Isolation of a Caulimovirus from Strawberry Tissue Infected with Strawberry Vein Banding Virus

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ABSTRACT

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Strawberry vein banding virus (SVBV), a semipersistent aphid-transmitted virus, was partially purified from infected strawberry tissue utilizing a step gradient consisting of a layer of 1.50 g/cm^3 CsCl in 30% sucrose. Semipurified SVBV collected from the gradient interface reacted heterologously with antiserum to the cabbage B strain of cauliflower mosaic virus (CaMV). Sedimentable antigenic activity was detected by ELISA in the 200S region of linear log sucrose gradients for both SVBV and CaMV after treatment with formaldehyde. In addition, chromatography of SVBV and CaMV on ECTEOLA (Cellex-E) ion exchange cellulose revealed that both eluted at approximately 0.18 M NaCl. Spherical virions about 45 nm in diameter were seen by serologically specific electron microscopy of semipure SVBV and CaMV extracts and typical caulimovirus cytoplasmic inclusions were observed by electron microscopy in ultrathin sections of SVBV infected strawberry leaves. These data indicate that SVBV is a legitimate member of the caulimovirus group.

Strawberry vein banding virus (SVBV) occurs in strawberries throughout eastern and western North America. A number of strains, including the most severe, occur in the Pacific coast beach strawberry, *Fragaria chiloensis* Duchesne. The virus is transmitted in a semipersistent manner by a number of aphid species, but most efficiently by the strawberry aphids *Chaetosiphon fragaefolii, C. jacobi,* and *C. thomasi* (5). In nature, the virus is known only in *Fragaria* spp.; however, experimental transmission to *Sanguisorba minor,* the garden burnet, was accomplished by leaf insert grafting and by the dark strawberry aphid, *C. jacobi.* Mechanical transmission has not been accomplished.

The disease is apparently of minor economic importance in commercial California strawberry plantings due to its sporadic occurrence and low incidence. In single infections, the virus is visually symptomless in commercial cultivars but it can reduce runner production, vigor, yield, and fruit quality. However, it may cause severe disease in complexes with other viruses (5).

In *F. vesca* L. indicator plants, the most prominent symptoms are yellowish banding of tissues along the veins of the first new leaves which are produced after infection. Later, symptoms may disappear and recur sporadically. Until recently, no information on the causal agent of this disease was known. Characteristic cytoplasmic inclusions and 40- to 45-nm virus particles recently were observed in ultrathin sections of strawberry leaf tissue infected with vein banding symptoms (8). As a result, SVBV tentatively was included in the caulimovirus group.

This paper describes a procedure for extracting semipure vein banding virus from strawberry tissue and presents further evidence for the inclusion of SVBV in the caulimovirus group (11).

MATERIALS AND METHODS

Purification. The purification scheme for SVBV was similar to those reported for cauliflower mosaic virus (CaMV) (13), dahlia mosaic virus (DaMV) (3), and carnation etched ring virus (CERV) (10). Since no herbaceous host is known, *F. vesca* L. var. *semperflorens* (Duchesne) Seringe tissue, infected with SVBV type strain (100 g), was homogenized in 400–600 ml of 0.5 M potassium phosphate buffer (pH 7.5) with 0.2% mercaptoethanol in a Waring Blendor. The homogenate was clarified by the addition of 25% chloroform-7% butanol followed by low speed centrifugation. The virus was precipitated from the aqueous phase with 8% polyethylene glycol (PEG) 6000 in the presence of 0.1 M NaCl. The large, gelatinous, viruscontaining low speed pellet was resuspended in 100 ml of water by slow stirring at 4 C for several hours or overnight. Further purification by differential centrifugation was unsatisfactory for strawberry extracts due to dense, viscous contaminating material. The virus was separated from most of this material by centrifugation on a step gradient consisting of a layer of 1.5 g/cm^3 CsCl in 30% sucrose (3 ml) and a layer of 30% sucrose (7 ml). Strawberry extract (15 ml) was layered above the sucrose and centrifuged in a Beckman SW 25.1 swinging bucket rotor for 2 hr at 24,000 rpm at 4 C. Opalescent material at the sucrose-CsCl interface contained semipure SVBV. Further purification was achieved either by sucrose density gradient centrifugation or by chromatography on ECTEOLA (Cellex-E) ion exchange cellulose from Biorad Laboratories (Richmond, CA 94804).

Enzyme-linked immunosorbent assay (ELISA). The microplate ELISA test conducted with antiserum to the cabbage-B strain of CaMV with a titre of 1:512 was performed according to Clark and Adams (4,6) except that the gamma globulins were purified by affinity chromatography on Affi-Gel Blue obtained from Biorad Laboratories (Richmond, CA 94804). Microtiter plates were coated with gamma globulin at 5 μg/ml in 0.05 M sodium carbonate buffer (pH 9.6) for 4 hr at 30 C. Viral antigens were added at approximately 1 μg/ml in 0.02 M phosphate, 0.15 M NaCl, 0.05% Tween-20 at pH 7.4 (PBS) with 2% polyvinyl pyrollidine-10 and 0.2% egg albumin and incubated 12 hr at 4 C. Alkaline phosphatase-coupled gamma globulin was added at 5 μg/ml and incubated 4 hr at 30 C. Absorbance at 405 nm was measured with a Beckman Model 35 spectrophotometer 1 hr after addition of substrate at 1 mg/ml in diethanolamine buffer (pH 9.8).

Sucrose density gradient centrifugation. Semipure virus from the sucrose-CsCl interface was analyzed by centrifugation on linear log sucrose density gradients (1) in water in a Beckman SW 50.1 rotor at 38,000 rpm for 45 min at 4 C. Gradients were analyzed on an ISCO Model 640 density gradient fractionator. Collected fractions were tested for CaMV antigenic activity by ELISA at a gamma globulin coating concentration of 5 μg/ml.

ECTEOLA ion exchange cellulose chromatography. Semipure SVBV and CaMV in 0.01 M Tris buffer (pH 7.5) were applied to 1.5

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cm × 5.0-cm ECTEOLA columns and eluted with a salt gradient ranging from 0.1 M to 0.5 M NaCl in 0.01 M Tris (pH 7.5). The column eluates were scanned at 254 nm with an ISCO model UA-5 absorbance monitor. Fractions were collected with an ISCO Model 272 fraction collector and analyzed by ELISA as described above.

Serologically specific electron microscopy. Semipure CaMV and SVBV preparations were dialyzed against 1% formaldehyde at 4 C and observed for virus particles by SSEM (2,6). Collodion coated 74-μm(200-mesh) copper grids were coated with CaMV gamma globulin diluted 1:100 in coating buffer (4) for 2 hr. Grids were rinsed with 0.01 M Tris buffer (pH 7.5) with 0.001 M EDTA and CaMV and SVBV samples, diluted 1:4 in the same buffer, were applied for 2 hr. After being rinsed with water, the particles were stained with 5–10 drops of aqueous uranyl acetate, drained, dried, and observed in a Phillips EM300 electron microscope.

Transmission electron microscopy of ultrathin sections. Leaf tissue of SVBV-infected *F. vesca* was prepared by the uranyl soak method which involved primary fixation with 4% glutaraldehyde in 0.08 M cacodylate buffer (pH 6.8) and dehydration through an ethanol series saturated with uranyl acetate (7). For comparison, some tissue also was postfixed overnight at 4 C in 1% osmium tetroxide buffered with cacodylate and dehydrated in acetone. All tissue was embedded in low viscosity epoxy resin (E. F. Fullam, Inc., Schenectady, NY 12301). Silver sections obtained by ultramicrotomy were expanded with chloroform vapor and placed on 38-μm (400-mesh) copper grids. The samples were stained with uranyl acetate followed by lead citrate and examined with a Phillips EM 300 electron microscope.

RESULTS

Virus isolation. The identification of caulimoviruslike inclusion bodies in SVBV-infected plants (8) provided the only presumptive evidence as to the nature of this virus disease. Our initial attempts to isolate virus from SVBV-infected plants by procedures developed for other caulimoviruses (3,10,13) yielded very viscous, impure preparations which, in immunodiffusion tests, reacted with antiserum prepared against the cabbage B strain of CaMV. However, virus produced in this manner was too impure for virus characterization and virus yield was too low for conventional analysis. By utilizing the CaMV antiserum in microplate ELISA tests, we were able to follow the progress of the purification of antigen related to CaMV. This approach allowed us to develop the sucrose-CsCl step gradient which optimized the recovery of an antigen free from the dense, viscous contaminants present in strawberry sap. The subsequent purification from other impurities, mainly proteins, was accomplished by chromatography on ECTEOLA cellulose and monitored by ELISA. The elution of CaMV antigenic activity from a column loaded with semipure SVBV extract (Fig. 1) at 0.18 M NaCl was essentially identical to the elution properties of purified CaMV. This fraction yielded purified material for subsequent analysis.

Sedimentation studies. Initial attempts to purify and identify a sedimentable nucleoprotein in SVBV extracts by rate zonal

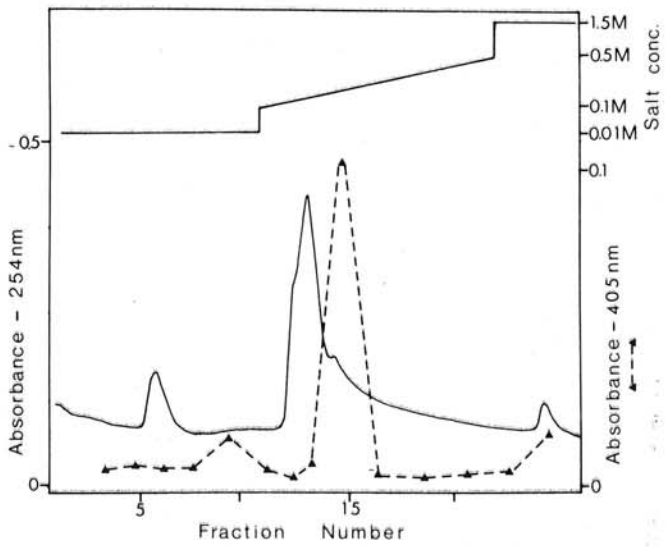

Fig. 1. Elution of a strawberry vein banding virus (SVBV) extract from a 1 × 5 cm column of Cellex E. The extract was loaded in 0.01 M Tris (pH 7.5) and eluted with a 40-ml linear gradient of 0.1 M to 0.5 M NaCl in 0.01 M Tris (top line). The elution profile was monitored at 254 nm (solid line) and fractions were tested for CaMV antigenic activity by ELISA which was recorded at 405 nm (dashed line).

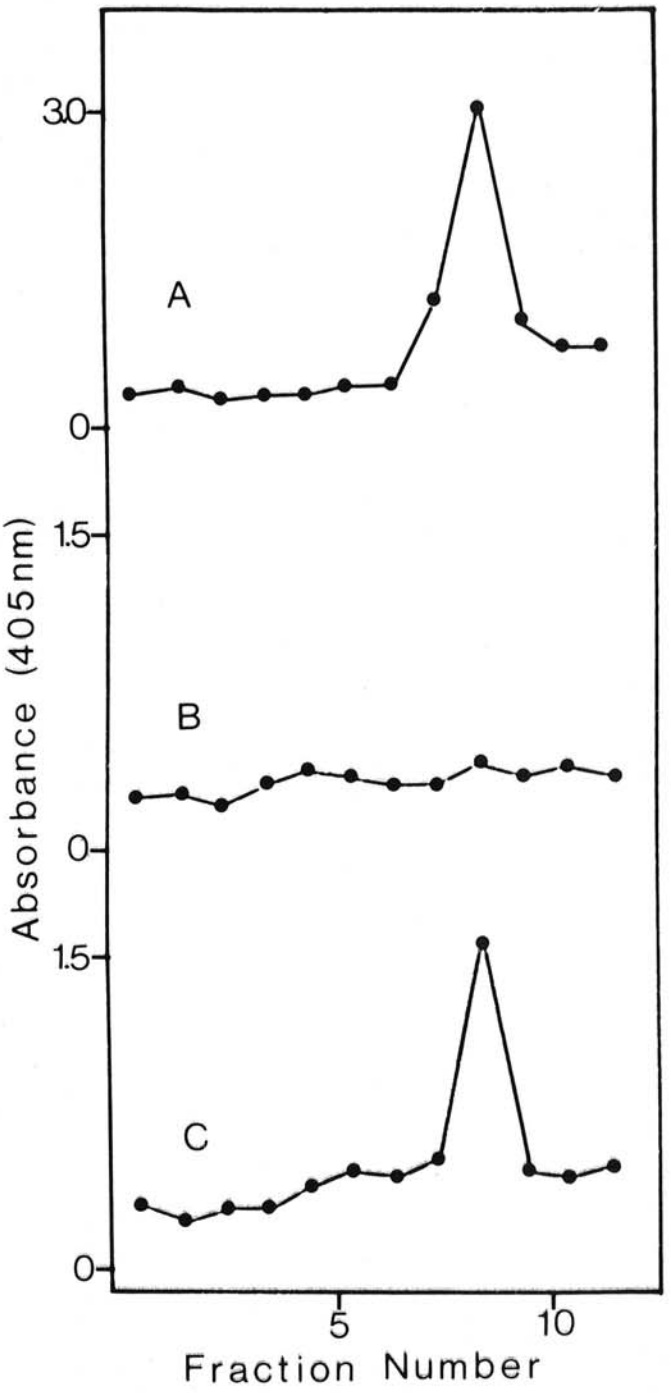

Fig. 2. Sedimentation of purified virus preparations: **A,** cauliflower mosaic virus (CaMV) treated with 1.0% formaldehyde; **B,** strawberry vein banding virus (SVBV); and **C,** SVBV treated with 1.0% formaldehyde after centrifugation in linear-log sucrose density gradients at 38,000 rpm for 45 min in an SW50.1 rotor. The gradient fractions were tested by ELISA for CaMV antigenic activity and recorded at 405 nm.

centrifugation in sucrose gradients, or equilibrium centrifugation in CsCl gradients, were generally unsuccessful. Specific sedimentable viral species could not be readily identified in the gradients. Centrifugation of purified virus in linear log sucrose gradients at conditions under which CaMV virus was readily detected (Fig. 2A) failed to reveal a comparable sedimentable antigen in the SVBV preparations (Fig. 2B). Only when the virus preparations were pretreated by dialysis against 1% formaldehyde at 4 C was 200S sedimentable antigen detected in the SVBV preparations (Fig. 4C) and under these conditions it co-sedimented with similarly treated CaMV (Fig. 4A) and untreated CaMV.

Serological relationships. The initial serological relationship of SVBV to CaMV was identified by immunodiffusion tests with antiserum to the cabbage-B strain of CaMV. To identify the nature of the serological relationship, purified virus preparations of CaMV, DaMV, and SVBV at 1 μg/ml were tested for ability to bind to microtiter plates precoated with CaMV gamma globulin at dilutions of 1/100, 1/400, and 1/1,600 (5.0, 1.25, and 0.31 μg/ml). The results (Fig. 3) indicated that both SVBV and DaMV behaved as heterologous antigens against CaMV antiserum when compared to the reactivity of an equal quantity of CaMV in the ELISA test. Although the dilution curves were similar for both heterologous viruses, it could not be established if the SVBV was more closely related to DaMV or CaMV without testing the reciprocal sera. Preliminary tests with DaMV sera (kindly supplied by A. A. Brunt) gave ambiguous results which would not allow for a definitive assignment of serological affinities for the three viruses. Evaluation of the serological relationships by ELISA will require the production of SVBV antiserum.

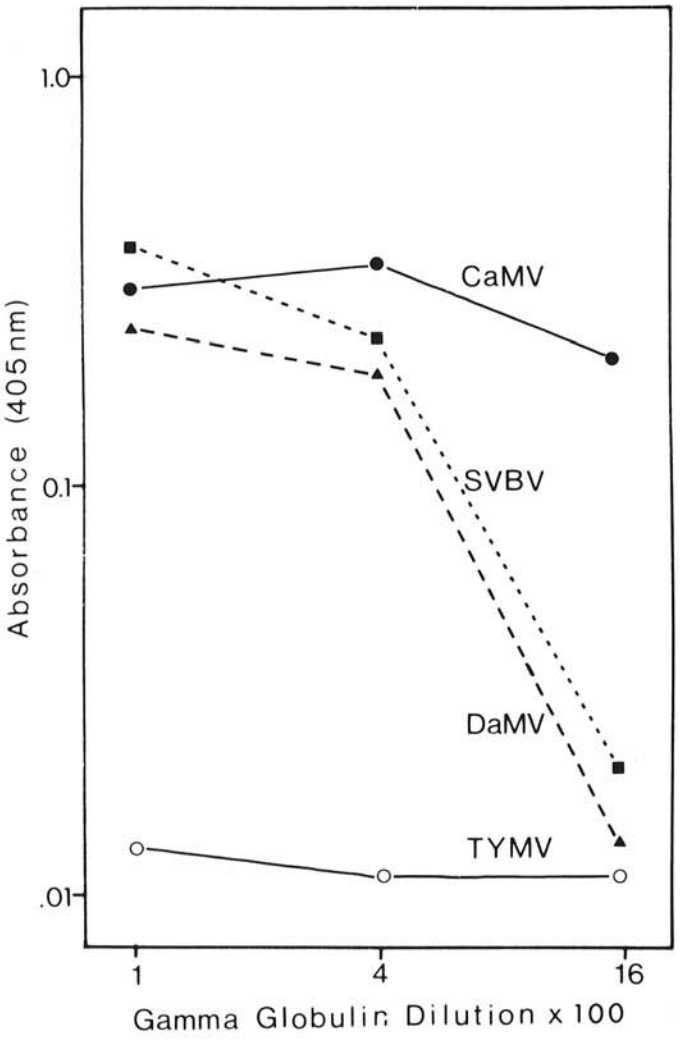

Fig. 3. The effect of CaMV gamma globulin coating concentrations on the reactivity of cauliflower mosaic virus (CaMV), strawberry vein banding virus (SVBV), dahlia mosaic virus (DaMV), and turnip yellow mosaic virus (TYMV) in a microtiter plate ELISA test. The plates were coated with CaMV gamma globulin at 5.0, 1.25, and 0.3 μg/ml for 4 hr at 30 C, incubated with viral antigens at 1 μg/ml for 12 hr at 4 C, enzyme coupled with CaMV gamma globulin at 5 μg/ml for 4 hr at 30 C and recorded at 405 nm 1 hr after the addition of substrate.

Electron microscopy. Initial attempts to identify 45-nm particles, which are typical of caulimoviruses, in SVBV preparations at the various stages of purification were unsuccessful. Although 200S sedimentable antigen, indicative of intact virus particles, could be consistently isolated from SVBV infected tissue, the concentration of virus was very low. However, when preparations of virus were adsorbed to grids by preparation procedures for serologically specific electron microscopy (2,6) with CaMV antiserum, low numbers of 45 nm particles indistinguishable from CaMV were observed (Fig. 4). Pretreatment of the virus preparations with 1% formaldehyde and positive staining with uranyl acetate allowed for more consistent identification of the virus particles.

To confirm the original observations of Kitajima et al (8) and to establish that other viruses were not likely involved in SVBV infection, infected leaf tissue was examined in ultra-thin sections. A sporadic distribution of virus inclusion bodies was observed in vascular parenchyma and mesophyll cells and various stages of inclusion body formation could be recognized (Fig. 5). The earliest recognizable stage was identified by clustering of free ribosomes in association with Golgi bodies followed by a dense accumulation of material in the center of the ribosome cluster (Fig. 5A). The more advanced stages of inclusion body formation often were found near the nucleus (Fig. 5B and 5C). Uranyl soak fixation revealed a fibrillar textured viroplasm in which ringlike 45-nm viruslike particles with a hollow center were embedded. Possible continuity of the fibrillar component of the viroplasm with nuclear membrane material was observed (Fig. 5B). Inclusion body formation appeared to involve the coalescence of smaller bodies in masses with larger numbers of virus particles (Fig. 5C) and fully mature inclusion bodies were several micrometer in diameter with a round-to-ovoid shape and defined borders (Fig. 5D and 5E). Virions were found both within the electron-dense matrix and within the clear lacunae which permeated the body. Virions within lacunae possessed discernible capsid projections (~10 nm) when the tissue was fixed by the uranyl soak method. Lacunae-located particles, excluding projections, possessed a diameter of 40 nm whereas matrix-located particles appeared somewhat larger (45 nm).

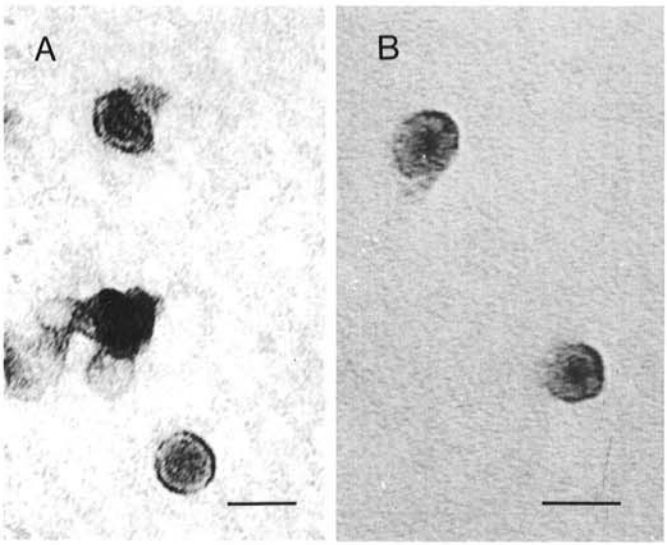

Fig. 4. Serologically specific electron microscopy of **A,** strawberry vein banding virus and **B,** cauliflower mosaic virus after fixation in 1% formaldehyde. The grids were precoated with CaMV gamma globulin at 5 μg/ml and then incubated with the virus preparations for 2 hr. After washing, the grids were stained with uranyl acetate. The bar represents 50 nm.

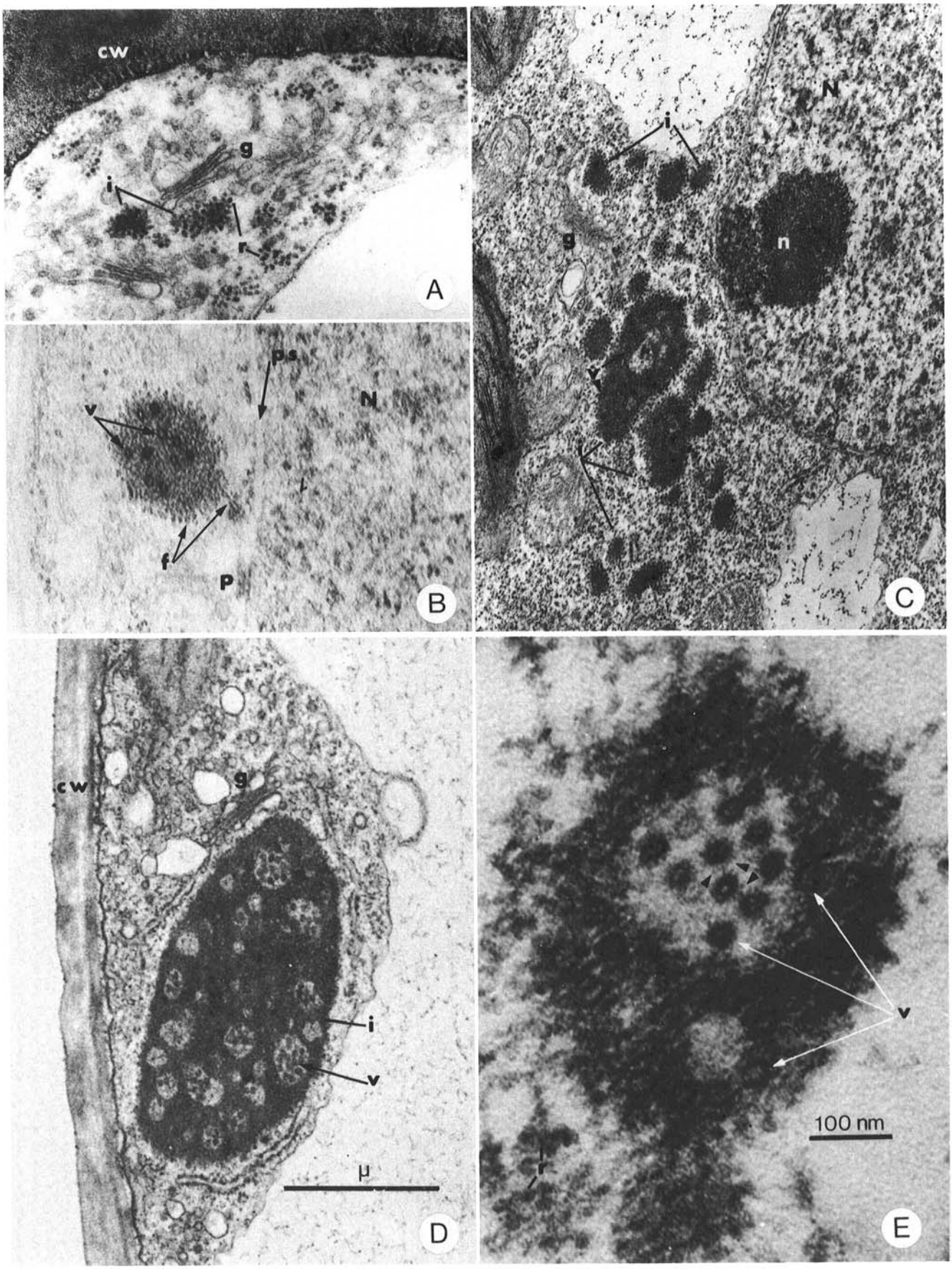

Fig. 5. Inclusion bodies in strawberry leaf cells exhibiting vein banding symptoms. *cw*, cell wall; *f*, fibrillar component of inclusion body; *N*, nucleus; *n*, nucleolus; *p*, nuclear pore; *ps*, perinuclear space; *r*, ribosomes; *v*, virions. Figs. 5A, B, and C: glutaraldehyde, osmium fixation. Figs. 5B and E: glutaraldehyde, uranyl soak fixation. **A,** Early stage in inclusion body formation associated with Golgi bodies and ribosome clusters. ×39,445. **B,** Later stage in inclusion body formation. Virions with densely staining capsids are enmeshed in a fibrillar mass which is connected with nuclear membrane-associated material. ×67,850. **C,** Inclusion body coalescence is shown as it frequently occurs in relationship to the nucleus and nucleolus. ×27,140. **D,** Section of a large, mature inclusion body with many electron lucent lacunae. Virions are present throughout the inclusion. ×29,210. **E,** Inclusion body with virions. Virions in lacunae have projections from their coats (arrowheads).×170,200.

DISCUSSION

Lack of progress in the isolation and characterization of many viruses infecting nonherbaceous vegetatively propagated plants, such as strawberries, is due to a number of difficult problems. Often, mechanical or vector transmission of these viruses to a suitable herbaceous host is extremely erratic or impossible. In addition, virus purification directly from the infected host usually is not possible by conventional procedures due to the dense, viscous nature of the host sap and the presence of materials such as tannins in the tissue. The development of the purification procedure described here, centrifugation on a sucrose-CsCl step-gradient and ion-exchange chromatography on ECTEOLA cellulose, has allowed for the isolation and identification of a strawberry virus directly from the infected plants, thus providing a possible solution to some of these problems.

The isolation of virus particles directly from strawberry tissue infected with vein banding disease is only presumptive evidence that the virus is the causal agent; the disease was not reproduced when strawberry plants were mechanically inoculated with the purified virus preparations. However, no other viruslike particles were isolated from SVBV-infected plants and transmission and electron microscopy studies have not indicated that more than one virus is involved in this disease.

The properties of the virus associated with strawberry vein banding disease all are consistent with its inclusion in the caulimovirus group (14). SVBV, like other caulimoviruses, is aphid transmitted in a semipersistent manner and has a limited natural host range. In nature, SVBV occurs only in *Fragaria* sp. which distinguishes it from other caulimoviruses. Sufficient virus was not obtained for a thorough physicochemical comparison to other caulimoviruses. However, the isolation of 45-nm particles with a sedimentation coefficient of 200 ± 10S is consistent with a caulimovirus grouping. In addition, SVBV reacted to CaMV antiserum in immunodiffusion tests and displayed a heterologous reaction, similar to the reactivity of DaMV, to CaMV antiserum in ELISA tests. Although a definitive statement about the relationship of the three caulimoviruses cannot be made from these results, they clearly support the caulimovirus nature of SVBV.

Further confirmation was obtained from observations of the development of caulimoviruslike inclusion bodies in SVBV infected tissue. The results are similar to those reported previously for SVBV (8) and DaMV (9). The substitution of uranyl acetate for osmium in postfixation revealed a unique spike structure not previously reported for other caulimoviruses. The hollow structure of the observed virions and the intense stain affinity of uranyl ions for DNA suggest that the viral DNA is associated with the capsid protein. This is essentially similar to the observations reported for DaMV in which the 40–45 nm particles possessed an indistinct outer coat surrounding a 10-nm electron-dense layer (9). This outer coat may correspond to the projection layer of SVBV resolved by the uranyl soak fixation method.

The identification of SVBV as a caulimovirus may have important implications for strawberry plant certification programs. The introduction into strawberry propagation programs of heat treatment combined with meristem-tip culture has helped to reduce but not to eliminate virus disease problems. This may be due in part to reliance upon graft transmission tests to strawberry indicator cultivars for detection of virus spread and reinfection. We know from previous work on meristem-tip culture of dahlia (12), that without prior heat treatment of infected source plants, DaMV may remain symptomless and undetectable in cultured plants for at least 10 mo. Whether this long latent period occurs with SVBV is unknown since meristem-tip cultured *F. ananassa* cv. 'Hood' plants which tested SVBV-free on *F. virginiana* UC12 indicator plants were discarded after 6 mo. Development of applicable ELISA tests may be the only method for early detection of such latent infections. Furthermore, application of rapid and sensitive tests such as ELISA would greatly facilitate routine indexing in plant certification programs and allow identification and study of field problems. Since the heterologous CaMV antiserum was unsatisfactory in detecting SVBV in crude extracts of infected strawberry, early diagnosis of latent infections and rapid field indexing may be feasible only after production of a good homologous SVBV antiserum. This could prove even more significant as preliminary experiments indicate that at least one strain of strawberry mottle disease is a virus complex in which one component is a caulimovirus.

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