Development of Cylindrical Inclusions in Potyvirus-Infected Protoplasts

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ABSTRACT

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The development of cylindrical inclusions in tobacco mesophyll protoplasts infected with the potyvirus tobacco vein mottling virus (TVMV) was examined. Bundles and pinwheels were observed in ultrathin sections of protoplasts collected 15–30 h after inoculation. In sections subjected to immunogold labeling with anti-TVMV cylindrical inclusion protein serum and gold-conjugated Protein A, the cylindrical inclusion protein was detected as early as 10 h after inoculation.

Potyvirus infections of plant cells result in the formation of morphologically distinctive inclusions referred to as cylindrical inclusions. These inclusions were initially thought to consist of virus particles (2,17), but this was later reported not to be the case (8). Cylindrical inclusions were shown to consist of protein (18) that was immunologically distinct from viral coat protein (19). Electrophoretic analysis indicated the size of the cylindrical inclusion protein (CI) to be approximately 70 kDa (10) and Dougherty and Hiebert (5) showed that CI is one of several proteins encoded by potyviral RNA.

Cylindrical inclusion morphology and development have been studied rather extensively and form the basis for the grouping of potyviruses into four taxonomic subdivisions (6,7). Yet the function of the cylindrical inclusion or of its constituent protein has not been determined. It was suggested that cylindrical inclusions might play a role in virus transport because they appeared to be associated with plasmodesmata (1,12). More recently, the CI has been shown to share amino acid sequence homology with proteins thought to be involved in virus replication (4), especially helicase-related proteins (11).

Almost all previous investigations of the structure and development of cylindrical inclusions have involved examination of infected leaf or root samples. Although these approaches have provided a number of elegant representations and descriptions of these extraordinary structures, they have not afforded the opportunity to examine the temporal aspects of inclusion formation within a particular cell. As part of an investigation of the function of the potyviral CI, we have attempted to detect and characterize this protein at the early stages of infection, during which its role in the establishment of infection is probably most significant. To this end, we have examined the development of cylindrical inclusions in tobacco mesophyll protoplasts inoculated with tobacco vein mottling virus (TVMV) RNA.

Many years ago Goffinet and Verhoyen (9) reported the presence of cylindrical inclusions in sections of tobacco mesophyll protoplasts 48 h after inoculation with potato virus Y. Here, we describe the detection of TVMV CI in sections of tobacco mesophyll protoplasts as early as 10 h postinoculation and the presence of cytoplasmic bundles and pinwheels, the longitudinal and radial orientations of cylindrical inclusions, respectively, in protoplasts infected for periods of up to 30 h.

MATERIALS AND METHODS

Virus and RNA purification. TVMV was propagated in *Nicotiana tabacum* L. 'Kentucky 14' and purified by previously published procedures (15,16). TVMV RNA was isolated from purified virus particles by sodium dodecyl sulfate sucrose-gradient centrifugation (15).

Isolation and inoculation of protoplasts. *N. tabacum* 'Xanthi' mesophyll protoplasts were isolated by slight modifications of the procedure described by Takebe et al (20). The maceration medium contained 0.5% Macerase (Calbiochem, La Jolla, CA) and 0.5% potassium dextran sulfate in 0.7 M mannitol (adjusted to pH 5.5). Mesophyll cells were treated with 1% cellulase (Onozuka R10, Yakult Honsha Co. Ltd., Japan) in 0.7 M mannitol (pH 5.5) at 25–30 C and shaken at a frequency of 80 excursions per minute.

Protoplasts were inoculated with TVMV RNA (25 μg of RNA per 1×10^6 protoplasts) using a Gene-Pulse Electroporation Unit (Bio-Rad, Richmond, CA) as described by Luciano et al (14). Electroporated protoplasts (1×10^6) were incubated in 10 ml of incubation medium (20) containing the antibiotics carbenicillin (100 μg/ml), cephaloridine (100 μg/ml), and nystatin (4 μg/ml) at 25 C under 35–55 μE m^{-2} s^{-1} fluorescent illumination.

Electron microscopy. Protoplast samples (2.5×10^5) for electron microscopy were fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, and incubated at room temperature for 30–60 min. The protoplasts were washed two to three times by centrifugation at 1,000 rpm for 3 s in an Eppendorf microfuge and resuspended in 0.1 M sodium cacodylate buffer, pH 7.4. Postfixing was accomplished by treatment with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer, pH 7.4, for 30–60

min at room temperature in the dark. After two to three washings with cacodylate buffer as described above, the protoplasts were dehydrated in a graded ethanol series (25–100%) and embedded in Spurr's medium. Sections were cut on a Porter Blum MT-2 microtome (Norwich, CN), mounted on grids, stained in uranyl acetate and lead citrate, and viewed in a Philips 400 electron microscope.

Preparation of protoplast samples for electron microscopy and immunogold labeling included treatment with 3% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, but omitted treatment

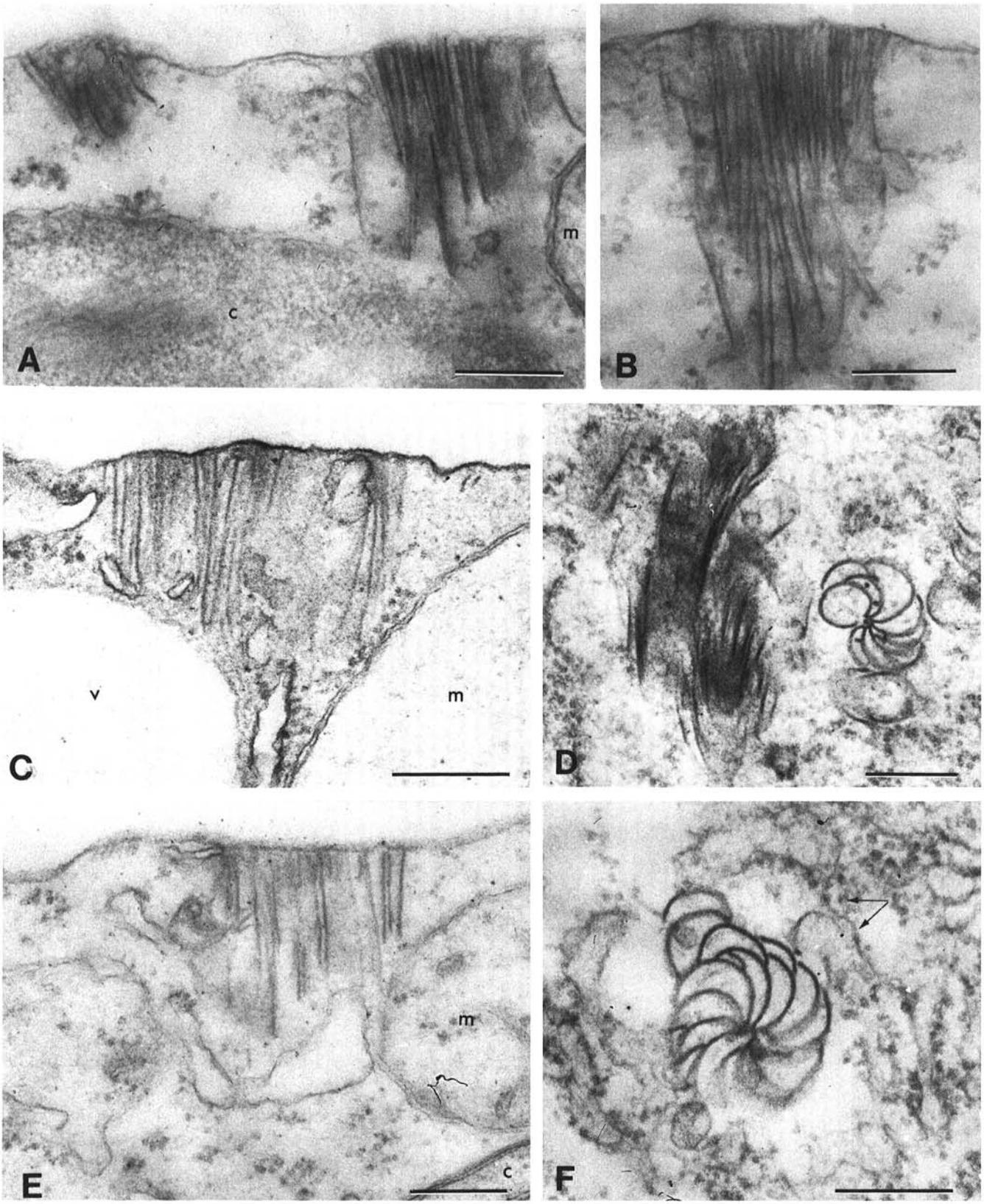

Fig. 1. Cylindrical inclusions in ultrathin sections of tobacco mesophyll protoplasts **A,B,** 15 h; **C,D,** 20 h; and **E,F,** 30 h after inoculation with tobacco vein mottling virus RNA. Bundles are in contact with the plasma membrane (A–C,E); other bundles and pinwheels are present in the cytoplasm (D,F). m = mitochondrion; c = chloroplast; v = vacuole; arrows point to ribosomes and endoplasmic reticulum. Bars = 30 nm.

with osmium tetroxide. Protoplasts were subjected to a graded ethanol series (25–70%) and embedded in L.R. White medium. Sections were stained in uranyl acetate only.

Immunogold labeling was accomplished by the method described by Tomenius et al (21) and involved the use of anti-CI immunoglobulin (Ig; produced by D. W. Thornbury, University of Kentucky) at a concentration of 9 μg of Ig per milliliter and gold-conjugated Protein A (10 nm gold particle, Sigma Chemical Co., St. Louis, MO).

RESULTS AND DISCUSSION

Cylindrical inclusions were observed 40–48 h postinoculation, but the cytoplasmic organelles in these protoplasts were not well preserved and the plasma membrane was often discontinuous. We then examined cylindrical inclusion development in protoplasts infected for periods of 15–30 h. The structural integrity of these protoplasts was indicated by the presence of mitochondria and chloroplasts with double membranes (Fig. 1A, C, and E), ribosomes and endoplasmic reticulum (Fig. 1F), and a continuous plasma membrane (Fig. 1A–C and E).

Bundles and pinwheels were observed as early as 15 h postinoculation as well as in sections of protoplasts incubated for periods of 20 and 30 h postinoculation. The bundles were usually observed in contact with the plasma membrane (Fig. 1A–C and E), but sometimes were detected in the cytoplasm with no apparent association with any membranous structure (Fig. 1D). Pinwheels were usually seen in the cytoplasm away from the plasma membrane (Fig. 1D and F) and occasionally in close proximity to ribosomes and endoplasmic reticulum (Fig. 1F).

To determine the subcellular location(s) in which cylindrical inclusions were initially produced, we conducted an immunocytological examination of protoplasts infected for periods of 15 or fewer hours. Bundles were evident in 13- and 15-h samples (Fig. 2A and B) and immunogold labeling verified the presence of the CI. In protoplasts collected 10 h postinoculation, there were areas in which CI-specific immunogold label was associated with the plasma membrane (Fig. 2C–E) and cylindrical inclusions were observed within these areas (Fig. 2C and E). In some sections, however, there were areas of immunogold label along the plasma membrane where cylindrical inclusions were not discernible (Fig. 2D). Such areas occurred randomly and always in association with the plasma membrane and thus probably contained cylindrical inclusions at early stages in their development.

Lawson et al (13) and Andrews and Shalla (1) noted the presence in sections of potyvirus-infected plant tissues of cylindrical inclusions in the cytoplasm and in association with the plasma membrane. However, they showed by examining serial sections that those inclusions that appeared to be located in the cytoplasm were actually in contact with the plasma membrane. These findings suggest that development of potyviral cylindrical inclusions occurs at the plasma membrane. Indeed, in the experiments reported here, the inclusions were always associated with the plasma membrane in protoplasts collected at a period after inoculation that we believe represents an early stage (10 h postinoculation) in cylindrical inclusion development.

Cylindrical inclusions have been reported to occur most frequently in close proximity to plasmodesmata (1,12), and the open central cores of some pinwheels have been shown to be aligned over plasmodesmata (12). As a result, plasmodesmata were proposed as the sites of pinwheel formation (12), and the association of cylindrical inclusions with plasmodesmata led to the suggestion that the inclusions are involved in the transport of virus from cell to cell in plant tissues (1,12). The formation of inclusions in protoplasts in a manner similar to that in leaf and root tissues might therefore be thought to argue against this suggestion since cell-to-cell movement of virus does not occur with isolated protoplasts.

We have shown that TVMV cylindrical inclusion development can be studied in tobacco mesophyll protoplasts. The use of

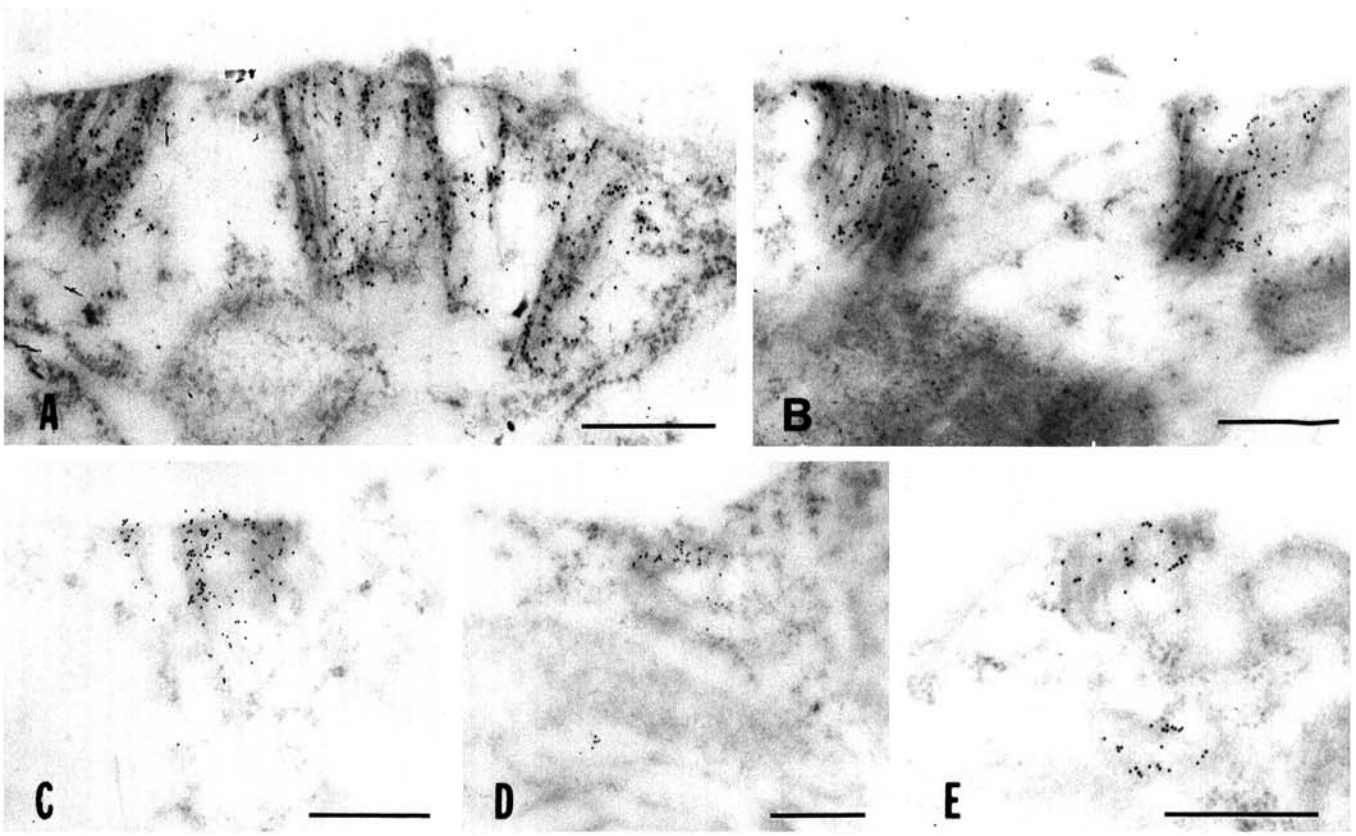

Fig. 2. Detection of tobacco vein mottling virus cylindrical inclusion protein (CI) in ultrathin sections of tobacco mesophyll protoplasts after treatment with anti-CI Ig and gold-conjugated Protein A. Immunogold labeled bundle formations were observed in contact with the plasma membrane **A,** 15 h; **B,** 13 h; and **C–E,** 10 h after inoculation. Bars = 50 nm.

protoplasts provides a system in which infection occurs in a near-synchronous manner and inclusions can be detected relatively soon after inoculation. With the ability to produce infectious transcripts of TVMV cDNA in vitro (3), we intend to generate and analyze mutations in the CI gene of TVMV RNA in order to map functional domains in the CI in relation to cylindrical inclusion development and, ultimately, to define the role the CI plays in potyvirus infections.

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