In Situ Localization of Barley Yellow Dwarf Virus Coat Protein in Oats

Petra H. Nass, Birute P. Jakstys, and Cleora J. D'Arcy

First and third authors: University of Illinois, Department of Plant Pathology, Urbana 61801; and second author: University of Illinois, Center for Electron Microscopy, Urbana 61801.
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ABSTRACT

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The expression of barley yellow dwarf virus (BYDV) coat protein was followed in situ by immunogold labeling of ultrathin sections. Samples of BYDV-PAV-IL–infected oat seedlings were taken 1–10 days postinoculation (dpi) and prepared for electron microscopy. Gold labeling was first detected in the host cytoplasm at 2 dpi. At 3 dpi, labeling had increased and was found mostly around or within regions that contained ribosome-associated filamentous material. No labeling was found over densely compressed filaments. By 4–5 dpi, viral coat protein also was detected in the nucleus. Viral coat protein was not associated with any other cellular components. The results suggest that viral coat protein is first expressed in the cytoplasm of the infected host cell. Since labeling was mostly found in or around regions of ribosome-associated filaments, it is proposed that coat protein is expressed by cytoplasmic ribosomes from viral RNA. Coat protein found in the nucleus during later stages of infection probably diffused into the nucleoplasm after disruption of the nuclear membrane.

Barley yellow dwarf viruses (BYDVs) cause significant economic losses worldwide because of damage to barley, wheat, and oats. Five strains of BYDVs have been recognized and named after their predominant aphid vector species: MAV (*Sitobion avenae* Fabricius), PAV (*Rhopalosiphum padi* L. and *S. avenae*), SGV (*Schizaphis graminum* Rondani), RPV (*R. padi*), and RMV (*R. maidis* Fitch). Aphids transmit the virus during the feeding process, when viral particles are released into the phloem of the host. BYDV infection remains restricted to the cells of the phloem tissue: phloem parenchyma, sieve elements, and companion cells. The infection leads to a premature necrosis of the phloem and causes external symptoms such as stunting and leaf chlorosis (5). The alterations of cellular ultrastructure caused by BYDV infection have been described in oats, *Avena byzantina* K. Koch and *A. sativa* L. (7–10); barley, *Hordeum vulgare* L. (15); barren brome, *Bromus sterilis* (21); and *Leersia oryzoides* (1). Infected cells are characterized by the presence of one or more of the following: viral particles, filamentous material in the cytoplasm or the nucleus, virus-induced vesicles, and a densely staining substance in the cytoplasm and plasmodesmata.

Gill and Chong (8–10) defined three phases of infection in oats based on alterations in the cytoplasm (early phase), nucleus (intermediate phase), and both the nucleus and cytoplasm (late phase). The phases differ for the five strains of BYDVs. The major changes during infection with BYDV-PAV, the viral strain used in this study, begin with the appearance of densely staining material in plasmodesmata. An electron-opaque amorphous substance and filaments, either free or within single-membrane vesicles, appear in the host cytoplasm. It has been shown recently that the filaments contain viral RNA (20). At the end of the early phase, filaments are visible in the nucleopores. During the intermediate phase of infection, the nuclear outline becomes distorted, and massive clumping of heterochromatin begins. Only in the late phase can viral particles be seen in cytoplasm and, later, in the nucleus. Based on these observations, Gill and Chong (8–10) suggested that the cytoplasm is the site of coat protein expression and viral assembly.

The site of viral coat protein expression has been identified for various plant viruses, such as alfalfa mosaic virus (26), red clover mottle virus (25), tobacco mosaic virus (22), and barley stripe mosaic virus (16–18) but has not been determined for any of the luteoviruses. The objective of the present study was to develop an in situ immunogold localization assay that would allow us to follow the expression of BYDV-PAV coat protein over time in infected oat phloem cells.

MATERIALS AND METHODS

Virus, vector, and host. The viral isolate used in this study was BYDV-PAV-IL (12). Colonies of viruliferous and virus-free *R. padi* were maintained on caged plants of *H. vulgare* cv. Hudson in growth chambers at 18–21 C with a 14-h day. Seedlings of the host plant *A. byzantina* cv. Coast Black were grown in the greenhouse at 18–21 C, 235–830 μE m^{-2} s^{-1}, with a 16-h day and transferred into growth chambers after infestation with aphids. Seven-day-old Coast Black oat seedlings were inoculated with BYDV-PAV-IL by approximately 30 viruliferous *R. padi* during a 48- to 72-h inoculation access period. Aphids were then fumigated for 10 min with Pratt's 4S Vapona (B.G. Pratt Company, Patterson, NJ).

Tissue preparation for electron microscopy. After the beginning of the inoculation access period, 1-mm^2 samples were taken daily for 10 days from the midrib and stem of the inoculated primary leaf. Tissue pieces were fixed overnight at 4 C in 3.5% paraformaldehyde and 0.5% glutaraldehyde in 50 mM sodium cacodylate buffer, pH 7.0, followed by 4 h in 1% osmium tetroxide at 4 C. After dehydration in a graded series of ethanol and propylene oxide, samples were embedded in Spurr's resin or a Spurr's/Medcast resin mixture.

Immunogold localization assay. *Primary and secondary antibodies.* Polyclonal primary antibodies against BYDV-PAV-IL coat protein subunits (4,11) were used at a concentration of 0.1 mg/ml in autoclaved PBS (50 mM sodium phosphate, 0.15 M sodium chloride, pH 7.2), containing 1.25% bovine serum albumin (BSA) and 5% normal goat serum (NGS) (Sigma Chemical Company, St. Louis). Primary antibodies were cross-adsorbed with sap from noninoculated, 4-wk-old host tissue. The sap was prepared by grinding healthy plant tissue in PBS at a ratio of 1:3 (w/v). To remove plant fibers the mixture was passed through cheesecloth. The sap was filtered through a 0.2-μm syringe filter, divided into 100-μl aliquots, and stored at −20 C. For cross-adsorbtion, antibodies were incubated at a concentration of 0.1 mg/ml for 2 h in a mixture of equal volumes of plant sap and blocking buffer. The mixture was centrifuged prior to use in a microfuge for 10 min at 11,000 rpm. Goat anti-rabbit immuno-

globulin conjugated to 10 nm of gold (Ted Pella, Redding, CA) was used as the secondary antibody. All solutions used in the procedure were passed through a 0.1-μm filter and stored as 1.5-ml aliquots at −20 C until use.

Labeling procedure. The procedure described previously (19) was modified as follows. All steps were performed at room temperature. Ultrathin sections were placed on formvar carbon-coated nickel grids. Grids were placed for 15 min onto droplets of 0.42 M sodium metaperiodate. Nonspecific binding sites were blocked for 30 min in PBS containing 2.5% BSA and 10% NGS. Sections were allowed to react with primary antibody on 25-μl droplets for 2 h and were subsequently washed twice for 10 min each on PBS-Tween and once on blocking buffer. Grids were removed after 30 min and washed twice for 10 min each on PBS-Tween and twice for 10 min on water. Sections were stained for 30 min with 4% uranyl acetate and for 1–5 min with 0.5% lead citrate and viewed with a Hitachi H-600 TEM at 75 kV (Nissei Sangyo American, Ltd., Mountainview, CA).

Controls. Noninoculated seedlings and plants infested with nonviruliferous *R. padi* were sampled and labeled as described above. In addition, polyclonal rabbit antibody against soilborne wheat mosaic virus or rabbit preimmune serum at 0.1 mg/ml was used as a negative control.

Quantification of labeling. Electron micrographs at magnifications between 45,000 and 150,000× were used to quantify the label deposits. One electron micrograph from each of 10 experiments was analyzed. A grid pattern was placed over subcellular profiles, and the number of gold particles per given square was counted. Label densities are expressed as gold particles per square micrometer. Electron micrographs did not always contain all cell organelles; therefore, the values given in Table 1 are averages of five to 10 experiments.

TABLE 1. Labeling densities (gold particles per square micrometer) in control and barley yellow dwarf virus-PAV-IL infected cells

	Cytoplasm	Nucleus	Cell wall	Vacuole	Mitochondria
Control	0.0 (0.0)	0.0 (0.0)	0.5 (0.3)[a]	0.6 (0.1)	0.2 (0.5)
4 dpi[b]	5.1 (3.6)	1.4 (1.1)	0.2 (0.3)	0.6 (0.2)	0.0 (0.0)
6 dpi	17.2 (7.2)	na[c]	na	na	na

[a]Values are averages of five to 10 experiments. Numbers in parentheses are the standard deviations.
[b]Days postinoculation.
[c]Not applicable.

RESULTS

Ultrastructure. The ultrastructural alterations and the sequence of events of BYDV-PAV infection in oats described in previous reports (7–10) also were identified in this study. By 1–2 days postinoculation (dpi), cells were at an early to intermediate stage of infection (Fig. 1). The host cytoplasm mainly contained filaments, a few virus-induced vesicles, and a densely staining amorphous substance. BYDV particles were first observed around the filamentous material at 2–3 dpi (Fig. 2). The particles became more numerous by 4–5 dpi and also were seen in the nucleus

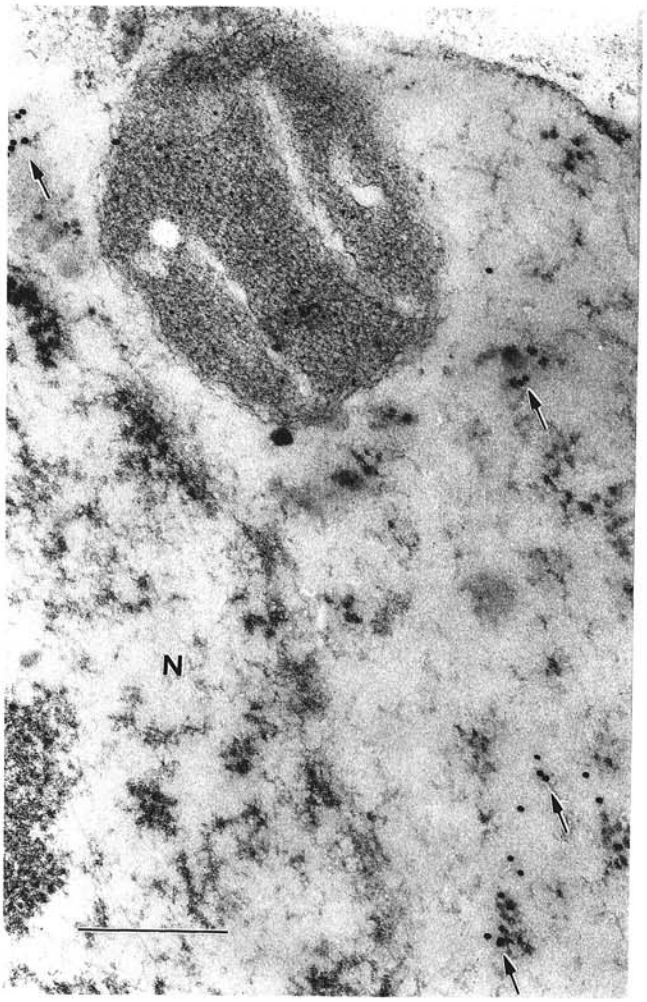

Fig. 1. Barley yellow dwarf virus-infected cell at intermediate stage of infection, 2 days postinoculation. Gold particles (arrows) are few and present only in small areas of the host cytoplasm. N = Nucleus. 66,500×. Scale bar = 300 nm.

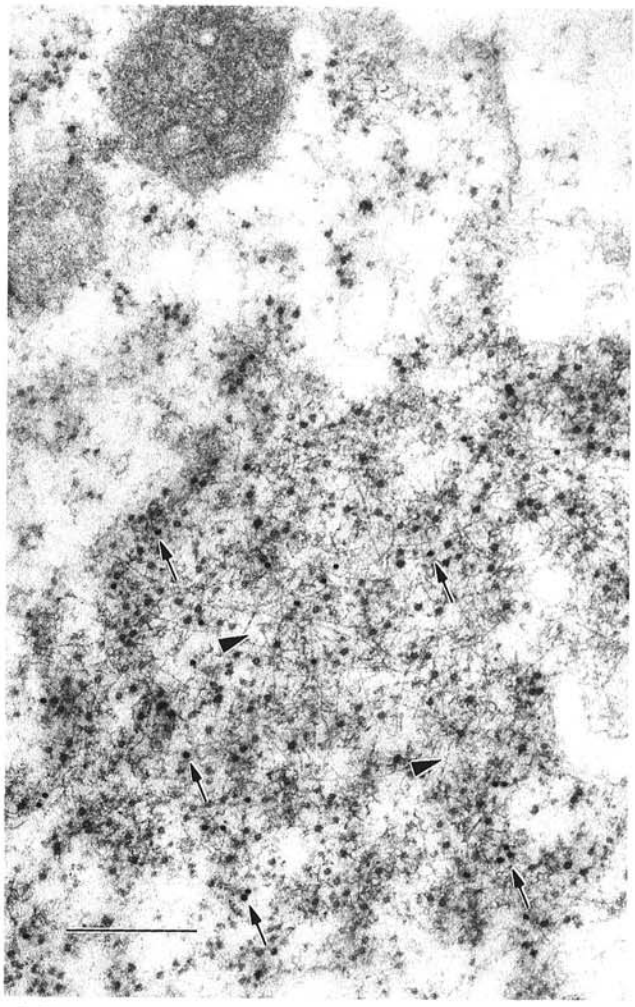

Fig. 2. Phloem companion cell at late stage of barley yellow dwarf virus infection, 3 days postinoculation. Numerous filaments (arrowheads) in the host cytoplasm are interspersed with viral particles and ribosomes. Gold labeling is occasionally associated with filaments (arrows). 57,500×. Scale bar = 300 nm.

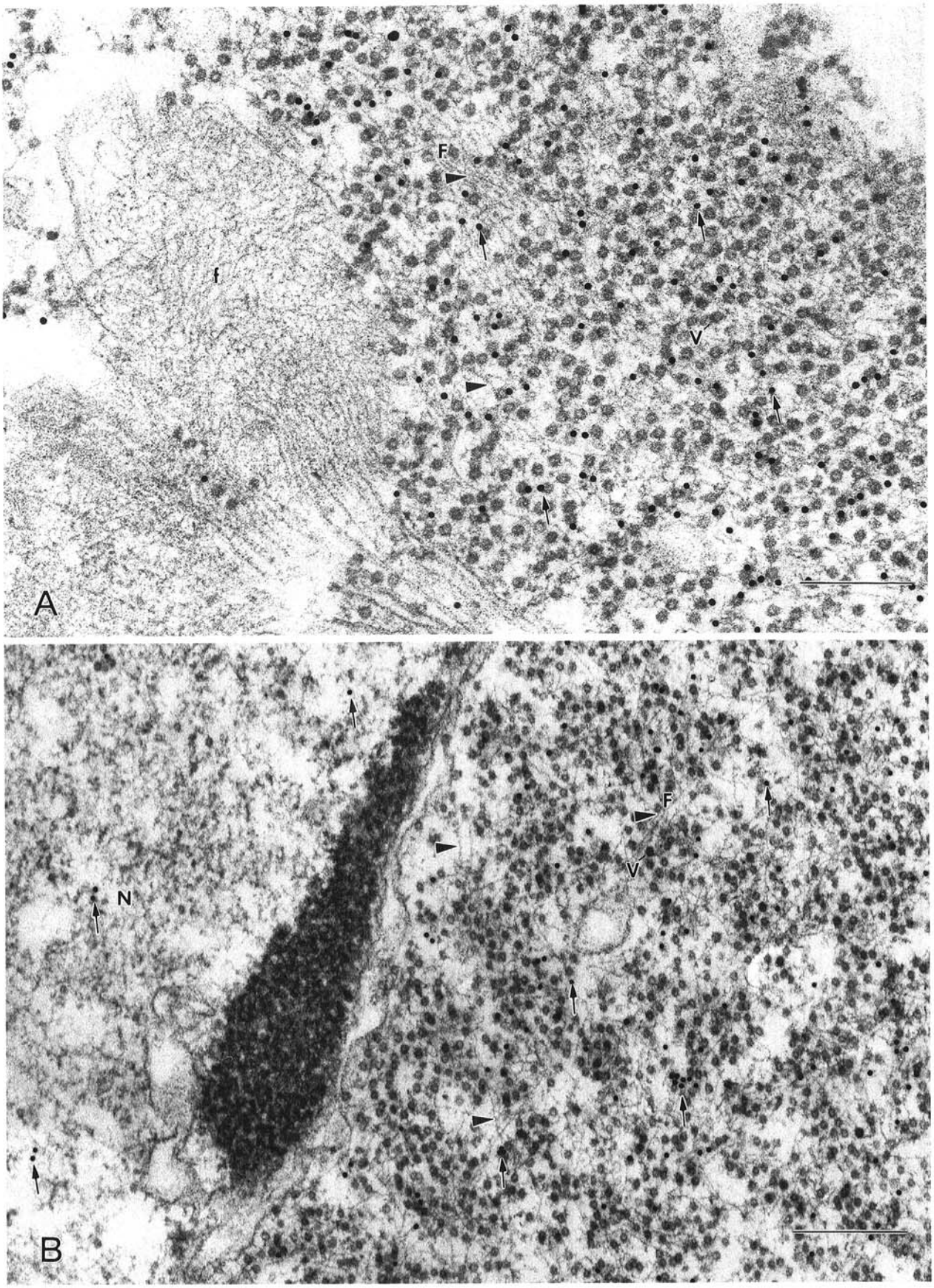

Fig. 3. A, Phloem cell at 5 days postinoculation (dpi) with barley yellow dwarf virus. Closely packed filaments (f) adjacent to viral particles (V), ribosomes and filaments (F, arrowheads). Gold labeling (arrows) was not associated with densely packed filaments. 105,000×. Scale bar = 200 nm. **B,** Immature sieve element at 4 dpi. Host cytoplasm contains large amounts of filamentous material (F, arrowheads) attached to ribosomes. Viral particles are present. Gold particles (arrows) appear in the nucleus (N) (arrows). 70,000×. Scale bar = 300 nm.

(Fig. 3B). At this stage, more filamentous material was evident and was often associated with ribosomes. The nucleus appeared deformed and was frequently surrounded by a subtended nuclear membrane. After 6 dpi, most infected cells were filled with viral particles and were devoid of identifiable cell organelles (Fig. 4).

Detection of viral coat protein. Viral coat protein was first detected in the cytoplasm of cells near the end of the intermediate stage of infection in BYDV-PAV-IL–inoculated plants. At this stage, only a few gold particles were observed in isolated regions of the cytoplasm (Fig. 1). Gold labeling became more frequent with the appearance of viral particles in the cytoplasm within or around regions that contained filamentous material (Fig. 2). Densely compressed filamentous material and filaments not associated with ribosomes were devoid of gold labeling (Fig. 3A). Cells at an intermediate stage of infection that contained areas with a few viral particles reached label densities with gold particles at $2.1/\mu m^2$. Gold-label densities of cells 4–10 dpi are summarized in Table 1. The number of gold particles in the cytoplasm increased to $5.1/\mu m^2$ once gold particles were found in the nucleus (Fig. 3B). The highest label density observed in the nucleus was $1.4/\mu m^2$. Cells completely filled with viral particles showed the highest label density ($17.2/\mu m^2$) (Fig. 4).

Initial experiments showed that it was necessary to cross-adsorb the polyclonal antibodies with healthy plant sap to achieve specific labeling of viral coat protein. Without cross-adsorption, indiscriminate gold labeling was found at concentrations of more than 20 gold particles per square micrometer. The use of cross-adsorbed antibodies eliminated most of the nonspecific labeling, and gold particles were only observed occasionally attached to the membranes of mitochondria, cell walls, or vacuoles of control treatments (Table 1). No viral coat protein was associated with other cell organelles, vacuoles, or the cell walls. No labeling of BYDV particles was seen in samples treated with soilborne wheat mosaic virus antibodies compared to sections exposed to BYDV-PAV-IL antibodies.

DISCUSSION

Viral coat protein of BYDV can be specifically detected in ultrathin sections of oats by immunogold labeling. The level of nonspecific labeling in noninoculated samples was low and comparable to that in similar studies (13,22). This allowed distinction between nonspecific and specific labeling in inoculated samples. Heterologous antibodies did not label BYDV particles, further demonstrating the specificity of the in situ immunogold assay.

Labeling was first observed in the cytoplasm of infected host cells, which indicates that viral coat protein is first accumulated in the cytoplasm of the infected phloem cell. A larger number of gold particles was first detected in the cytoplasm, and label densities remained higher in the cytoplasm than in the nucleus. This result supports the conclusion drawn by Gill and Chong (9) from their observation that viral particles appeared first in the cytoplasm of BYDV-PAV–infected oats. The close association of viral protein with areas containing loosely packed and ribosome-bound filamentous material strongly supports the conclusion that the filaments consist of viral RNA, as has been demonstrated recently (20). Therefore, it is suggested that viral coat protein is expressed from plus-strand BYDV RNA by host ribosomes in the cytoplasm and enters the nucleus only after the disruption of the nuclear membrane during the late phase of infection.

The results of this study confirm observations made in earlier studies (7–10,15) concerning events during BYDV infection. All stages of infection, as well as all alterations reported to be induced by BYDV, were observed.

The fact that viral particles could first be observed within areas containing filamentous material is inconsistent with Gill and Chong (8–10), who observed that viral particles appeared first around the nucleus. The discrepancy might be explained by the different viral isolates used in these studies. Viral particles appear within a short time frame and can be detected within 1 day.

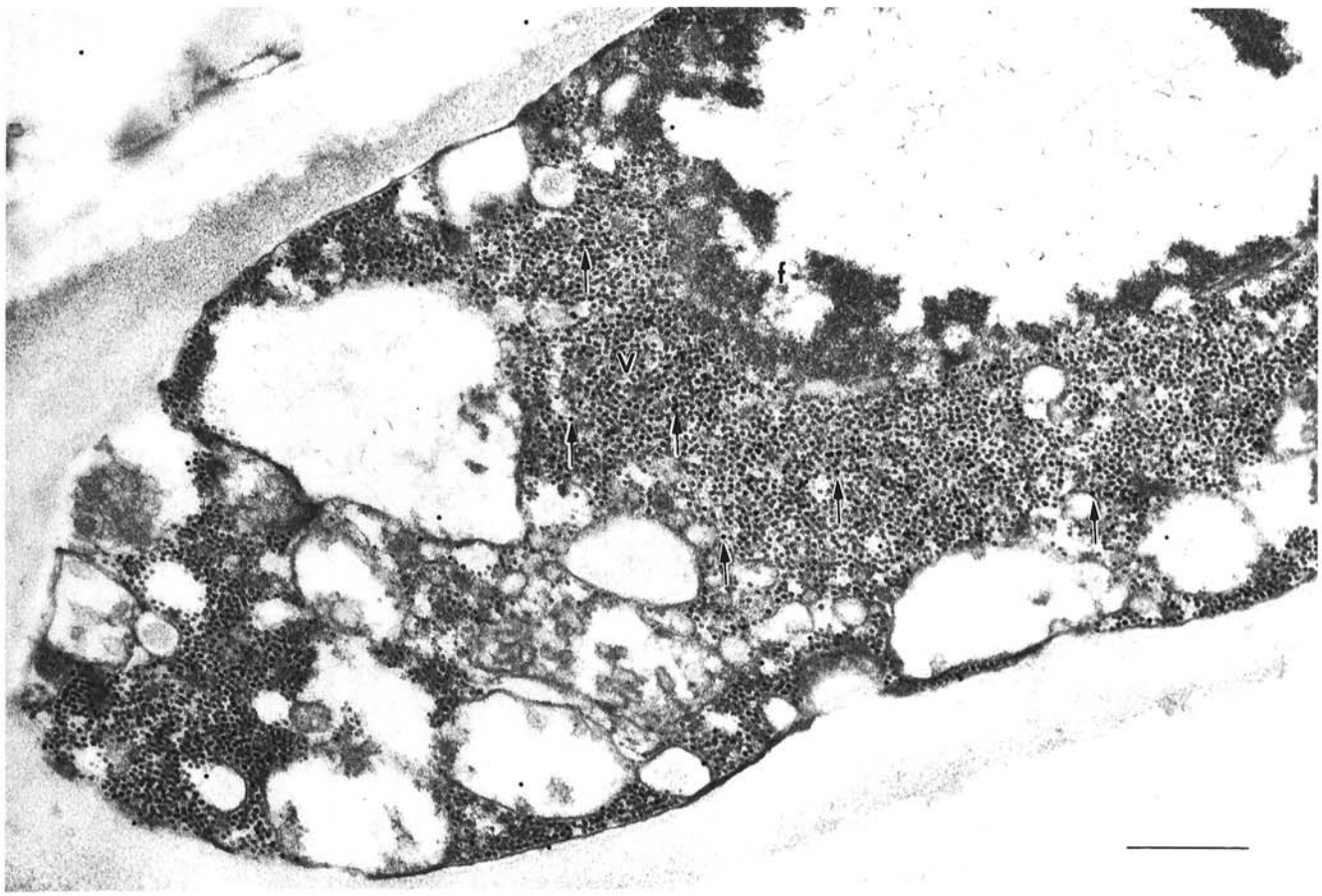

Fig. 4. Cell at 10 days postinoculation with barley yellow dwarf virus completely filled with viral particles (V). Densely staining filamentous material (f) is present in the center of the cell. Arrows indicate gold labeling. 33,000×. Scale bar = 500 nm.

Sections that represented this stage were rare and did not always contain a nuclear membrane, and therefore, it is possible that we might have overlooked this stage. However, the observation that gold labeling also was detected first in areas around the filamentous material and not around the nucleus suggests that viral particles of BYDV-PAV-IL were assembled first, and therefore can be seen first, in these areas and not around the nuclear membrane.

Although it has been shown for other groups of plant viruses that coat protein is accumulated in the cytoplasm (25,26), this is the first report for any of the luteoviruses. Viral particles of potato leafroll luteovirus (PLRV) were detected first and only in the host cytoplasm of infected potato *Solanum tuberosum* (24), suggesting that PLRV coat protein also is accumulated in the cytoplasm of the infected cell. In sugar beet, *Beta vulgaris* (6), and pennycress, *Thlaspi arvense* (3,14), infected with beet western yellows luteovirus (BWYV) virions were closely associated with the nucleus at an early stage of infection, and it has been suggested that the nucleus might play a major role in virus multiplication (6,12). BWYV is more closely related to BYDVs in subgroup II than to those in subgroup I to which BYDV-PAV-IL belongs (23). The sequence of events during virus infection, as well as some of the cytopathological alterations caused by BYDVs subgroup II, such as RMV or RPV, closely resemble those caused by BWYV and differ from those caused by subgroup I viruses. For example, virus particles of BWYV, BYDV-RPV, and BYDV-RMV usually are seen first in the nuclei of infected phloem cells. In situ localization studies with these viruses will be necessary to gain further understanding of the differences in the infection process. Current studies in our laboratory have demonstrated the usefulness of the in situ labeling method for the detection of the 17-kDa viral RNA binding protein (20) and the 50-kDa readthrough protein (2) of BYDV-PAV-IL. Future research will be conducted on localization and temporal production of the BYDV-PAV RNA polymerase.

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