Rapid Communication

Localization of the N-terminal domain of cauliflower mosaic virus coat protein precursor

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Abstract

Cauliflower mosaic virus (CaMV) open reading frame (ORF) IV encodes a coat protein precursor (pre-CP) harboring an N-terminal extension that is cleaved off by the CaMV-encoded protease. In transfected cells, pre-CP is present in the cytoplasm, while the processed form (p44) of CP is targeted to the nucleus, suggesting that the N-terminal extension might be involved in keeping the pre-CP in the cytoplasm for viral assembly. This study reports for the first time the intracellular localization of the N-terminal extension during CaMV infection in *Brassica rapa*. Immunogold-labeling electron microscopy using polyclonal antibodies directed to the N-terminal extension of the pre-CP revealed that this region is closely associated with viral particles present in small aggregates, which we called small bodies, adjacent to the main inclusion bodies typical of CaMV infection. Based on these results, we propose a model for viral assembly of CaMV.
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Keywords: Cauliflower mosaic virus; Coat protein; Electron microscopy; Assembly; Small inclusion bodies

Introduction

Cauliflower mosaic virus (CaMV), the type member of the *Caulimoviridae* (reviewed in Hohn and Fütterer, 1997), is a double-stranded (ds) DNA virus of 8 kb. CaMV coat protein (CP) is expressed from viral open reading frame (ORF) IV as a precursor (pre-CP) harboring an N-terminal extension. CaMV CP is involved in assembly (Chapdelaine and Hohn, 1998), packaging of the viral RNA (Guerra-Peraza et al., 2000), and delivery of the viral genome to the nucleus (Karsies et al., 2002; Leclerc et al., 1999). These various CP functions are probably regulated by several events, including CP maturation by viral- and host-encoded proteases, which cleave the N- and C-terminal extensions to generate the three major forms of CP (p44, p39, and p37) (Torruella et al., 1989); CP phosphorylation on $S^{82,86,88}$ by host casein kinase II (Chapdelaine et al., 2002); interaction with TAV (ORF VI product) (Himmelbach et al., 1996); the protein degradation signal located in the N-terminal extension (Karsies et al., 2001); and retention of pre-CP in the cytoplasm through an unknown mechanism involving the N-terminal extension (Karsies et al., 2002). Although the function of the N-terminal extension is still unknown, we suspect it is essential for the virus life cycle. We hypothesize that it plays a key role in the retention of CP in the cytoplasm for viral assembly by acting as an anchoring domain. To gain further insight into the role of the N-terminal extension, we investigated its intracellular localization in CaMV-infected plants. Our results lead us to propose a model of CaMV capsid assembly.

Results

Purification of the N-terminal extension of CaMV pre-CP

To produce the N-terminal extension peptide of CaMV pre-CP, we used the IMPACT TWIN system (New England Biolabs, Beverly, MA). The N-terminal 76 amino acids of

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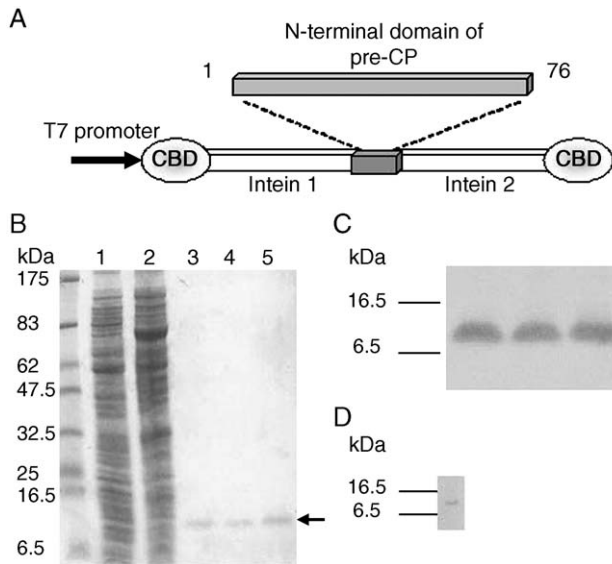
Fig. 1. Purification of the N-terminal domain of CaMV capsid protein precursor. (A) Schematic representation of the construct used to produce the N-terminal domain of pre-CP. The first 76 N-terminal amino acids of the precursor were inserted between the two self-cleaving inteins (Inteins 1 and 2) attached to the chitin-binding domain (CBD). The CBD allows the fusion protein (CBD-Intein 1-Pre-CP N-terminal extension-Intein 2-CBD) to bind to chitin beads. (B) Coomassie blue-stained 10% tricine SDS-PAGE gel showing purification of N-terminal extension peptide. The position of prestained molecular marker (kDa) are shown on the left. Expression of the Intein-Pre-CP-N-terminal-extension fusion protein in *E. coli* ER2566 cells. Lanes: 1, uninduced cell extract; 2, induced cell extract (the full-length precursor is the most prominent band); 3–5, elution fractions following pH- and temperature-dependent cleavage of Intein 1 (pH 7 at 25 °C) and thiol-induced cleavage of Intein 2 (40 mM DTT). (C) Immunodetection of the purified protein using anti-pIV$^{1-362}$ antibody; Lanes 3–5. (D) Western blot showing that the N-terminal protein (residues 1–76) is indeed recognized by the mouse antibody raised against it.

the CaMV pre-CP were cloned between intein 1 and intein 2 of the expression vector pTWIN1 (Fig. 1A). This system facilitated purification of a stable and soluble N-terminal extension using self-cleavage and an affinity tag to chitin.

Intein 1 is engineered to cleave at its C-terminus in a pH- and temperature-dependent manner and Intein 2 undergoes thiol-induced N-terminal cleavage. The overexpressed protein (pIV$^{1-76}$) was affinity-purified using a resin covalently bound to chitin, and the N-terminal extension was released by a temperature/pH shift and addition of dithiothreitol. The protein was analysed by SDS-10% PAGE (Fig. 1B) and Western blot (Fig. 1C). A protein of the predicted mass (8.4 kDa) was obtained and recognized by an antibody generated against pIV$^{1-362}$ (previously described by Chapdelaine and Hohn, 1998). A second passage of the eluted fractions over the resin allowed removal of partially spliced or unspliced products. Purified pIV$^{1-76}$ was injected into a mouse and a rat to produce specific IgGs. The polyclonal antibodies thus produced specifically recognized the recombinant N-terminal extension (Fig. 1D). However, due to nonspecific binding to other plant proteins, antibody purification was essential to avoid nonspecific labeling in subsequent electron microscopy studies. Antibodies were purified by affinity to pIV$^{1-265}$ on immunoblot (Harlow and Lane, 1988). The purified recombinant N-terminal extension (Fig. 2A) was specifically recognized by the purified antibodies (Fig. 2C) without reacting with the processed form (p44) of CP found in the purified virus (Fig. 2C). As expected, the anti-pIV$^{1-362}$ antibody reacted with proteins pIV$^{1-265}$ and pIV$^{1-76}$ (Fig. 2B). The anti-N-terminal antibody is specific for the N-terminal extension, making it an ideal tool with which to study the localization of the extension in planta.

Infection of plants

Brassica rapa plants were inoculated with 1 μg of linearized CaMV DNA (*Sal*I digest of an infectious isolate of CaMV cloned in plasmid pBR322). Local symptoms appeared 15 days post inoculation (dpi). Symptoms were systemic after 30 days and typical electron-dense inclusion bodies containing virus particles were observed by 30 dpi at the ultrastructural level (see Figs. 3B–E).
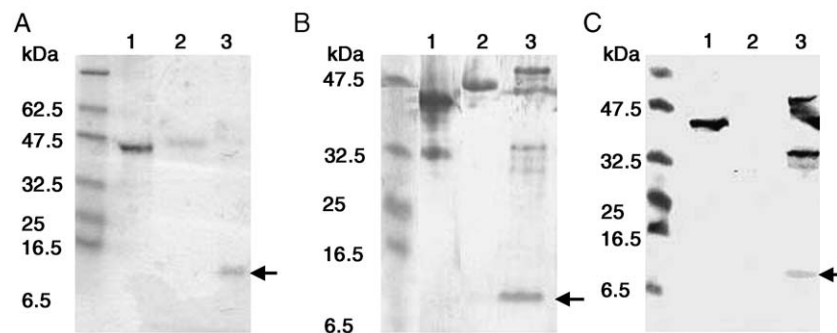

Fig. 2. Western analysis of CaMV proteins from the purified virus and recombinant capsid protein. (A) Coomassie blue-stained SDS-PAGE gel. Lanes: 1, pIV$^{1-265}$ expressed in *E. coli* and purified from inclusion bodies; 2, purified virus (only the p44 form was present); 3, N-terminal extension protein (pIV$^{1-76}$) purified using the IMPACT-TWIN system. Proteins were transferred onto nitrocellulose and blotted using an antibody directed against the capsid precursor anti pIV$^{1-362}$ (B) or a rat antibody against the N-terminal extension protein (C). The positions of size markers (kDa) are indicated on the left of each blot. Arrows indicate the position of the N-terminal extension protein.
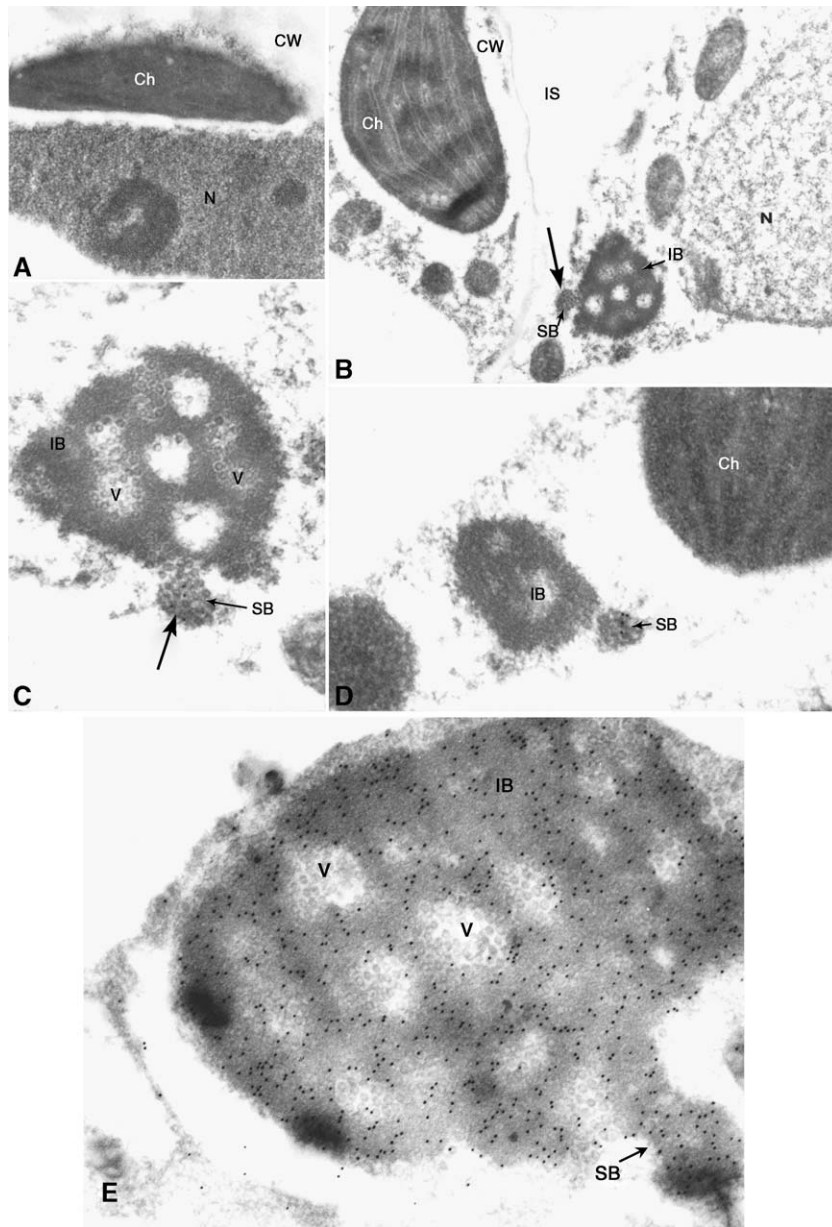
Fig. 3. Transmission electron micrographs of healthy and CaMV-infected *B. rapa* leaf tissue, collected 30 days after infection. CW, cell wall; Ch, chloroplast; IB, inclusion body; IS, intracellular space; N, nucleus; SB, small body and V, virus. Immunogold labeling was performed on thin sections with the purified mouse polyclonal antibody (anti-N-terminal domain) as primary antibody followed by colloidal gold (10 nm)-conjugated goat anti-mouse IgG + IgM. (A) Sections from healthy plant controls are free of labeling (×40 000). (B) Localization of the N-terminal domain of CaMV pre-CP. A small body (SB) structure neighboring the larger electron-dense inclusion body (IB) is specifically labeled (arrow) (×30 000). (C) Enlargement of B showing the deposition of gold particles over the small body, particularly over the virions (arrow) (×80 000). (D) Another infected cell, showing labeling of the small body (×100 000). (E) Immunogold labeling with pVI antibody: the inclusion body matrix is labeled. Deposition of gold particles over the small body can also be seen (×60 000).

Intracellular localization of the N-terminal domain of pre-CP in infected turnip leaf cells

Immunogold labeling was performed on thin sections of infected leaves at seven different times postinfection. The purified antiserum produced against the N-terminal extension was used to localize the N-terminal extension of pre-CP in infected plant leaves. In sections from control leaves (healthy plants) (Fig. 3A), no labeling was detected, indi-cating the absence of any endogenous substrate for the antibody. At early times in the infection process (1, 3, 6, 10 15, and 20 dpi), inclusion bodies were not found within the host cells and all sections examined were free of labeling (data not shown). By 30 dpi, gold particles were found to specifically accumulate over small dense structures, which we have named "small bodies". A delay of 30 days is rather a long period to trigger an infection. This is probably due to the use of DNA instead of virions for inoculation, DNA

being less infectious than CaMV particles. It is also possible that some plant cells could not support CaMV replication because they got damaged during bombardment. As illustrated in Figs. 3B–D, small bodies (arrows) neighbored larger inclusion bodies containing viruses and were specifically labeled. The N-terminal extension appeared to be located at the virion surface. Specificity of labeling was assessed by omission of the primary antibody. This resulted in loss of deposition of gold particles over the small bodies (data not shown). Likewise, no labeling was observed over the host cell organelles, cell walls, and cytoplasm. The N-terminal extension of pre-CP was associated only with viral particles seen inside small bodies. Specificity of the labeling with the antibody raised against the viral pVI protein (TAV) was also assessed. The pattern was found to be similar to that previously described (Giband et al., 1984), with pVI being present in both the large and small inclusion body matrices (Fig. 3E). We have observed specific labeling of small inclusion bodies with the antibodies more than 30 times on 10 different sections made from 10 different blocks. These results are very reproducible and the labeling was very specific. The small inclusion bodies were always associated with large inclusion bodies and always labeled with the N-terminal antibodies. We did not find small inclusion bodies under our conditions that was not labeled with the antibodies. The small bodies contain between two and eight gold particles with an average of five gold particles, whereas the large inclusion was never labeled. We also observed large inclusion bodies in some cells that were not labeled and where small inclusion bodies were absent.

Discussion

The assembly process of CaMV remains only partially understood. The Gag protein of the animal retrovirus family, a model for CaMV and the plant pararetrovirus group, is known to anchor in the plasma membrane before assembly and encapsidation (Ono et al., 2000). By analogy, it is possible that CaMV also anchors its CP to a specific site where the protein accumulates and where assembly and encapsidation is triggered. Several lines of evidence suggest that the cytoplasmic inclusion bodies are the site of viral protein accumulation and virion assembly (Hohn and Fütterer, 1991; Shepherd, 1976). Previous electron microscopy studies have demonstrated the association of virus particles with the inclusion bodies (Giband et al., 1984; Shepherd et al., 1976). The interaction between the CP (pIV) and TAV, the viral inclusion body protein (pVI) seems to play a role in CaMV assembly (Himmelbach et al., 1996; Kobayashi et al., 1998). However, the involvement of TAV in assembly is so far only speculative. Our objective was to identify a cellular compartment that could be important for accumulation of the CaMV pre-CP.

As proposed by Karsies et al. (2002), pre-CP is produced and remains in the cytoplasm during the CaMV replication cycle. p44, a processed form of the capsid starting at aa 77, harbors a nuclear localization signal (NLS), which is of key importance for nuclear targeting of CP transiently expressed in plant protoplasts (Karsies et al., 2002; Leclerc et al., 1999). However, in such experiments, pre-CP remained in the cytoplasm (Karsies et al., 2002). The N-terminal extension of the pre-CP appears to change the localization of the protein from nuclear to cytoplasmic. In this report, we used immunogold electron microscopy to reveal localization of pre-CP in planta to small inclusion bodies (small bodies) located in close proximity to the cell membrane. The small bodies are specifically labeled with antibodies directed towards the N-terminal extension of pre-CP, and are closely associated with viral particles. This result suggests that processing of pre-CP by the virus-encoded protease occurs after assembly of virus particles. The large inclusion bodies were free of labeling, which suggests that only mature virus particles are found in the large inclusion bodies. We did not find the free N-terminal peptide in our Western blot assay. Because it is always associated with the small inclusion bodies, we suggest that the N-terminal extension is mostly present as part of the pre-capsid protein. Antibodies directed to TAV (pVI) also labeled the small inclusion bodies, suggesting that the interaction between TAV and the pre-CP is important early in assembly. Events before the formation of the small bodies are unclear because we could not detect labeling in any other structure of the infected cell. We propose that the N-terminal extension targets the pre-CP to an assembly site, presumably the plasma membrane of the infected cell and, upon accumulation of the protein in the anchoring sites, triggers assembly of the virion. Assembly is followed by aggregation of the particles into small inclusion bodies with the help of TAV (ORF VI product). Small inclusion bodies in close proximity to larger inclusion bodies were also observed in an electron microscopy study in Dahlia mosaic virus (DMV; also member of the *Caulimoviridae* family) infected leaves (Kitajima et al., 1969). They suggested that the virus particles are assembled in the inclusion bodies and that the Golgi might be associated with viral infection. For both virus, internal membranes such as cell membrane (CaMV) or Golgi (DMV) seem to be important for the assembly process and the formation of small inclusion bodies.

According to our observations, the following scheme of events can be proposed for viral assembly (Fig. 4). As infection progresses, the CaMV pre-CP protein accumulates at specific sites, possibly the plasma membrane. The N-terminal extension may operate as an anchoring domain for the initiation of viral assembly. In a wide sense, we propose homology of function with the viral matrix protein of HIV. In HIV-1, myristoylation of the matrix domain of Gag polyprotein is crucial to ensure its targeting to the plasma membrane, where assembly and budding occur (Gelderblom et al., 1989; Pal et al., 1990). Although the N-terminal extension of pre-CP does not share sequence homology with the HIV-1 matrix protein, we propose that it plays an
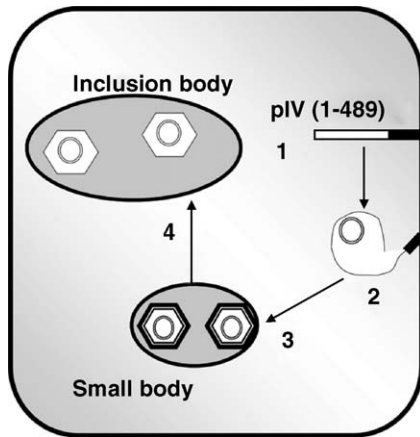
Fig. 4. Proposed model for CaMV assembly. As infection proceeds, the coat protein precursor (amino acids 1–489) is synthesized and retained in the cytoplasm by the N-terminal extension of the pre-CP (black box at the N-terminus of the pre-CP) (Step 1). The coat protein precursor accumulates in a cytoplasmic compartment, presumably the plasma membrane. The N-terminal extension may act as an anchoring domain. As coat protein concentration increases, RNA or reverse-transcribed DNA is available to trigger viral assembly (Step 2). The small bodies containing the N-terminal extension and viruses formed (Step 3) and the N-terminal extension is then cleaved by the viral protease (Step 4). Finally, the small bodies fuse to form a larger inclusion body (IB). Fusion process may be mediated by the viral pVI protein.

analogous role in the viral life cycle. Through this mechanism, the viral coat protein accumulates until a specific concentration is reached and genomic RNA or reverse-transcribed DNA is available to trigger viral assembly. The immature virion then gathers in small bodies containing the N-terminal domain of CaMV CP. The viral CP is finally cleaved by the viral protease, removing the N-terminal extension from the precursor. The pre-CP is also processed at its C-terminal domain and it is still unknown if the cleavage is regulated in the same manner as the N-terminal extension. Following the final processing, the newly formed mature virions in small bodies fuse and contribute to the expansion of the large inclusion bodies. We propose that the pVI–pIV interaction might be important for the regulation of the progression from small to large inclusion bodies because the N-terminal and C-terminal domains of pre-CP were shown to interfere with this interaction (Himmelbach et al., 1996). Our data suggest that pVI is present in the small inclusion bodies. How the interaction between pVI and pIV differs in the large and in the small inclusion bodies, and how this interaction is regulated, remains unclear. We are currently investigating this question.

Materials and methods

Plant material and infection

B. rapa plants (var "Just Right") were grown in a greenhouse at 20 ± 2 °C with a 16 h photoperiod. Two-

week-old turnip plants were infected with an infectious CaMV clone (isolate Cabb-B-S; a kind gift from T. Hohn), excised by digestion with *Sal*I from cloned DNA in the vector pBR322. After ligation, 1 μg of viral DNA previously precipitated with 1.6-μm gold microcarrier was injected into plant cells by particle bombardment [Helios Gene Gun (Bio-Rad)] in accordance with the manufacturer's instruction manual. The virus was purified as described previously (Hull et al., 1976).

Production of antibodies against the N-terminal domain of CaMV pre-CP

The N-terminal domain of CaMV pre-CP was amplified by PCR using the clone (isolate Cabb-B-S) containing the whole genome of CaMV in pBR322 as a template. Specific PCR primers incorporated *Nco*I and *Xho*I restriction sites at the N- and C-terminal ends, respectively. The fragment was cloned in frame in the pTWIN-1 vector (New England Biolabs) between the two self-cleaving inteins (Intein 1, Ssp DnaB intein and Intein 2, Mxe Gyr A intein) with chitin-binding domain, under the control of the T7 promoter. Expression and purification of the protein were essentially as described by the manufacturer (NEB) for N- and C-terminal intein fusions. Briefly, *E. coli* ER2566 cells containing the plasmid were grown for 3 h in 2XYT medium with 100 μg ampicillin at 37 °C to an $A_{600} = 0.5–0.7$. Proteins were induced with 1 mM IPTG overnight at room temperature. The cells were pelleted, followed by resuspension in buffer B2 (20 mM Hepes, pH 7, 500 mM NaCl, 1 mM EDTA) and lysed using a French Press. The cell lysate was centrifuged and the supernatant applied to a chitin bead column equilibrated with 10 volumes of buffer B2. Unbound proteins were washed off with the same buffer, after which self-cleavage of Intein 1 in buffer B2 (pH 7 at 25 °C overnight) and Intein 2 in buffer B3 at 4 °C (20 mM Hepes, pH 8.5, 200 mM NaCl, 1 mM EDTA, and 40 mM DTT) were induced. The protein was eluted in the presence of 40 mM DTT in buffer B3. The recovered protein was analyzed on 10% SDS-PAGE (Schagger and von Jagow, 1987) and electroblotted onto nitrocellulose at 400 mA for 45 min. Blots were probed using rabbit anti-pIV[1–362] antibodies (raised against amino acids 1–362 of the coat protein; Chapdelaine and Hohn, 1998) or mouse (rat) polyclonal antibodies produced against the N-terminal extension (see below). Binding of primary antibody was detected with HRP-conjugated secondary antibodies (Amersham Pharmacia) and chemiluminescent immunodetection reagent as described by the manufacturer (ECL system, Amersham).

A mouse and a rat were immunized intraperitoneally with 150 μl (30 μg) of the N-terminal protein emulsified with an equal volume of Freund's adjuvant. Two additional injections at 2-week intervals were administrated with Freund's incomplete adjuvant and a third was administrated without adjuvant. The presence of antibodies against the protein was

determined by ELISA and Western blot. The antibody was purified by immunoblot against the protein corresponding to amino acids 1–265 of the viral coat protein cloned in the vector pET3d, overexpressed in *E. coli* BL21, and purified as previously described (Chapdelaine and Hohn, 1998). pIV$^{1-265}$ protein was isolated from bacterial inclusion bodies that were solubilized in 8 M urea and renatured by dialysis against TBS. The purified antibody was collected in 100 mm glycine, pH 2.5, and neutralized in 1/10 volume of 1 M Tris (pH 8.0).

Tissue processing for transmission electron microscopy

Leaf samples were collected at 1, 3, 6, 10, 15, 20, and 30 dpi. Noninfected plants were used as a control. After fixation by immersion in 3% (w/v) paraformaldehyde in 0.1 M cacodylate buffer (pH 7.2) at room temperature for 3 h, samples were dehydrated in a graded ethanol series and embedded in L. R. White resin (Hard Grade acrylic resin, Canemco supplies). Ultrathin sections (90–100 nm) were cut with a diamond knife (RMC-MT 7000) and collected on Formvar-coated nickel grids. Sections were processed for immunocytochemical labeling.

Immunocytochemical labeling

For indirect labeling, sections were blocked for 30 min in one drop of 0.01 M sodium phosphate-buffered saline (PBS) containing 1%(w/v) skim milk powder pH 7.2 at room temperature. They were then transferred to a drop of primary antibody (mouse anti-N-terminal domain of pre-CP) in PBS-1% skim milk powder milk for 2 h. Grids were washed with PBS (pH 7.2) plus 0.05% Tween 20 and incubated on a drop of colloidal gold (10 nm)-conjugated goat anti-mouse IgG + IgM (British Biocell) diluted 1:20 in PBS–1% skim milk powder (pH 7.2) for 1 h. Grids were finally washed with PBS–0.05% Tween 20, rinsed with distilled water, and contrasted with 3% uranyl acetate before examination with a JEOL 1200 EX electron microscope (JEOL Co., Japan) at 80 kV. Specificity of labeling was assessed with polyclonal antibody specific for the viral pVI protein (1:500) and omission of the first antibody.

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