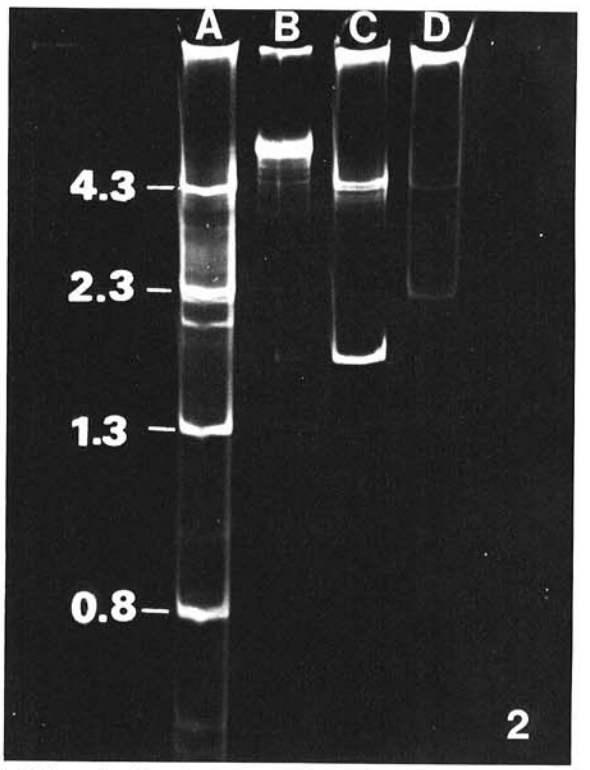
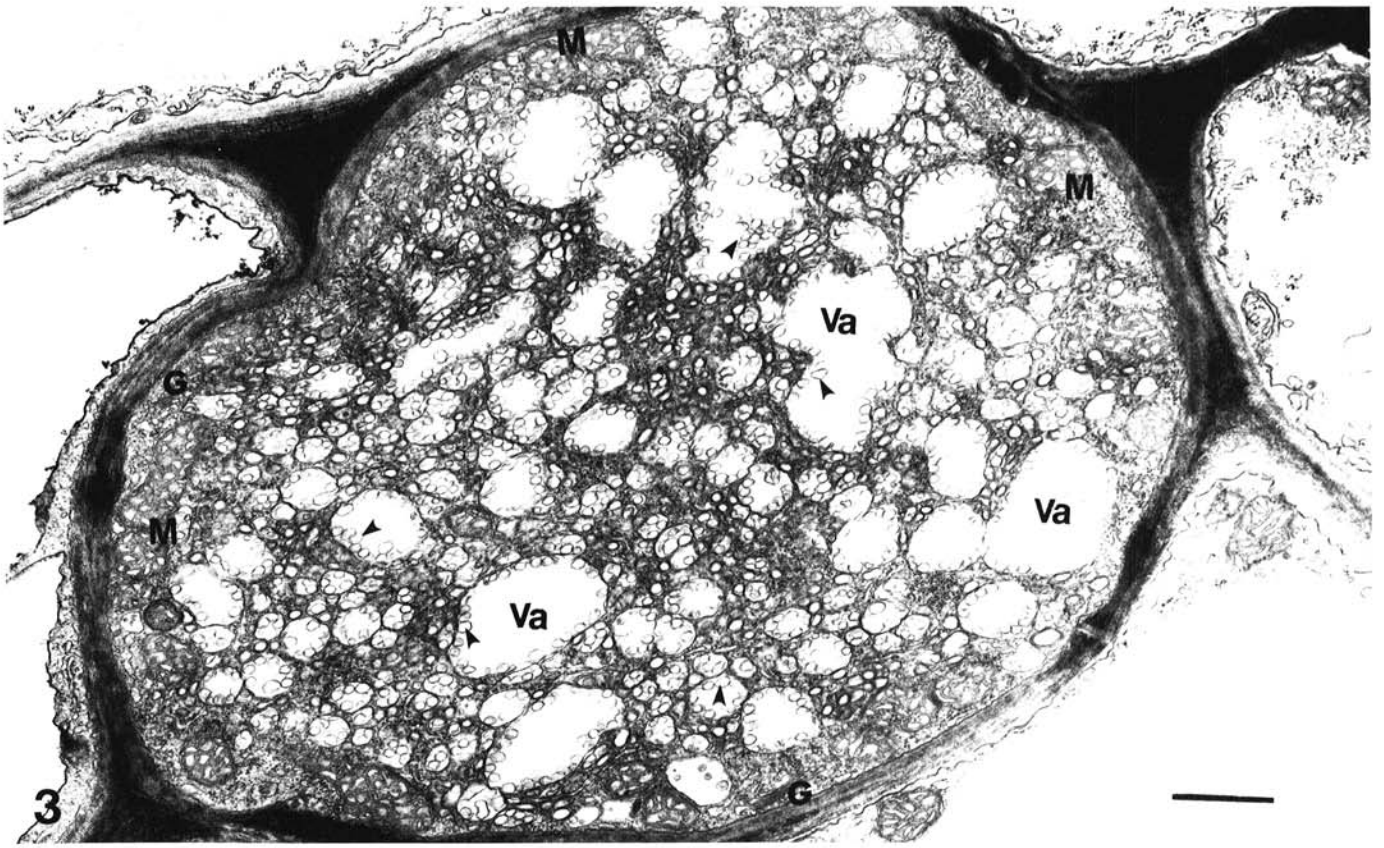
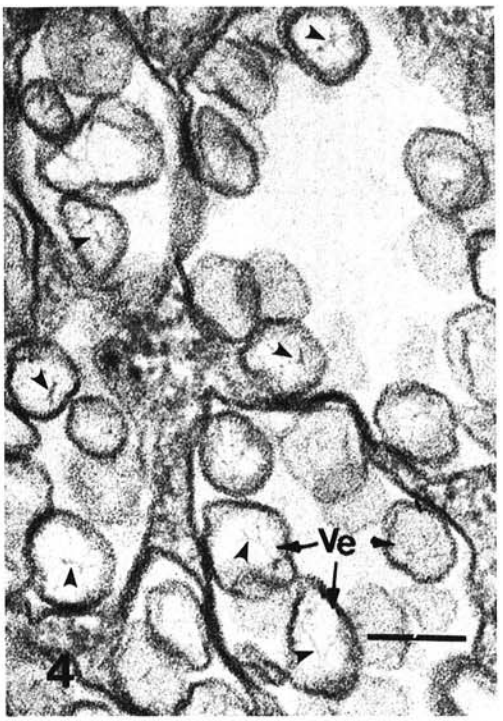
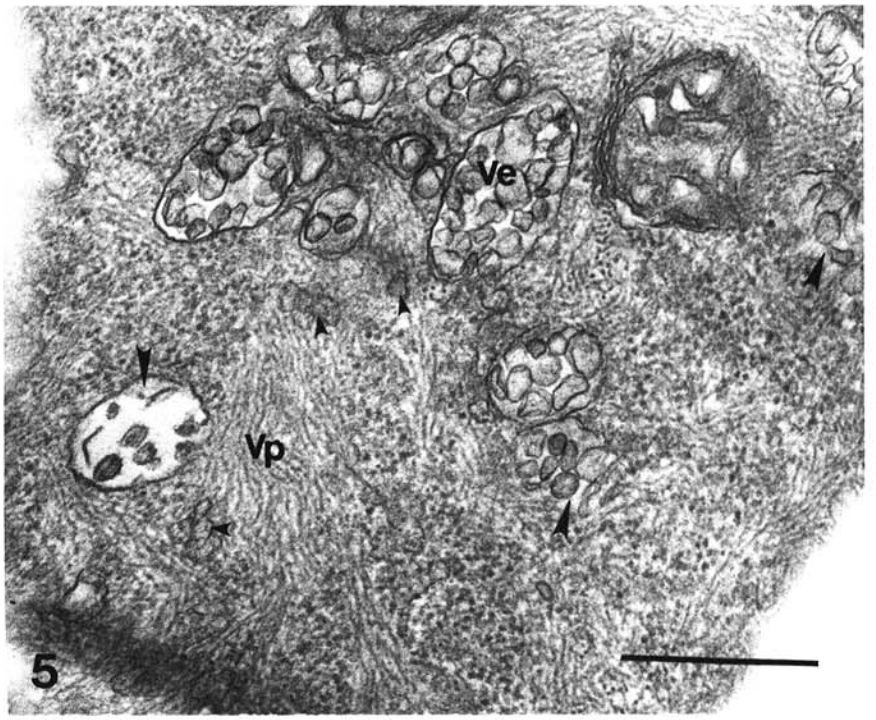


Fig. 2. Polyacrylamide gel electrophoresis (6%) of double-stranded RNA (dsRNA). Lane A, relative mass standards ($\times 10^6$) (mixture of dsRNA of tobacco mosaic virus, cucumber mosaic virus, and tobacco necrosis virus). Lane B, beet yellows virus. Lane C, *Diodia* vein chlorosis virus. Lane D, lettuce infectious yellows virus. Samples were analyzed by electrophoresis in a 6.5 cm × 9.5 cm × 0.8 mm mini-slab gel for 4 hr at 100 V.

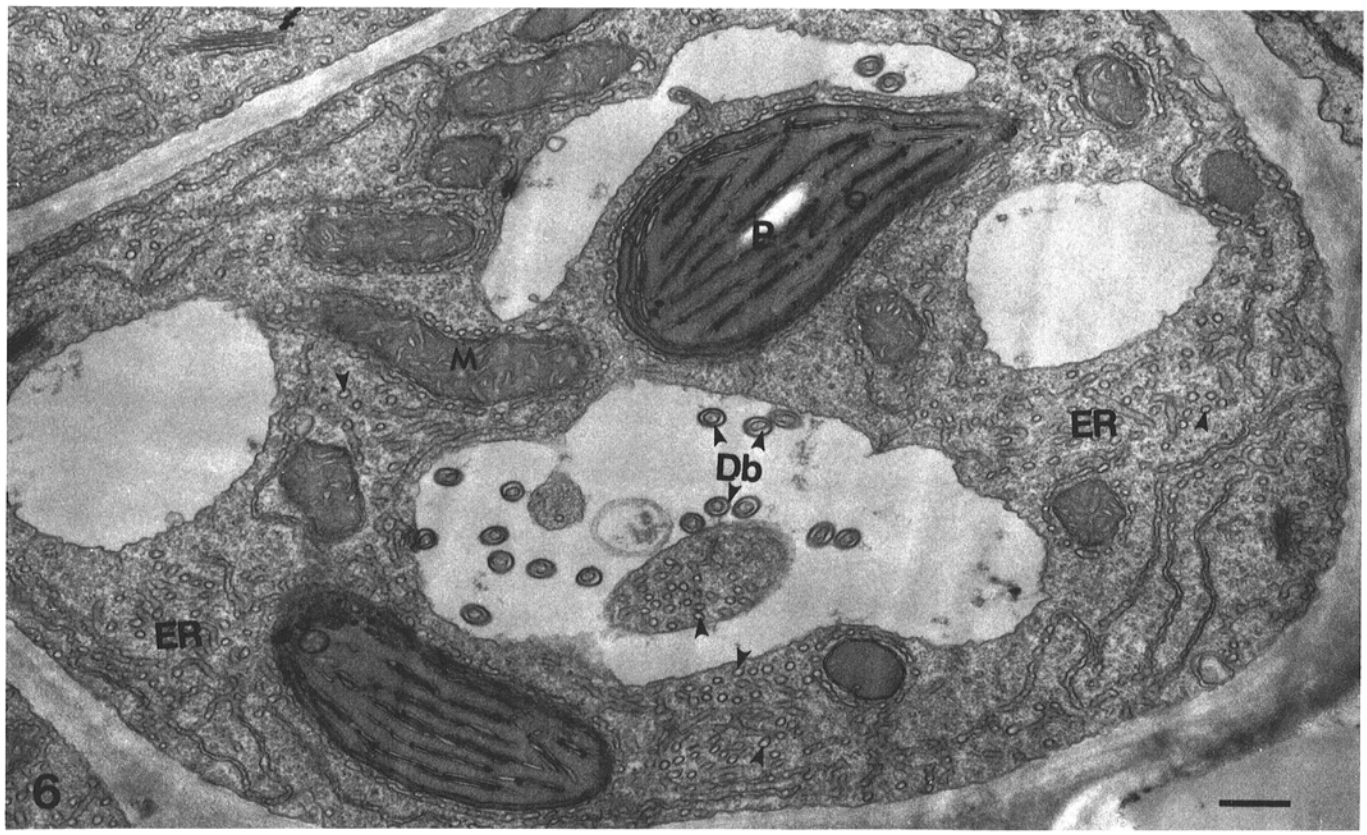
undetermined length and occurred mainly in parenchymatic cells associated with the phloem, including companion cells and developing young sieve elements (Fig. 5). Many cells containing virus particles also contained membranous circular vesicles of approximately 60–150 nm in diameter (Figs. 3–5). The vesicles, in most cases, occurred in electron-lucent vacuoles of various sizes, which were devoid of other cytopathic components (Fig. 3). These vesicles were formed apparently by invagination or budding of a small portion of the surrounding vacuole membrane into the lumen (Fig. 3). In large vacuoles, the budded vesicles often were

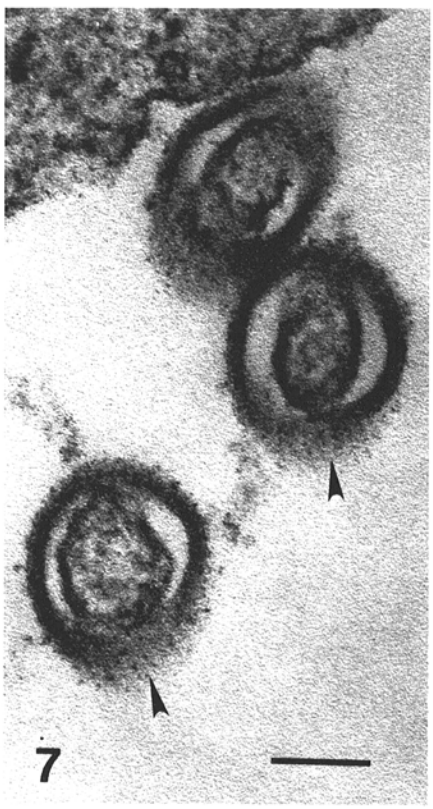


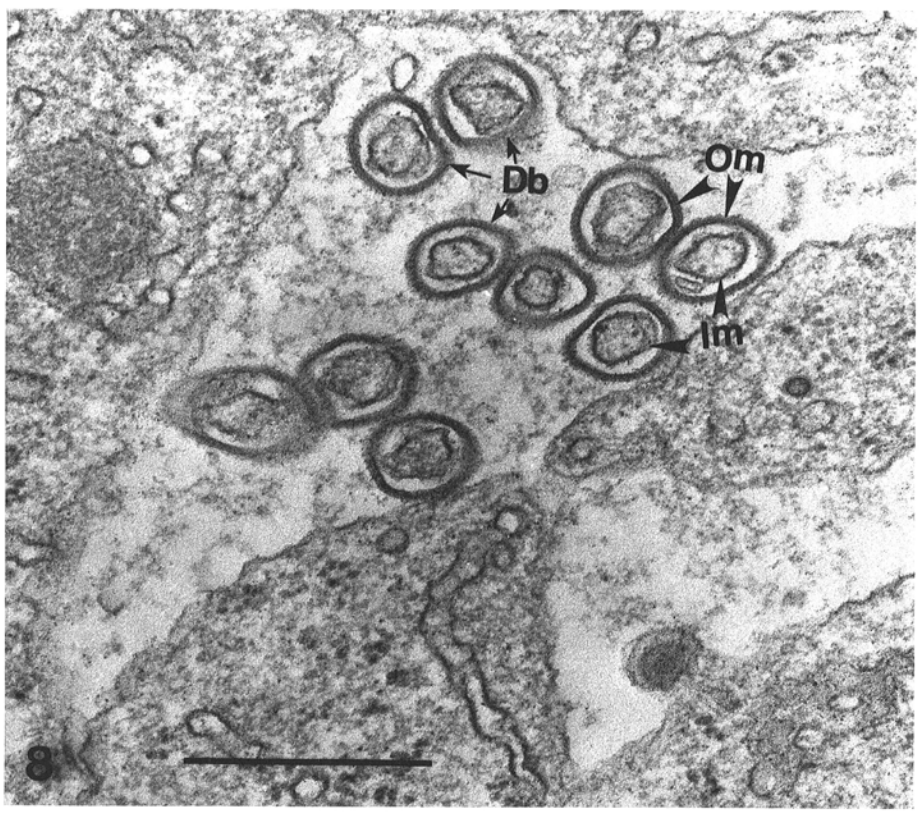




Figs. 3–5. 3, Low magnification of a phloem companion cell filled with membranous vesicles (arrowheads) in electron-lucent vacuoles (Va) of various sizes. Large-cell organelles such as mitochondria (M) and Golgi bodies (G) are restricted to the periphery of the cell. Bar represents 1,000 nm. **4,** Higher magnification of an area in Figure 3, showing the presence of fine fibrils (arrowheads) in the vesicles (Ve). Bar represents 100 nm. **5,** Cytoplasm of a phloem parenchyma cell containing aggregates of long, flexuous virus particles (Vp). The particles are associated with many vacuoles containing vesicles (Ve) with fibrils. Some vacuoles (large arrowheads) appear to be disintegrating as evidenced by a discontinuation and fragments of the vacuole membrane. Some vesicles (small arrowheads) apparently released from disintegrated vacuoles are in direct contact with virus particles. Bar represents 500 nm.







Figs. 6–8. 6, Low magnification of a xylem parenchyma cell containing greatly proliferated tubular endoplasmic reticulum (ER) in the cytoplasm and the double membrane-bound bodies (Db) in the vacuole. Transverse sections of the proliferated ER appear as homogeneous spheres (arrowheads). The ER surrounding large organelles such as mitochondria (M) and plastids (P) also are shown. Bar represents 500 nm. **7,** Higher magnification of three double membrane-bound bodies. The outer membranes of the bodies are sectioned somewhat obliquely, exhibiting spikelike structures (arrowheads). Bar represents 100 nm. **8,** A group of the double membrane-bound bodies (Db) in the vacuole. The outer membrane (Om) is much thicker and tauter than the inner vesicular membrane (Im). The inner vesicle's contents are similar to those of the cytoplasmic matrix but are not similar to the contents of the fine fibrils, such as those shown in Figure 4. Bar represents 500 nm.

aligned in a single row throughout the internal surface (Fig. 3). A higher magnification of these vesicles revealed that they all contained densely stained fibrils (Fig. 4) similar to those usually interpreted as nucleic acid (12). In some cells, most of the cytoplasm was occupied by densely packed vacuoles with vesicles and other cytoplasmic components such as mitochondria, plastids, and Golgi bodies, which were restricted to the periphery of the cell (Fig. 3). Some vesicle-containing vacuoles seemed to be disintegrated, exhibiting fragmentation and discontinuation of the vacuole membrane. The vesicles in such vacuoles appeared to have been released into the ground cytoplasm (Fig. 5).

Virus particles usually occurred in the cytoplasm as aggregates of various sizes. Random distribution of a few particles also was observed occasionally. In large aggregates, longitudinally sectioned particles were often parallel to one another, exhibiting a wavy form (Fig. 5). Virus particles rarely occurred in the vesicle-containing vacuoles (Figs. 3 and 4). The particles were present, however, in the ground cytoplasm between the vacuoles (Fig. 5). The vesicles in the vacuoles, therefore, were not associated directly with virus particles, and those that were not confined to the vacuoles were mostly in direct contact with virus particles (Fig. 5).

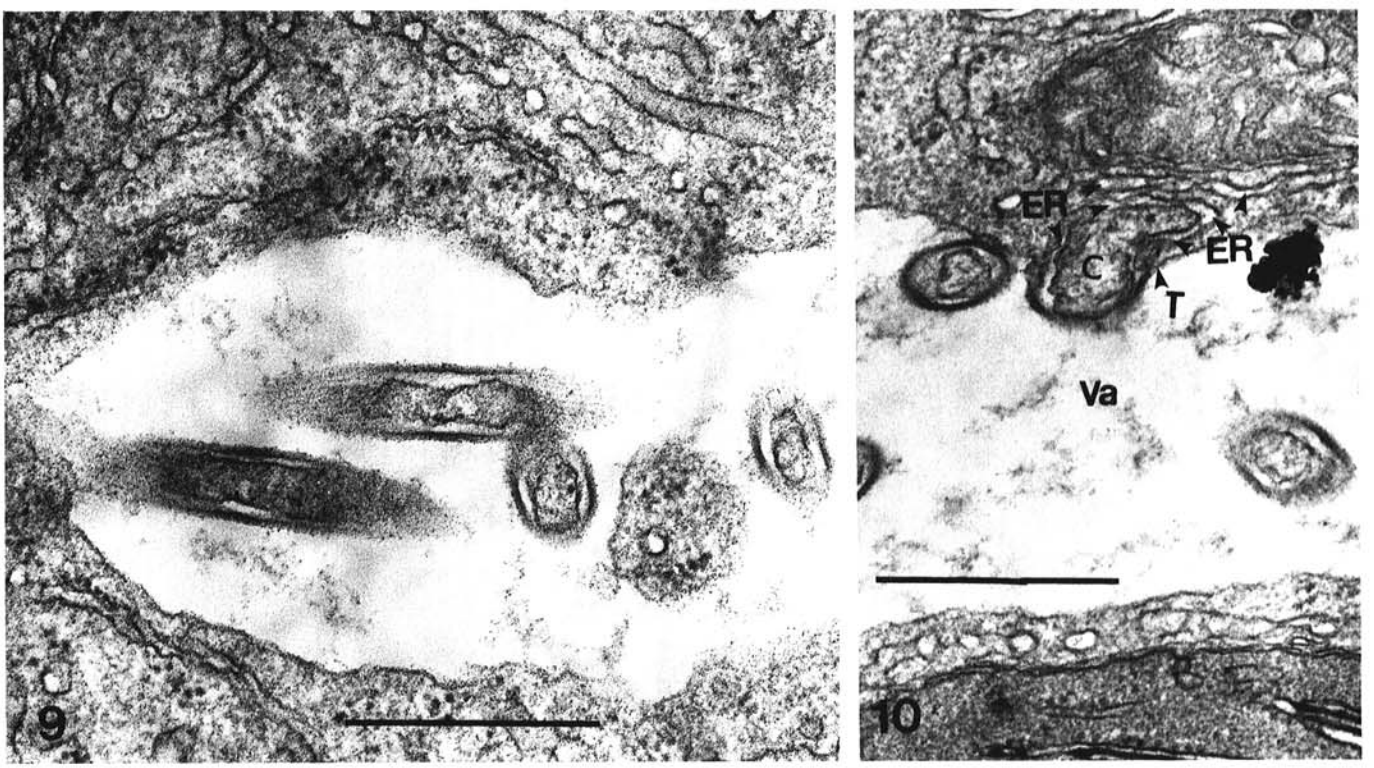
Although many phloem parenchymatic cells contained virus particles and associated fibril-containing vesicles, which are characteristic of closterovirus infection, other parenchymatic cells, especially those associated with tracheary elements, in the same vascular bundle exhibited cytopathic effects, which have not been reported to be associated with closterovirus infection. These were the occurrence of greatly proliferated membranes of smooth endoplasmic reticulum in the cytoplasm and double membrane-bound bodies within the vacuoles (Fig. 6). The membranes of the smooth endoplasmic reticulum were tubular, as evidenced by transverse sections in which they appear as small spheres (Fig. 6). Unlike those of fibril-containing vesicles associated with virus particles, these spheres were somewhat similar in size and shape with an average diameter of approximately 60 nm. These tubular membranes were consistently associated with the periphery of other large organelles such as plastids and mitochondria (Fig. 6). Neither virus particles nor fibril-containing vesicles occurred in the cells that contained proliferated endoplasmic reticulum and double membrane-bound bodies, however.

The double membrane-bound bodies appeared in most cases as a circular profile (Figs. 6–8) and consisted of the outer membrane surrounding the inner membranous vesicle. The outer membrane had a taut contour with spikelike structures and was approximately twice as thick as the inner vesicular membrane, which was loose and undulated (Figs. 7 and 8). The spikelike structures were more conspicuous when the bodies were somewhat obliquely sectioned (Fig. 7). An electron-lucent gap separated the outer membrane and the inner vesicular membrane, and the inner vesicle's contents were similar to those of cytoplasmic matrix (Fig. 8). Although most of the double membrane-bound bodies observed were spherical or oval in shape, somewhat elongated forms also were observed occasionally (Fig. 9). Some of the spherical bodies observed could have been transverse sections of such elongated forms.

The double-membrane-bound bodies apparently were produced by budding off a portion of the cytoplasm into the vacuole, as depicted in Figure 10. A small region of endoplasmic reticulum was appressed tightly to the inner surface of the tonoplast, resulting in the formation of a budded area of cytoplasm into the vacuole (Fig. 10). The budded area, therefore, was bound by three membranes: the tonoplast and two layers of endoplasmic reticulum separated by its cisterna. The tonoplast and one of the two layers of endoplasmic reticulum facing toward the tonoplast were so tightly appressed together that they appeared as one thick membrane (Figs. 7, 8, and 10). When these budded areas were pinched off into the vacuole, they accompanied small portions of the cytoplasm, which appeared as membranous vesicles in sections surrounded by the other layer of the endoplasmic reticulum (Figs. 8 and 10).

DISCUSSION

This study presents evidence that vein-clearing and chlorosis symptoms occurring on *D. virginiana* are caused by a virus with



Figs. 9 and 10. 9, High magnification of four double membrane-bound vesicles, two of which were sectioned somewhat longitudinally and appear tubular. Bar represents 500 nm. **10,** A double membrane-bound body appears to be in the process of budding through the tonoplast (T) of the central vacuole (Va). A small region of endoplasmic reticulum (ER) is tightly appressed to the inner surface of the tonoplast and is bulging into the vacuole. C = cytoplasm. Bar represents 500 nm.

a particle morphology of long, flexuous rods. The cytopathology of DVCV is strikingly similar to that induced by many closteroviruses, including whitefly-transmitted cucumber yellows virus (23) and LIYV (8,14). The major cytopathic changes found in DVCV-infected tissues were the presence of virus particles with an average diameter of 12 nm confined mainly to phloem cells and the occurrence of the membranous, fibril-containing vesicles in the cytoplasm. These cytopathic effects are important tools for diagnosing viruses in the closterovirus group because the symptomatology and host-range studies of closteroviruses are of limited diagnostic value because these viruses are not readily sap transmitted from their original host and symptoms are not always distinctive (19).

The fibril-containing vesicles induced by most closteroviruses have a characteristic morphology and have been referred to as "BYV type" (13) because they are all similar to those induced by beet yellows virus, the type member of the closteroviruses (9). The origin of the BYV-type vesicles has not been established fully because of the lack of spatial association with any particular cell organelle. Esau and Hoefert (9), however, postulated that the vesicles originate "de novo" as receptacles of the fibrils that might be viral RNA; however, the vesicles associated with closteroviruslike particles in the DVCV-infected cells of this study were clearly derived from the vacuolar membrane. In addition, mitochondrial involvement in the genesis of virus-induced vesicles, associated with closteroviruslike particles, has been reported in grapevine leafroll disease (17). Thus, the virus-induced vesicles associated with closterovirus infection are not the BYV type alone but vary depending upon the particular viruses.

The double membrane-bound bodies observed in this study have not been reported with other closteroviruses. Their unique structure, consistent occurrence only in cells of DVCV-infected leaves, and association with abnormally proliferated endoplasmic reticulum strongly suggest that these bodies are virus induced, although their functional significance in viral pathogenesis is unclear. The fact that the cells containing virus particles and fibril-containing vesicles are phloem-associated paranchyma cells, including companion cells, whereas those having double membrane-bound bodies are primarily xylem-associated parenchyma cells may suggest that these two types of cells differ in ability to support virus replication. Furthermore, the occurrence of the double membrane-bound bodies only in the central vacuole along with the fact that no virus particles and/or fibril-containing vesicles occurred in cells with the bodies suggest that the production of these bodies may represent a resistance mechanism to virus replication.

The bandedwinged whitefly has been shown to transmit DVCV to healthy *Diodia* plants. Currently, only two other closteroviruses have been reported to be transmitted by whiteflies: cucumber yellows virus, transmitted by *T. vaporariorum* (Westwood) (23), and LIYV, transmitted by *B. tabaci* (8). The bandedwinged whitefly, which transmitted DVCV in this study, has not been reported previously to be a vector of any plant virus.

Extraction and analysis of dsRNA yielded three dsRNA bands. It is assumed that the double-stranded form is the replicative form of the single-stranded RNA of the virus (6). Some viruses, such as those belonging to the closterovirus group, have many minor dsRNA, which are diagnostic for this group (5). Evaluation of the results obtained from the dsRNA of DVCV, as well as LIYV, is difficult, although the dsRNA appear to be similar to that of the closterovirus group because of the number of bands and their M_r.

Successful purification of these closteroviruses has been limited to LIYV. Purification of DVCV was only minimally successful. This is probably due to the low-titer, phloem-limited nature of the virus and the lack of a more suitable host plant for virus purification. Oxidation products were abundant in extracts of infected *Diodia* and may have contributed to difficulties in purification.

Although infectivity of purified virions has not been demonstrated, ultrastructural studies along with analysis of the dsRNA indicate a close, although not identical, relationship with closteroviruses. Further studies are required to completely characterize this virus. However, our evidence suggests that DVCV is a member of the closterovirus group, and we propose that whitefly-transmitted closteroviruses be categorized under a separate subgroup, as suggested by Duffus et al (8).

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