Association of Virions and Coat Protein of Tobacco Vein Mottling Potyvirus with Cylindrical Inclusions in Tobacco Cells

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ABSTRACT

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The course of infection with tobacco vein mottling potyvirus (TVMV) in tobacco protoplasts and leaves was studied by electron microscopy with immunogold labeling. In sections of protoplasts inoculated with TVMV RNA 10, 21, or 45 h prior to fixation, antibodies to TVMV coat protein (CP) were bound to cylindrical inclusions and individual virions usually associated with these inclusions. Also, in sections of leaves from plants inoculated with TVMV 5, 14, or 21 days prior to fixation, antibodies to TVMV CP were bound to cylindrical inclusions and aggregates of virions in the cytoplasm. In protoplasts and leaves, the specific labeling of CP on the cylindrical inclusions increased significantly with time postinoculation, and in most cases, these inclusions were associated with the rough endoplasmic reticulum. Association of TVMV virions and CP with cylindrical inclusions at early stages of infection suggests that these inclusions may be involved in potyviral protein synthesis and virion assembly in addition to their possible role in viral RNA replication.

Recent studies at the molecular level have begun to elucidate the nature and some of the functions of the gene products of potyviruses (reviewed by Dougherty and Carrington [4] and Riechmann et al [14]). Immunogold labeling at the ultrastructural level allowed the localization of some potyvirus gene products in various cytoplasmic and nuclear inclusions present in infected cells (2,9). Little is known, however, about where these gene products are produced in the cell; most ultrastructural studies have involved infected leaves (5,10), fixed a few to several days postinoculation (p.i.), in which the time of infection of individual cells is unknown. Inoculation of protoplasts, on the other hand, results in near-synchronous infection and, thus, allows investigation of the temporal aspects of ultrastructural and cytochemical changes that occur early in the infection process. Recent studies (12,15), using antisera to the P3 and cylindrical inclusion (CI) proteins of tobacco vein mottling potyvirus (TVMV), have allowed the detection of these gene products in tobacco mesophyll protoplasts as early as 10 h p.i. Both CI and P3 proteins were associated only with the cylindrical inclusions, and specific labeling with the P3 protein apparently increased with time from 10 to 45 h p.i. (15). However, no immunocytochemical studies on potyvirus coat protein (CP) in protoplasts have been reported.

Thus, the immunocytochemical study reported here focuses on the localization of TVMV CP in infected protoplasts 10–45 h p.i. Infected leaves, 5–21 days p.i., also were examined to compare the localization of TVMV CP in protoplasts and leaf tissue. A brief report on the present work has been published (1).

MATERIALS AND METHODS

Preparation of samples for transmission electron microscopy (TEM). Tobacco leaf mesophyll protoplasts were isolated and inoculated by electroporation with TVMV RNA, as previously described (11,12). Samples of 2×10^5 inoculated or mock-inoculated protoplasts were processed for TEM after incubation for 10, 21, or 45 h p.i.

Tobacco leaves were processed for TEM at 5, 14, or 21 days p.i. For the 5-day samples, young leaves (~7 mm wide) were aphid-inoculated with TVMV and processed for TEM 5 days later; aphid inoculation was used to avoid the mechanical injury to these leaves that would be expected after mechanical inoculation. For the 14- and 21-day samples, however, systemically infected leaves were used; these were obtained by mechanically inoculating young tobacco plants with purified TVMV. The first uninoculated leaves to show systemic symptoms over the entire leaf were processed 14 and 21 days p.i.

Pelleted protoplasts and small (1×2 mm) pieces of infected or control leaves were processed for TEM by one of two methods. For immunogold labeling, samples were fixed in 0.1–1% glutaraldehyde plus 2% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.2), dehydrated in graded ethanol to 70%, and embedded in LR White resin (Electron Microscopy Sciences, Ft. Washington, PA) at 50–52 C (13). For better ultrastructural preservation of leaf tissues and protoplasts, without immunogold labeling, other samples were prefixed in 2.5–3% glutaraldehyde in 0.1 M cacodylate buffer, postfixed in 1% osmium tetroxide, dehydrated in an ethanol-acetone series, and embedded in Spurr's medium at 70 C.

Immunogold labeling of sections. Thin sections of leaves or protoplasts fixed in glutaraldehyde-paraformaldehyde and embedded in LR White were mounted on uncoated or collodion-carbon coated, 200-mesh nickel grids. Mounted sections were floated for 30 min on blocking buffer (1% bovine serum albumin [BSA] + 0.1% Tween 20 in 0.1 M Tris-HCl, 0.85% NaCl [TBS], pH 7.5), then for 1 h on primary antiserum diluted, as indicated below, in TBS-BSA buffer (0.01% BSA + 0.1% Tween 20 in 0.1 M TBS, pH 7.5). After washing in several running drops of TBS-BSA buffer, sections were floated for 30 min on goat anti-rabbit IgG-colloidal (10 nm) gold (Sigma Chemical Co., St. Louis) diluted to 1/25 in TBS-BSA buffer. Sections were washed in running drops of buffer, followed by deionized water, and stained for 5 min in uranyl acetate and 2 min in lead citrate, before being examined with a Hitachi 600 electron microscope at 75 kV.

Primary antisera were produced in rabbits against TVMV virions or TVMV CP; both were diluted 400-fold for immunogold labeling. Because these two antisera gave similar results, they will both be referred to as antibodies to TVMV CP. Antisera to TVMV CI, also produced in rabbits (12), were diluted 100- or 400-fold. As a control, sections of infected leaves or protoplasts were incubated with preimmune rabbit serum at the same dilutions as the primary antisera used. Sections of uninfected leaves or mock-inoculated protoplasts, incubated with the primary antiserum before gold labeling, were examined as an additional control.

Quantitation of labeling. For each time period p.i., 30–40 sections from infected leaves, or from pelleted protoplasts, were immunolabeled and examined. Two methods were used to determine the intensity of gold labeling. With sections from infected leaves, electron micrographs (45,000–75,000×) were used. The number of gold particles per 0.09-μm^2 area of section was determined for three structures (virion bundles, cylindrical inclusions, and background cytoplasm) in ~50 areas per structure from several micrographs (Table 1). Because of the scarcity of cylindrical inclusions encountered in sections of protoplasts, particularly those processed at 10 h p.i., all the cylindrical inclusions detected in protoplast sections were examined to determine the intensity of gold labeling. This was done directly on the microscope screen, at 15,000× with a 10× binocular, by counting the number of gold particles per 0.09 μm^2 over most of the area of the inclusion. These areas were measured with reference lines on the microscope screen, calibrated at 30,000×, with negatively stained tobacco mosaic virus particles as a size standard. Statistical analysis, using chi-square or Student's t test, was performed on the above data to determine if differences in labeling intensity between various times p.i. were significant.

RESULTS

TVMV-infected protoplasts. In sections of protoplasts fixed after incubation for 10, 21, or 45 h p.i. (Fig. 1A–E), gold-labeled TVMV CP antibodies were bound to the cylindrical inclusions (Fig. 1A, B, and E) with individual virions associated with these inclusions (Fig. 1A and D) and with parts of the cytoplasm close to the tonoplast (Fig. 1A). Association of virions or virion-like particles with the cylindrical inclusions was observed in both osmium-fixed, Spurr-embedded protoplasts (Fig. 1C) and glutaraldehyde-paraformaldehyde–fixed, LR White-embedded samples (Fig. 1B, D, and E) at 10–45 h p.i. In these samples, however, some virions were either unlabeled or only labeled at one end (Fig. 1D and E), probably because such virions were either completely or partially embedded in the LR White sections and, thus, had not been exposed at either or both ends to the primary antibodies or gold conjugate.

TABLE 1. Intensity of gold labeling on virion bundles, cylindrical inclusions, and background cytoplasm, using antibodies to tobacco vein mottling potyvirus coat protein (TVMV CP) or TVMV CI, in sections from tobacco leaves fixed 5 or 21 days postinoculation (p.i.) with TVMV[a]

Antibody/ Structure	Mean no. of gold particles/0.09 μm^2 (±S.E.)[b]	
	5 days p.i.	21 days p.i.
TVMV CP:		
Virion bundles	...[c]	57.9 ± 4.39
Cylindrical inclusions	1.9 ± 0.22	6.3 ± 0.22
Cytoplasm	0.2 ± 0.05	0.1 ± 0.01
TVMV CI:		
Cylindrical inclusions	22.7 ± 0.97	22.9 ± 0.91
Cytoplasm	0.0 ± 0.01	0.1 ± 0.05

[a] Leaf pieces were fixed in glutaraldehyde plus paraformaldehyde and embedded in LR White.
[b] Standard error.
[c] Only small bundles of somewhat loose virions were found; thus, it was difficult to determine labeling intensity per 0.09 μm^2.

The percentage of cylindrical inclusions labeled with CP and the intensity of labeling on these inclusions increased from 10 h (Fig. 1E) through 45 h p.i (Fig. 1A and B). Thus, in protoplasts fixed 10 h p.i., 20% of the cylindrical inclusions observed was unlabeled, 20% was weakly labeled (one to three gold particles per 0.09 μm^2), and 60% was moderately labeled (more than three gold particles per 0.09 μm^2). In protoplasts fixed 45 h p.i., percentages of unlabeled, weakly labeled, and moderately labeled cylindrical inclusions were 3, 18, and 79%, respectively. The difference in the amount of labeling of cylindrical inclusions between 10 and 45 h of incubation of infected protoplasts was statistically significant ($\chi^2 = 7.2$, $P < 0.05$). Using antibodies to TVMV CI protein, all the cylindrical inclusions detected at 10–45 h p.i. were labeled.

The occurrence of cylindrical inclusions in sections of protoplasts after incubation for 10 h p.i. was very rare (36 inclusions were detected in ~4,000 protoplast-sections, i.e., 40 sections × 100 protoplasts per section) compared to that in protoplasts incubated for 45 h p.i. (355 inclusions in ~1,000 protoplast-sections). Most of the cylindrical inclusions detected in protoplasts after incubation for 10 h p.i. were relatively small and were associated with the plasma membrane and/or rough endoplasmic reticulum (Fig. 1E). Larger inclusions detected at 21 and 45 h p.i. were associated mainly with the rough endoplasmic reticulum, but some also were close to the plasma membrane (Fig. 1A–D).

TVMV-infected leaves. In sections of infected leaves processed 5, 14, or 21 days p.i., gold-labeled TVMV-CP antibodies were bound to the cylindrical inclusions and to bundles of virions in the cytoplasm (Fig. 2A). CP-specific gold label was either distributed at random on the cylindrical inclusions or was linearly arranged parallel to their longitudinal axes (Fig. 2A), indicating association of TVMV CP and/or virions with these inclusions. Intensity of CP-specific labeling on the cylindrical inclusions increased significantly with time from 5 to 21 days p.i. ($t = 8.18$, $P < 0.001$). At 21 days p.i., however, the intensity of CP labeling on these inclusions was still much lower than that on virion bundles (Table 1). The cylindrical inclusions were more intensely labeled with TVMV-CI antibodies (Fig. 2B), with no significant difference in intensity between 5 and 21 days p.i. ($t = 0.17$; Table 1).

The TVMV-CP antibodies used in this study did not react with TVMV-CI protein when tested in Western blots (D. Thornbury, *personal communication*).

Using antibodies to TVMV CP or TVMV CI on sections of infected leaves or protoplasts, there was no specific gold labeling on any of the organelles in infected cells, including the nucleus, mitochondria, endoplasmic reticula, chloroplasts, microbodies, cell vacuoles, or cell walls. Nor did specific gold labeling occur when sections from infected leaves or protoplasts were incubated with preimmune rabbit sera (Fig. 2C) nor when sections from uninfected leaves or mock-inoculated protoplasts were incubated with CP or CI protein antibodies.

DISCUSSION

We have used TEM and immunogold labeling to investigate the subcellular localization of virions and viral CP during the course of infection of tobacco cells with TVMV. Protoplasts were used in an attempt to examine the early stages of infection, and we were able to detect virions and TVMV CP in protoplasts incubated for 10 h p.i. Protoplasts incubated for shorter periods were not examined because little or no TVMV CP was detected by enzyme-linked immunosorbent assay (11), and no TVMV virions or cylindrical inclusions had been found by TEM (12) in protoplasts prior to 10 h p.i. Even after incubation for 10 h p.i., thousands of protoplast-sections had to be examined by TEM to find a reasonable number of cylindrical inclusions, as experienced here and in a previous investigation (15).

Association of the cylindrical inclusions with virions or virion-like particles (3,5,6,10), with CP (9), and with the endoplasmic reticulum (3,10) has been observed previously in potyvirus-infected leaves but not in protoplasts. Additionally, the increase of CP labeling on the cylindrical inclusions with time p.i. has

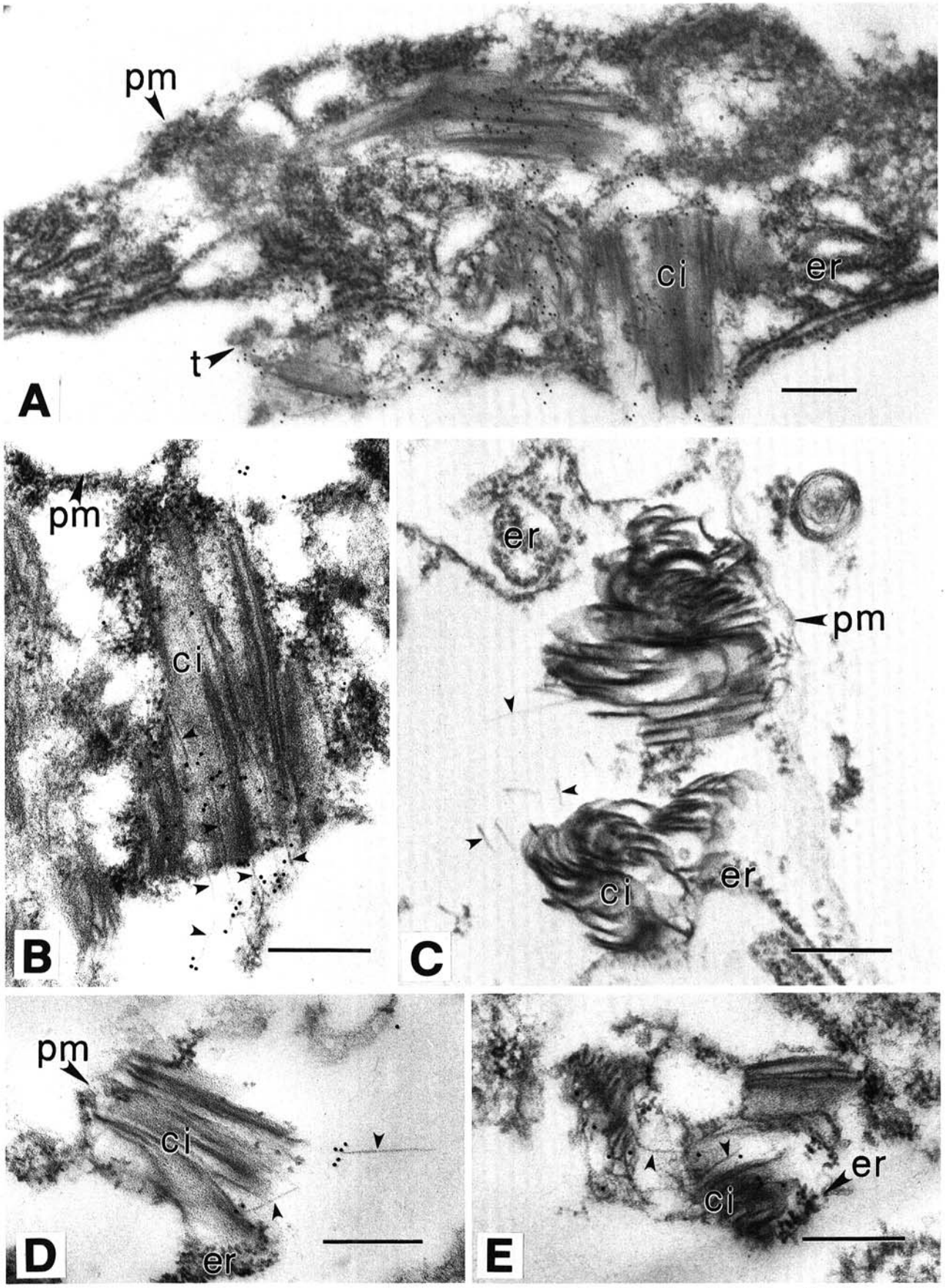

Fig. 1A–E. Sections of protoplasts fixed after incubation for 45 (**A and B**), 21 (**C and D**), or 10 h (**E**) postinoculation with tobacco vein mottling potyvirus (TVMV) RNA. **A, B, D, and E,** are from glutaraldehyde-paraformaldehyde–fixed, LR White-embedded samples; sections were incubated with antibodies to TVMV coat protein; gold label is associated with cylindrical inclusions (ci), with virions (small arrowheads) associated with ci, and with parts of the cytoplasm close to the tonoplast (t). **C,** is from an osmium-fixed Spurr-embedded sample; virion-like particles (small arrowheads) are associated with cylindrical inclusions, and these inclusions are associated with rough endoplasmic reticulum (er) and plasma membrane (pm). Bars = 300 nm.

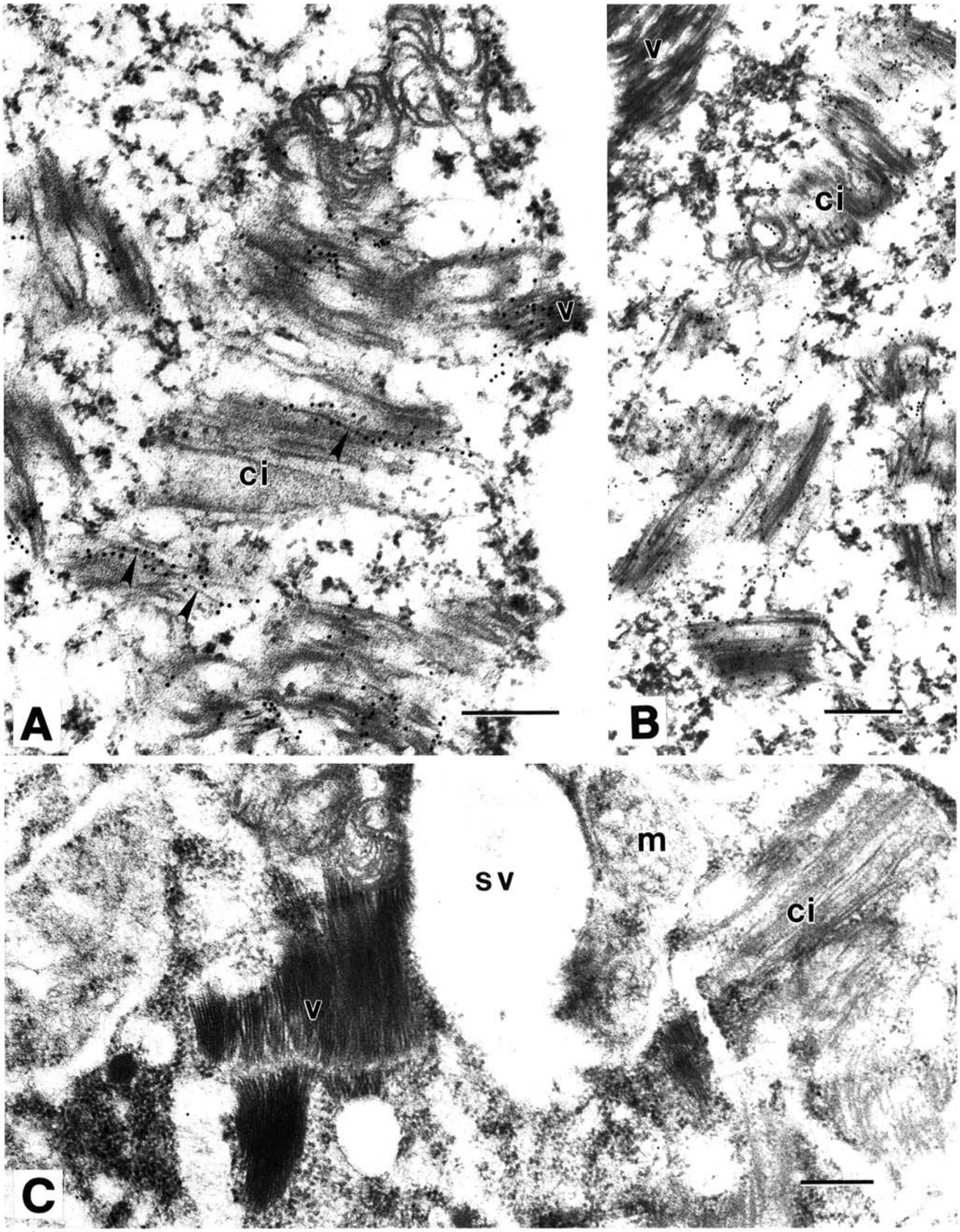

Fig. 2A–C. Sections of cells from systemically infected leaf tissue fixed in glutaraldehyde-paraformaldehyde and embedded in LR White, 21 days postinoculation with tobacco vein mottling potyvirus (TVMV). **A,** A section of a mesophyll cell incubated with antibodies to TVMV coat protein; gold label is associated with a small bundle of virions (v) and with cylindrical inclusions (ci); some gold particles are linearly arranged, probably indicating virions associated with these inclusions (arrowheads). **B,** A section incubated with antibodies to TVMV cylindrical inclusion protein; gold label is associated with ci but not with v. **C,** A section incubated with preimmune serum; no gold labeling was found on v or ci. m = mitochondrion; sv = secondary vacuole. Bars = 300 nm.

not been reported previously in leaves or protoplasts.

The association of TVMV CP with cylindrical inclusions at the early stages of infection in protoplasts and the increase with time p.i. of CP-specific labeling on these inclusions suggest that potyviral CP may be synthesized in association with cylindrical inclusions. Because at least two other TVMV proteins, P3 (15) and CI (12) also are associated with cylindrical inclusions at early stages in the infection process, it might be reasonable to consider whether synthesis of all the potyviral proteins might occur in, on, or near these inclusions, which are usually associated with the rough endoplasmic reticulum. Additionally, association of TVMV virions with the cylindrical inclusions at 10–45 h p.i. suggests that these inclusions may be involved in virion assembly. With the demonstration of helicase activity of the CI protein of a potyvirus (7,8), the replication of potyviral RNA also is likely to involve cylindrical inclusions. Taken together, these various schemes suggest a subcellular complex involving the cylindrical inclusions as the site of synthesis of each of the potyviral proteins, the replication of potyviral RNA, and the production of progeny virus particles.

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