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Grapevine Fanleaf Nepovirus P38 Putative Movement Protein Is Located on Tubules *In Vivo*

Christophe Ritzenthaler, Anne-Catherine Schmit, Pierre Michler, Christiane Stussi-Garaud, and Lothaire Pinck

Institut de Biologie Moléculaire des Plantes du CNRS et Université Louis Pasteur, Laboratoire de Virologie, 12 rue du Général Zimmer, 67084 Strasbourg Cedex, France.
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In *Chenopodium quinoa* cells infected with grapevine fanleaf virus, tubular structures are formed which protrude from or penetrate the cell wall and which contain viruslike particles. These structures are thought to be involved in cell-to-cell spread of the virus. Immunogold cytochemistry using immunoaffinity-purified antibodies permitted the location of the P38 putative movement protein of grapevine fanleaf virus (GFLV) in or on these tubular structures. We have shown by light and electron microscopy that similar structures are formed at the surface of *C. quinoa* protoplasts, in the absence of cell wall and consequently of plasmodesmata. These tubules, although not abundant, measured 40 to 60 nm in diameter and up to 30 μm in length. By immunofluorescence and immunogold-silver staining, P38 was localized along these structures. Together with P38 involvement in tubule formation in planta, this supports the idea that P38 protein has a role in the cell-to-cell spread of GFLV. These studies also revealed that P38 is very abundant in the cytoplasm of infected protoplasts. This subcellular location, although unusual for a movement protein, is, however, in agreement with previous subcellular fractionation experiments with P38 protein in *C. quinoa* plants.

Additional keywords: cell-to-cell communication, plant virus, cell wall.

Establishment of systemic infection requires the virus to spread from the initially infected cell to the rest of the plant. Virus spread from an infected cell into neighboring uninfected cells, also termed cell-to-cell or short-distance movement, represents the first step in this process and involves virus-encoded movement protein(s) (MP). Two mechanisms, both inducing potential modification of plasmodesma, have been described so far for cell-to-cell movement of plant viruses: The tobacco mosaic virus (TMV)-like mechanism (for recent review see Citovsky and Zambryski 1993) and the cowpea mosaic virus (CPMV)-like mechanism, which involves formation of tubules through which virions are transported to the adjacent cell (McLean et al. 1993).

Grapevine fanleaf virus (GFLV) belongs to the plant nepovirus genus within the *Comoviridae* family (Ward 1993). Members of this family are characterized by polyhedral viral particles, a plus-sense bipartite genome, the presence of a small covalently genome-linked protein (VPg) at the 5′ end and a poly(A) sequence at the 3′ OH extremity of virus RNA. The nematode transmissibility of nepoviruses differentiates them from comoviruses.

Corresponding author: L. Pinck; E-mail: pinck@medoc.U-strasbg.fr

The large RNA of the F13 isolate of GFLV (RNA1, 7,342 nucleotides (nt) (Ritzenthaler et al. 1991) encodes a 253-kDa polyprotein (P1) involved in viral replication (Viry et al. 1993) and polyprotein processing (Margis and Pinck 1992; Margis et al. 1994; Margis et al. 1991). RNA2 (3,774 nt; Serghini et al. 1990) encodes a 122-kDa polyprotein (P2) and is needed for viral spread in planta (Viry et al. 1993). Upon in vitro translation, polyprotein P2 is cleaved by the RNA1-encoded 24-kDa proteinase into a 28-kDa N-terminal protein (P28), a 38-kDa protein (P38), and the carboxy-terminal coat protein (CP) (Margis et al. 1993). Comparison of the genome organization of GFLV with other como- and nepoviruses suggests that the P38 domain, just upstream of the CP, plays a role in cell-to cell movement (Goldbach et al. 1990).

For CPMV, a nonstructural protein (48 kDa) has been identified as viral MP and is required together with the CP for cell-to-cell movement (Wellink and van Kammen 1989; Wellink et al. 1993). In plants infected with como-, nepo-, and caulimoviruses (for review see Francki et al. 1985) and with one bipartite geminivirus (Kim and Lee 1992), tubular structures containing viruslike particles are often found protruding through the cell wall into the cytoplasm of adjacent cells. Immunogold labeling studies revealed that the tomato ringspot nepovirus (TomRSV) 45-kDa protein (Wieczorek and Sanfaçon 1993), the red clover mottle comovirus (RCMV) 43-kDa protein (Shanks et al. 1989), the cauliflower mosaic caulimovirus (CaMV) 46-kDa protein (P1, Linstead et al. 1988), the CPMV 48-kDa proteins (van Lent et al. 1990) and, recently, the tomato spotted wilt tospovirus NSm protein (Kormelink et al. 1994), were detected in infected plants in association with such tubular structures, indicating that these proteins play a role in viral cell-to-cell spread via the formation of tubules. More recently, studies performed with CPMV (van Lent et al. 1991) and with CaMV (Perbal et al. 1993) allowed the identification of extracellular tubular structures containing viruslike particles extending from the surface of infected protoplasts. For CPMV, it has been demonstrated that the 48-kDa protein by itself is sufficient to induce these structures in protoplasts from host and nonhost plant species (Kasteel et al. 1993; Wellink et al. 1993).

Using immunoaffinity-purified anti-P38 antibodies, we showed recently that P38 putative MP of GFLV corresponds to a stable final maturation product of polyprotein P2 in vivo and that P38, although mainly cytosolic is also associated with crude membrane and cell wall fractions (Ritzenthaler et al. 1994).

In this study, we show that tubular structures are formed in GFLV-infected *Chenopodium quinoa* leaf cells and protoplasts and that P38 protein is involved in the formation of these structures. Moreover, we confirm the cytosolic location of P38 in infected cells. These results strongly support the idea that P38 protein of GFLV is involved in cell-to-cell spread of the virus probably via the formation of tubular structures, in much the same way as for CPMV and CaMV, but also suggest that P38 may have other functions.

RESULTS

Ultrastructural modifications in GFLV-infected *C. quinoa* leaf cells.

Comparison of aldehyde/osmium-fixed Epon-embedded (see Materials and Methods) leaf tissue from infected and healthy plants allowed characterization of some virus-induced ultrastructural modifications: Chloroplasts were not significantly damaged, but the mitochondria were often disorganized. Tubular structures containing viruslike particles, although not abundant (on average, one per section) were clearly visible in the mesophyll or epidermal cells originating from regions of leaves exhibiting mosaic symptoms (Fig. 1A–E). Most of these tubules were protruding from the cell wall into the cytoplasm through or close to plasmodesmata (Fig. 1A–C). At this stage of infection (14 days postinoculation), tubules were generally sheathed with cell wall material and a double layer of plasma membrane (Fig. 1A). The plasma membrane of neighboring cells seemed to be continuous through the cell wall and along the protruding tubules. Occasionally, a multiple array of tubules containing rows of isometric particles aligned in parallel direction (Fig. 1D) were seen in the cytoplasm. Some tubulelike cytoplasmic strands containing viruslike particles and extending into the vacuole (Fig. 1E) were also observed in some rare occasions. Membranous vesicles, apparently due to plasmalemma outgrowth within the cytoplasm (Figs. 1A, B and 2Bi,ii) were often observed in association with or close to tubular structures.

Intracellular localization of the P38 protein in GFLV-infected leaves.

To determine the localization of P38 within the cell and its possible association with modifications observed in infected tissues, sections of (i) aldehyde-fixed LR-white-embedded, or (ii) aldehyde/osmium-fixed Epon-embedded leaf material were probed with anti-P38 antibodies and gold conjugate (see Materials and Methods). The first fixation and embedding procedure resulted in heavy labeling of cell wall projections within the cytoplasm (Fig. 2Ai, ii), which resemble the tubules described in Figure 1A–C. In healthy tissues, no such structures could be detected and only limited background labeling was observed (not shown). Therefore, one can assume that the labeling was due to the presence of P38 on those tubulelike structures. However, under these conditions of fixation and embedding, the ultrastructure was barely visible and we were never able to detect viruslike particles within the labeled structures. Unambiguous interpretation of these micrographs not being possible, labeling was carried out on aldehyde/osmium-fixed Epon-embedded sections. This procedure allowed a low but reproducible labeling on the tubules (Fig. 2Bi), on the cell wall close to plasmodesmata (Fig. 2C), in the cytoplasm in the vicinity of tubules (Fig. 2B) and on tubule-associated vesicles (Fig. 2Bii). The labeling was detected on two successive sections at the same place, the gold particles being visible on a tubule on the first section (Fig. 2Bi) and on a vesicle associated with the same tubule on the second section (Fig. 2Bii). If present, the labeling found in healthy tissues was much lower, sparse, and not associated with particular cellular structures (Fig. 2D, E).

These in situ experiments, clearly suggest the close association of P38 with tubules containing viruslike particles. However, due to the limited labeling observed in Epon-embedded material, and the absence of precise ultrastructural details in LR-white-embedded material, further studies were performed in *C. quinoa* protoplasts.

Immunofluorescent and immunogold silver-staining (IGSS) of the P38 protein in GFLV-infected protoplasts.

Mock- and GFLV RNA-transfected *C. quinoa* protoplasts, harvested 40 h postinoculation, were labeled with anti-P38 antibodies and FITC-linked secondary antibodies. Infected protoplasts were easily recognizable by their strong green fluorescence using the fluorescein excitation filter of 490 nm associated to the emission filter of 520 nm (Fig. 3a(i), b). In Figure 3a, the same cell was controlled for nucleus and chlororplast preservation. DNA was labeled using the Hoechst dye and analyzed with excitation/emission filters of respectively 338/460 nm, and the autofluorescence of chlorophyll was observed under excitation/emission filters of 545/>575 nm. P38 protein was apparently present over the whole cytoplasm as judged from the diffuse staining of the cells (Fig. 3a(i), b), vacuoles and organelles such as nuclei or chloroplast were never labeled. Anti-P38 antibodies also identified thin fluorescent threads emerging from the surface of the protoplasts (Fig. 3a(i), b(i)). These structures identified as tubules in CaMV and CPMV-infected protoplasts were very variable in length (<5 to >40 μm) and in number (1 to 10) at the cell surface with an average number of one to two tubules per infected cell. Only a small proportion (approximately 10%) of the fluorescent protoplasts were observed with tubular structures at their surface. No such structures were seen in uninfected protoplasts.

The same types of experiments were carried out using immunogold silver-staining (IGSS). Unlike the protoplasts used for immunofluorescence studies, which were fixed in ethanol, those used for IGSS were treated with aldehydes. To allow penetration of the antibodies in the tissues, membranes were briefly permeabilized with detergent as described in Materials and Methods. These protoplasts were thus suitable for electron microscopy (EM) studies. Using this method, infected protoplast (indicated by a star in Fig. 3c), unlike mock-inoculated protoplasts (data not shown) or protoplasts that probably escaped viral infection (Fig. 3c(i)), were specifically labeled. Depending on the duration of silver enhancement, infected cells appeared almost unstained to dark brown (Fig. 3c, 4-min enhancement) under light microscopy. Silver-

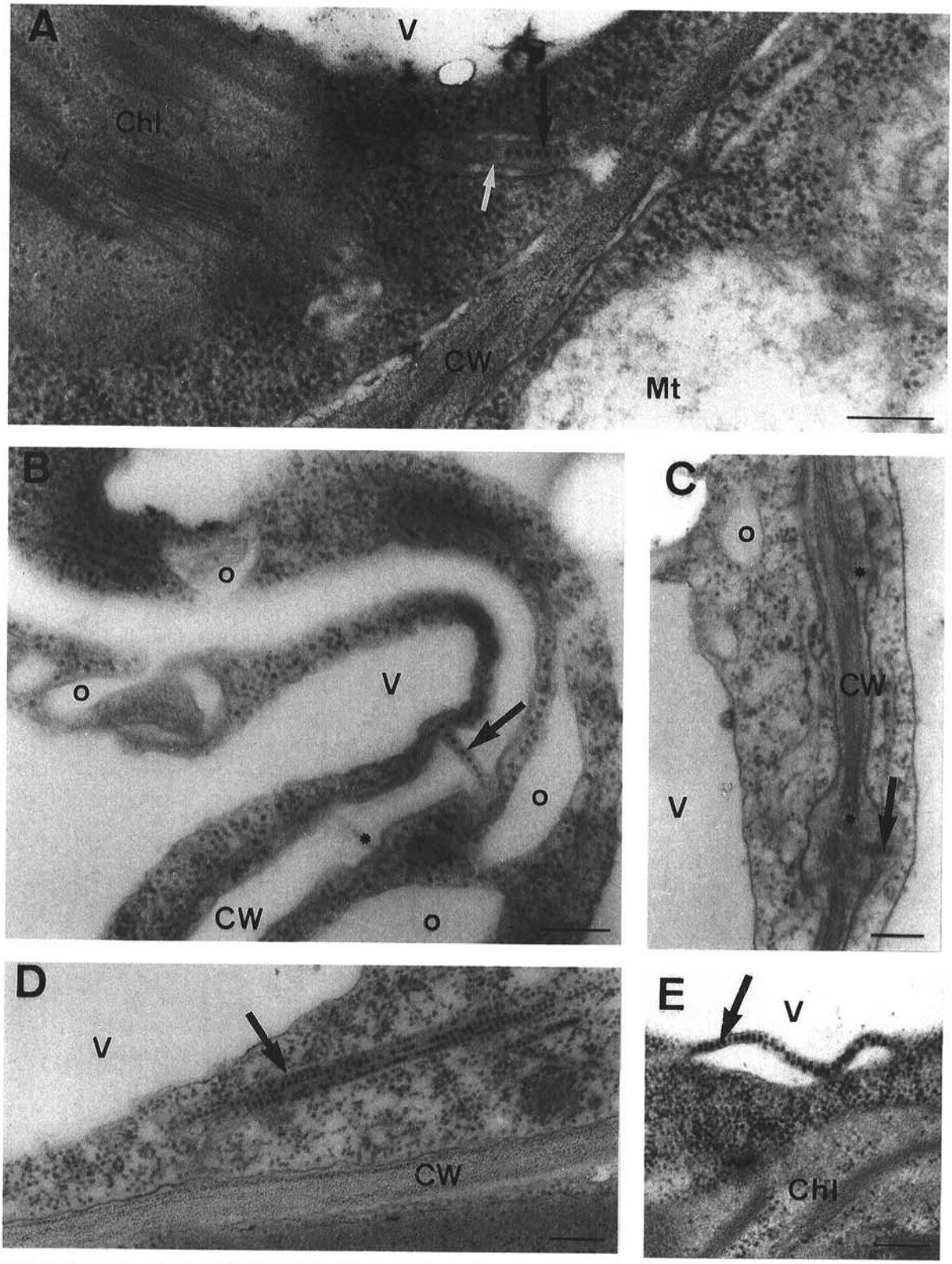

Fig. 1. Electron micrographs of cytopathic effects induced by grapevine fanleaf virus (GFLV) in systemically infected *Chenopodium quinoa* leaves, 14 days postinoculation. Leaf tissues were aldehyde/osmium-fixed and Epon-embedded. Viruslike particles are aligned in tubular structures (arrows, **A–C**) protruding through the cell wall (CW) within or close to plasmodesmata (stars). They are also localized within the cytoplasm **(D),** and cytoplasmic strands resembling to tubules were observed emerging in the vacuole **(E).** Chl, chloroplasts ; CW, cell wall ; Mt, mitochondria ; V, vacuole. Solid circles (o) membranous vesicles; stars (*), plasmodesmata; white arrow, plasma membrane. Bar markers represent 200 nm.

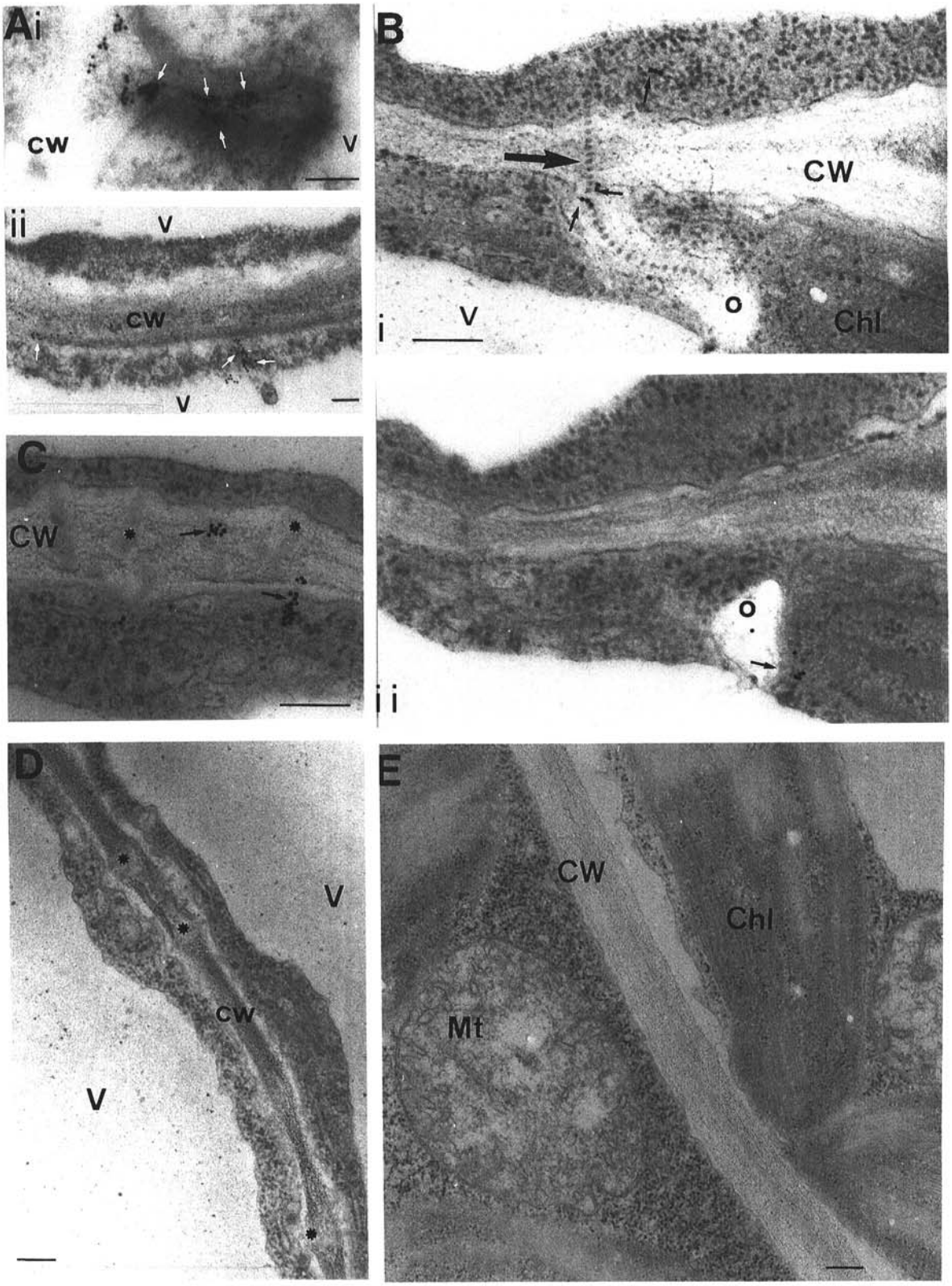

Fig. 2. P38 immunogold labeling of grapevine fanleaf virus (GFLV)-infected **(A-C)** and healthy **(D-E)** leaf tissues observed in electron microscopy. Gold particles (small arrows) are localized in the cell wall (A,C), superimposed to tubules (big arrows; B) and close to plasmodesmata (stars, C). In the controls (D,E) we failed to detect gold particles. (Ai, ii): Aldehyde fixation/LR white embedding. (B-E): Aldehyde osmium fixation/Epon embedding. Bi and Bii are two consecutive sections showing a gold labeled tubule and a labeled tubule associated vesicle, respectively. Chl, chloroplasts; CW, cell wall; (o), membranous vesicle ; Mt, mitochondria, V, vacuole. Bar markers represent 200 nm.

magnified gold particles also allowed the detection of tubular structures at the surface of some infected protoplasts (Fig. 3c(ii)). The percentage of infection and of protoplasts with tubules observed by this method were similar to those obtained by immunofluorescence. Due to the low proportion of protoplasts presenting tubules upon GFLV infection, IGSS is a useful alternative to other methods described so far for the observation of tubules in EM (Perbal et al. 1993; Wellink et al. 1993).

Protoplasts showing tubulelike structures were first observed and then precisely localized and processed for EM as described in Material and Methods. Low magnification EM observations showed that cell organization and organelles were well preserved contrarily to membranes, probably due to detergent treatment (Fig. 4A). Intense labeling was found in association with cytoplasmic material (Fig. 4A,B) and all along the tubules protruding from infected cells (Fig. 4C,D). Investigations at higher magnification confirmed the localization of P38 within the cytoplasm but not in the chloroplasts and mitochondria, and also revealed tubularlike structures within the cytoplasm as suggested by the shape and size of the labeled structure (arrow in Fig. 4B). The tubules emerging from the surface of the protoplasts (Fig. 4D), as measured by EM, were 40 to 60 nm in diameter and up to 20 μm in length. A globular structure about 1.3 μm in width and 2.5 μm in length (indicated by a star in Fig. 4D), was also observed along one tubule. Similar structures were described in CPMV-infected protoplasts and tubules and were shown to contain virus particles (van Lent et al. 1991). Unfortunately, we were not able to distinguish viruslike particles within the tubules nor within the globular structures.

DISCUSSION

Despite numerous reports on the association of (putative) MPs of several viruses with tubular structures containing viruslike particles in planta, to date no nepovirus protein has been shown to induce such structures in protoplasts. Prior to determining if P38 protein is involved in tubule formation in vivo, we sought to determine if such tubules were formed in GFLV-infected *C. quinoa* plants.

In aldehyde/osmium-fixed Epon-embedded infected leaf tissue, typical cytopathic effects of nepovirus infection were observed. Formation of membranous vesicles are features that are common to many nepo- and comovirus infections (de Zoeten et al. 1974; Harrison et al. 1974; Wellink et al. 1988; White and Seghal 1993). It has been suggested that these structures are the site of viral RNA synthesis (de Zoeten et al. 1974) and more recently, Wellink et al. (1988) demonstrated that they contain CPMV RNA1-encoded nonstructural proteins. On the other hand, tubular structures containing viruslike particles may well be characteristic of all viruses that move from cell-to-cell according to the CPMV-like mechanism (McLean *et al.* 1993). In comparison to previous reports concerning the occurrence of tubular structures in plant infected by como- and nepoviruses, those we observed in GFLV-infected *C. quinoa* leaf cells were rare (Babini and Bertaccini 1982; de Zoeten et al. 1982; Peña-Iglesias and Rubino-Huertos 1971; Wellink et al. 1988). Among the different cytopathic effects observed, the most intriguing was probably the close parallel arrays of tubularlike structures containing isometric particles within the cytoplasm. These aggregates are probably of tubular origin since they were similar in size and ultrastructure in their "empty" portions when compared to typical single cytoplasmic tubules.

Our results on immunogold labeling of GFLV-infected *C. quinoa* leaves revealed close association of P38 with tubules, suggesting the possible involvement of this protein in the formation of these structures. Similar data were obtained with the 45-kDa protein of TomRSV, another nepovirus (Wieczorek and Sanfaçon 1993). As for TomRSV and despite the absence of such structures in healthy leaf tissues and the high labeling we observed, viruslike particles were never seen within the tubules when tissue were embedded in LR white resin.

In the case of CaMV (Perbal et al. 1993) and CPMV (Kasteel et al. 1993; van Lent et al. 1991; Wellink et al. 1993) tubular structures have been visualized emerging from protoplasts, thus in the absence of plasmodesmata, suggesting that tubules might be de novo structures rather than plasmodesmatal outgrowths. Immunofluorescence and IGSS studies clearly show for the first time with a nepovirus that tubules are formed in protoplasts and that the P38 protein is a structural component of these structures since labeling is present over the whole length of the tubules. These observations are in agreement with the immunogold labeling of ultrathin sections of plant tissues presented in this paper. Although conclusive proof can only be provided from analysis of mutation/complementation experiments, it is most likely that P38 is involved in cell-to-cell movement and thus constitutes the GFLV MP.

With respect to the precise function of P38, the similarity between the tubular structures formed upon GFLV infection and those induced by other tubule-inducing viruses, confirmed the subgrouping of nepoviruses within the CPMV-like subgroup of plant MP(s). As for CaMV (Perbal et al. 1993), and despite the high level of expression of P38 as judged from labeling of infected protoplasts, tubules were not abundant (rarely more than two), contrary to what is observed with CPMV (van Lent et al. 1991; Wellink et al. 1993). This could be attributed to differences in the ability of different MP's to form tubules in vivo. However, further work will be needed to confirm this hypothesis since the differences might also be related to the type of tissue or plant examined.

Until now, it is not known if the MP is the sole component of the tubules or whether host proteins are needed and whether plasmodesmata are essential for their assembly. Recent observations have shown that tubules are formed and protrude from insect cells infected with recombinant baculovirus carrying the MP of TSWV (Kormelink et al. 1994). This suggests that either no host proteins are needed for tubule formation and/or elongation in vivo or, if such proteins are required, they belong to a highly conserved family of proteins within the animal and plant kingdom.

Prior to the experiments described in this paper, subcellular fractionation experiments indicated that P38 is associated with cell wall and membrane fractions but is mainly found within the cytoplasm of infected cells (Ritzenthaler *et al.* 1994). Indirect evidence for this cytosolic localization came from the presence of tubulelike structures in the cytoplasm of infected cells as seen in Figure 1D,E. Conclusive proof of this localization was provided by immunofluorescence studies and

EM observations on IGSS treated protoplasts. Micrographs of infected protoplasts clearly showed fluorescence or gold particles over the whole cytoplasm with the exception of chloroplasts, mitochondria, and the vacuole. Together with the fact that P38 has been shown to be stable in vivo (Ritzenthaler et al. 1994), these features are "atypical" for a MP and to our knowledge unique.

MATERIALS AND METHODS

Inoculation of *C. quinoa* and protoplast transfection.

Young *C. quinoa* were mechanically inoculated with 10 µg of GFLV in 50 mM phosphate buffer, pH 7.0 (Ritzenthaler et al. 1994). Protoplasts were isolated (Hans et al. 1992) and transfection carried out with 2 µg of viral RNA (Ritzenthaler et al. 1994). Controls consisted of healthy plants and mock-inoculated protoplasts.

Antibodies to the P38 of GFLV.

Affinity-purified immunoglobulins against P38 of GFLV were obtained as described by Ritzenthaler et al. (1994). Briefly, a recombinant 6HisP38 protein was expressed and purified from *E. coli*. Antiserum against this protein was obtained by injecting a rabbit with the purified recombinant protein. The purified 6HisP38 protein linked to a CNBr-

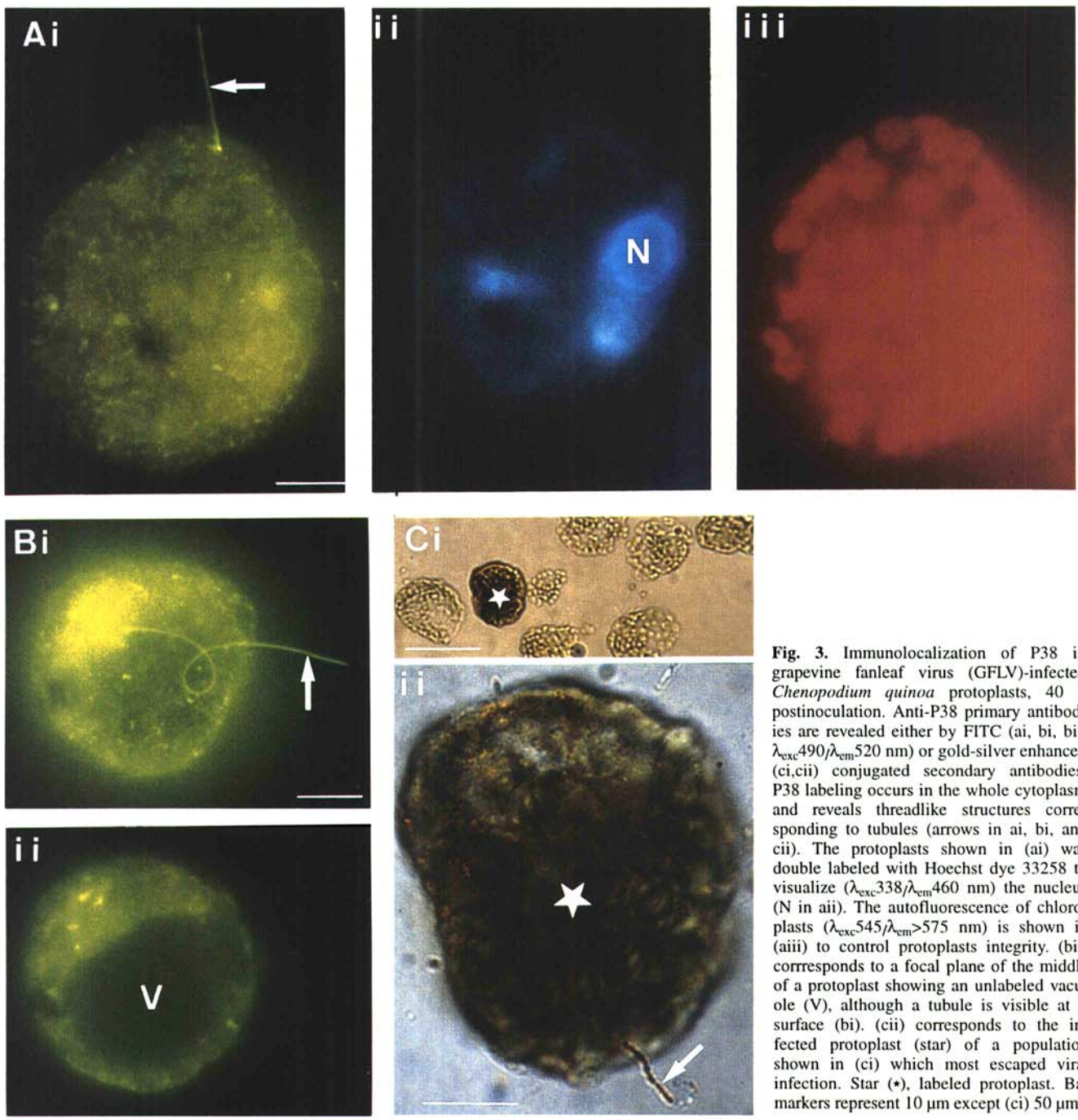

Fig. 3. Immunolocalization of P38 in grapevine fanleaf virus (GFLV)-infected *Chenopodium quinoa* protoplasts, 40 h postinoculation. Anti-P38 primary antibodies are revealed either by FITC (ai, bi, bii, $\lambda_{exc}490/\lambda_{em}520$ nm) or gold-silver enhanced (ci,cii) conjugated secondary antibodies. P38 labeling occurs in the whole cytoplasm and reveals threadlike structures corresponding to tubules (arrows in ai, bi, and cii). The protoplasts shown in (ai) was double labeled with Hoechst dye 33258 to visualize ($\lambda_{exc}338/\lambda_{em}460$ nm) the nucleus (N in aii). The autofluorescence of chloroplasts ($\lambda_{exc}545/\lambda_{em}>575$ nm) is shown in (aiii) to control protoplasts integrity. (bii) corrresponds to a focal plane of the middle of a protoplast showing an unlabeled vacuole (V), although a tubule is visible at it surface (bi). (cii) corresponds to the infected protoplast (star) of a population shown in (ci) which most escaped viral infection. Star (•), labeled protoplast. Bar markers represent 10 µm except (ci) 50 µm.

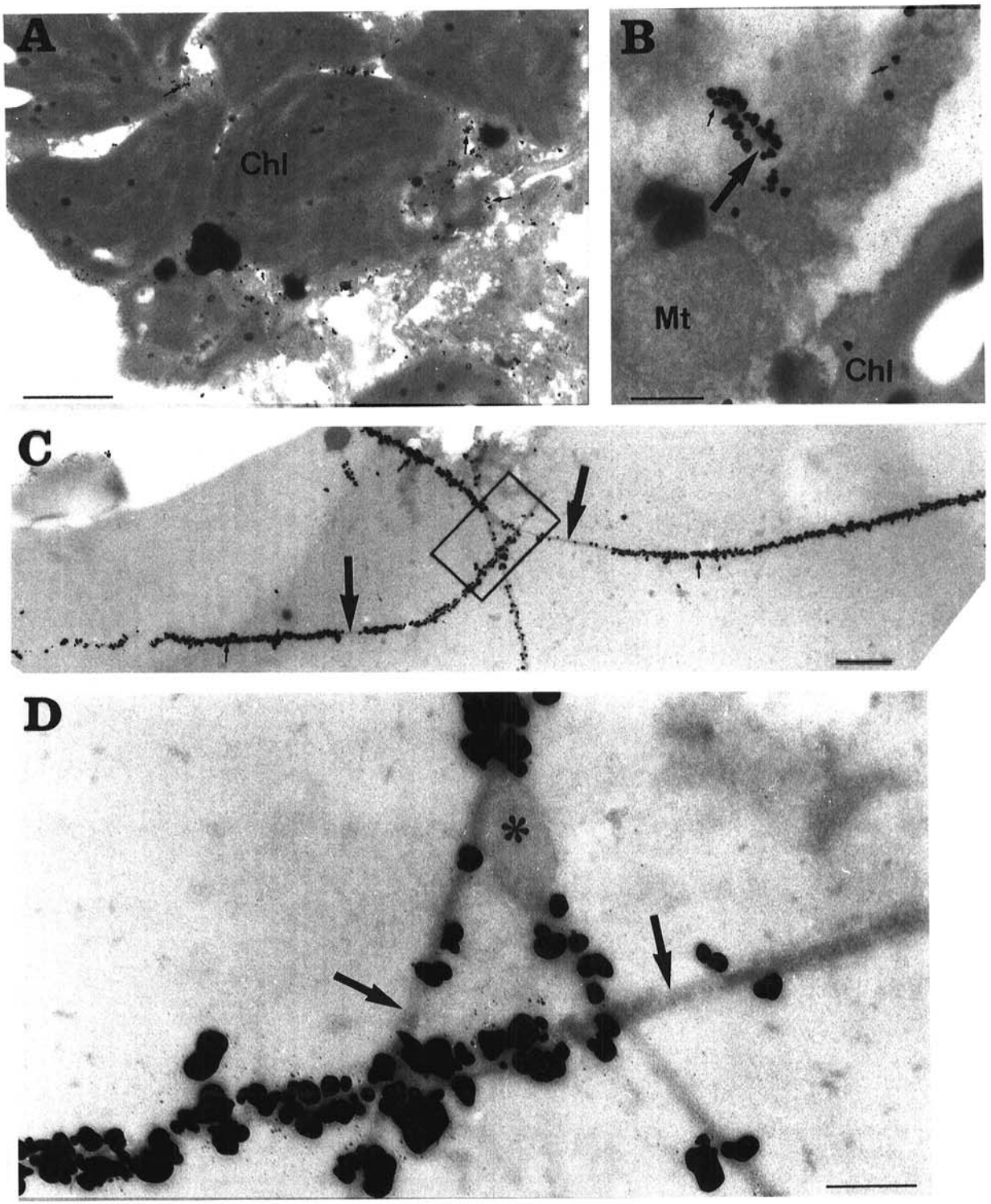

Fig. 4. Immunoelectron micrography of grapevine fanleaf virus (GFLV)-infected protoplasts, 40 h postinoculation. Cells were aldehyde-fixed, immunolabeled with anti P38 antibodies, revealed with gold-conjugated secondary antibodies and silver enhanced for 1 min **(A,B)** or 4 min **(C,D)** before Epon embedding. **A,** Low magnification electron micrograph of a labeled protoplast; particles (small arrows) are found in the cytoplasm, but never within the vacuole, mitochondria (Mt), or chloroplasts (Chl). **B,** In the same section observed at higher magnification, P38 protein is associated with a tubular-like structure (big arrow) within the cytoplasm. **C,** Tubules (big arrow) emerging from a protoplast shown at low magnification. **D,** Detail of the outlined aera in C. Note the swollen structure (star) at the connection between two tubules. A, C: bar markers represent 1 µm. B, D: bar markers represent 200 nm.

Sepharose 6B resin allowed the purification of the anti-6HisP38 antiserum by affinity chromatography. The affinity-purified antibodies immunoglobulins, referred to as anti-P38 antibodies in this paper, were shown to be highly specific for P38 (Ritzenthaler et al., 1994) and were used at a 1:1,000 dilution for in situ experiments.

Immunogold labeling of ultrathin sections of *C. quinoa* leaves.

Samples from healthy and systemically infected leaves showing typical GFLV symptoms were collected 14 days postinoculation. This time corresponds to the period where maximum P38 level is observed in infected tissues (Ritzenthaler et al. 1994). Control samples were taken from the equivalent leaves of uninfected plants. The tissue samples were fixed with 2.5% glutaraldehyde in 100 mM phosphate buffer, pH 7.2, (PB) for 7 h at 4°C and washed in the same buffer. Secondary fixation, when performed, was for 1 h in 0.5% osmium tetroxide. After dehydration by successive changes of graded ethanol followed by propylene oxide, aldehyde/osmium- and aldehyde-fixed samples were embedded in Epon and LR-white (London Resin), respectively.

Ultrathin sections were cut using a Ultracut E microtome (Reichert) and were mounted on Formvar-coated nickel slot grids. The grids were incubated for 3 h on drops of diluted anti-P38 antibodies in PB containing 0.05% Tween-20 (PBT). After four washes with PBT, sections were incubated for 1 h with 16 nm gold conjugated goat-IgG directed against rabbit-IgG (referred to as gold conjugate in the text) diluted 1:150 in PBT, washed twice in PBT, and three times in water prior to uranyl acetate and lead citrate staining.

Immunofluorescence labeling of *C. quinoa* protoplasts.

Mock-inoculated (control) or infected protoplasts were labeled mainly as described by van Lent et al. (1991). Briefly, protoplasts harvested 40 h postinoculation, were applied to poly-L-lysine (M_r 30,000 to 70,000) coated coverslips and were fixed with distilled ethanol for 20 min. After incubation with anti-P38 antibodies for 4 h at 4°C, the protoplasts were further incubated for 1 h at 37°C in the dark with fluoresceine isothiocyanate-conjugated goat-IgG raised against rabbit-IgG (FITC-conjugate) diluted 1:250 in PBS (16 mM Na_2HPO_4, 4 mM NaH_2PO_4, 150 mM NaCl, pH 7.4). Finally, the preparation was counterstained with Hoechst 33258 dye at 0.1 µg/ml in distilled water for 5 min and mounted in Citifluor (Touzart et Matignon, France).

Immunogold-silver staining (IGSS) of *C. quinoa* protoplasts.

Mock-inoculated and infected protoplasts were fixed for 30 min in 4% paraformaldehyde/0.1% glutaraldehyde in PB or for 1 h in 2.5% glutaraldehyde adjusted with 0.6 M mannitol to the same osmolarity as the protoplast media. The protoplasts were then washed in a large excess of PBS and permeabilized for 5 min in 0.2% Triton X-100. After 3 washes in the same buffer, the protoplasts were incubated with the primary antibody overnight at 4°C in PBS containing 1% bovine serum albumin (BSA), washed, and incubated for 1 h at room temperature with the gold conjugate, diluted 1:150 in PBS 1% BSA. After three washes for 20 min in PBS, preparations were treated for 10 min in 1% glutaraldehyde in the same buffer and washed twice with PBS and distilled water for 2 min before silver enhancement. Enhancement was done using the IntenSE BL Kit (Amersham) for 0 to 4 min, immediately followed by immersion of the coverslips in a large excess of water to stop the silver intensification (Schmit and Lambert 1987).

Resin embedding of the IGSS protoplasts for electron microscopy.

Protoplasts were first observed by light microscopy. Those presenting tubules at their surface were precisely localized on the surface of the coverslip and pictures were taken. The protoplasts were then postfixed with 0.1% OsO_4 for 20 min, washed for 10 min in distilled water, dehydrated in ethanol series, and propylene oxide. A 2-mm layer of Epon resin was poured on the coverslip for final flat embedding. Polymerization was performed for 48 h at 55°C. Disks of resins containing tubule-decorated protoplasts, were excised, glued on an LR white block and the rest of the coverslip removed by heating with a soldering iron. Serial ultrathin sections were made, stained with uranyl acetate/lead citrate, and observed. The tubules were generally detectable on the two first ultrathin sections.

Light and electron microscopy.

Fluorescent labeled preparations were examined on a Leitz Orthoplan microscope equipped for epifluorescence with an HBO100/W2 lamp and 63x/1.30 oil fluorescence lens, or on a Leitz DMRB microscope equipped for epifluorescence with an HBO 50/W2 lamp and 63x/1.40 oil PH3 PL APO/0.17 IC lens.

A Zeiss universal microscope equipped with a differential interference contrast Nomarski system was used with a Zeiss planapo 63x/1.40 oil phase contrast Ph3 Apo 40/1.00 oil objective.

The electron microscopy (EM) observations were made on a Hitachi H600 transmission electron microscope at 75 kV.

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