

Association of Bamboo Mosaic Virus (BoMV) and BoMV-Specific Electron-Dense Crystalline Bodies with Chloroplasts

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This research was supported in part by grants from Academia Sinica and National Science Council project NSC 80-0211-B-001-17. We thank Ling-Ling Wu for technical assistance.

Accepted for publication 9 July 1991 (submitted for electronic processing).

ABSTRACT

Lin, N. S., and Chen, C. C. 1991. Association of bamboo mosaic virus (BoMV) and BoMV-specific electron-dense crystalline bodies with chloroplasts. *Phytopathology* 81:1551-1555.

Infection of green bamboo (*Bambusa oldhamii*) with bamboo mosaic virus (BoMV), a possible member of the potexvirus group, is associated with the formation of a large number of unusual electron-dense crystalline bodies (EDCBs) and virion aggregates in the infected cells. The infection sequence was studied using leaf tissues prepared for electron microscopy. At an early stage of infection, before BoMV virion formation, EDCBs that lacked clear internal structure were detected in the chloroplasts and were used as infection initiation markers. At a later stage, BoMV virions were observed to be attached to the chloroplasts, whereas EDCBs containing ribosomelike beads occurred free and abundantly in the cytoplasm. In cells at a late stage of infection, masses of BoMV virions were readily

observed in the cytoplasm and vacuoles. However, the EDCBs were restricted to the cytoplasm and nuclei. Only the EDCBs observed in the cytoplasm were closely associated with the BoMV virions. Immunogold labeling showed that the localization of capsid protein during infection corresponded with the localization of virions by ordinary electron microscopy, except that immunogold-labeled virions or BoMV antigens also were labeled along the plasma membrane during the early stage of infection. No virions or viral antigen were detected in other organelles. The EDCBs were not labeled using the antiserum to BoMV capsid protein, indicating that EDCBs do not contain BoMV antigenic determinants identical to those of BoMV virions.

Additional keywords: immunoelectron microscopy.

Bamboo mosaic virus (BoMV) has properties that suggest it is a member of the potexvirus group (7,10,13,14). Most cultivated species of bamboo with rhizomes of the pachymorph type in Taiwan are susceptible to bamboo mosaic (14), and in two major cultivated species, green bamboo (*Bambusa oldhamii* Munro) and Ma Chu (*Dendrocalamus latiflorus* Munro), field incidences of more than 70% of the plants have been observed (N. -S. Lin, unpublished data). Characteristic symptoms include mosaic on the leaves and brown internal streaking of shoots and young culms. Thus, emerging shoots have a hard texture and are of low quality for eating and canning. Bamboo is normally vegetatively propagated, and the use of nonindexed, infected plants as propagating materials greatly aids in the spread of the disease. Bamboo mosaic is now considered to be a limiting factor in the production of vegetable bamboo in Taiwan.

Electron microscopic examination of systemically infected bamboo leaves and local lesions on inoculated *Chenopodium amaranticolor* and *Gomphrena globosa* revealed virus aggregates in the cytoplasm and the vacuoles of infected cells (9). In addition, some peculiar electron-dense crystalline bodies (EDCBs) were present in either the cytoplasm or the nucleus.

Because the BoMV antigen is unevenly distributed in the leaf tissues, as shown by direct tissue blotting on nitrocellulose membranes (15), the sequence of cytopathogenic effects may be elucidated by observation of a gradient of cells from uninfected to those that are heavily infected. We report here that the undeveloped EDCBs in the chloroplasts can be used as infection initiation markers. BoMV virions also first appear at the surface of the chloroplast membrane. This association of virions and virus-specific EDCBs with chloroplasts has not previously been reported for the potexvirus group.

MATERIALS AND METHODS

Virus and host. BoMV was isolated from infected green bamboo showing mosaic symptoms. Sap inoculum (w/v, 1:10) was pre-

pared by grinding infected young leaves in sterilized distilled water. After three successive local lesion passages through *Chenopodium quinoa* Willd., the virus was maintained and propagated in barley (*Hordeum vulgare* L. 'Larker') (N. -S. Lin and Y. -H. Hsu, unpublished).

Healthy bamboo plants were derived from shoot-tip cultures in the case of green bamboo (kindly provided by Dr. L. C. Huang, Institute of Botany, Academia Sinica, Nankang, Taipei, Taiwan, R. O. C.) (8) and from seed-germinated seedlings in the case of Ma Chu. These plants were demonstrated to be BoMV-free before use by immunoelectron microscopy (22) and an enzyme-linked immunosorbent assay (20). The single-lesion virus isolate was then back-inoculated onto both leaf surfaces of healthy plants by sap inoculation. The inoculated plants were kept in a greenhouse in a temperature range between 26 and 34 C and showed typical mosaic symptoms.

Virus purification. One hundred grams of BoMV-infected barley leaves, collected 10 days after inoculation, was ground in 300 ml of 0.5 M borate buffer, pH 9.0, containing 1 mM EDTA. After filtration through cheesecloth, the filtrate was centrifuged at 12,000 g for 10 min in a Hitachi RPR10-2 rotor (Hitachi America Ltd., Tarrytown, NY). To the supernatant, 4 M K_2HPO_4 and 2 M $CaCl_2$ were added to 1% and 2%, respectively, and the mixture was subjected to another low-speed centrifugation. Triton X-100 and PEG 6000 were added to the supernatant to provide a final concentration of 2% and 6%, respectively. The mixture was stirred for 30 min at 4 C and centrifuged at 12,000 g for 10 min. The pellet was resuspended in 0.05 M borate buffer, pH 8.0, containing 1 mM EDTA (BE buffer) and was centrifuged through a 5-ml cushion of 20% sucrose at 143,000 g for 1 h in a Beckman Ti50 rotor (Beckman Instruments, Fullerton, CA). The partially purified virus was centrifuged in $CsCl$ (40% w/v) in BE buffer at 76,700 g in a Hitachi RP70T rotor for 12 h at 4 C. The virus band was withdrawn, diluted in BE buffer, and centrifuged at 143,000 g for 1 h. The purified virus was finally resuspended in 5 ml of BE buffer and stored at -20 C. The yield of purified virus, as determined by ultraviolet absorption, was usually 15-20 mg from 100 g of infected barley leaves assuming A (0.1%, 260 nm) = 3 (13). Purity of virion preparations was

confirmed by the presence of a single protein band in sodium dodecyl sulfate (SDS)-polyacrylamide gel.

Polyacrylamide gel electrophoresis of protein and preparation of antiserum. Purified BoMV was disrupted in protein dissociation buffer (60 mM Tris-HCl, pH 8.8, 2% SDS, 2 mM EDTA, 5 mM DTE, and 10% Ficoll) (12) and analyzed in a 12% polyacrylamide gel using a discontinuous buffer system (11). After electrophoresis, the gel was placed in 0.25 M KCl at 4 C for 30 min. The major capsid protein band corresponding to protein of M_r 28,000 was excised, and the protein was eluted and concentrated as described by Brakke et al (2).

Antiserum to gel-eluted capsid protein (anti-BoMV-CP serum) was prepared in New Zealand white rabbits as described by Lin et al (19) and was used as a 1:200 dilution in phosphate-buffered saline in the immunoelectron microscope studies. The preimmune rabbit serum was used at the same dilution as controls.

Electron microscopy. Healthy green bamboo plants, at about four- to six-leaf stage, were inoculated with BoMV sap inoculum and maintained in a growth chamber at 27–29 C under 16 h

of daily exposure to $50 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{sec}^{-1}$ illumination. Emerging young leaves that had not yet unrolled were sampled about 2 wk post-inoculation. Before the leaf tissues were fixed, the rolled leaves were cut transversely with a razor blade and the cut surfaces were directly blotted onto nitrocellulose membranes that were then immunostained for the BoMV antigen as described previously (15). Only those young leaves that reacted positively for BoMV were processed for electron microscopy. Leaf tissues were then cut into small pieces, fixed with glutaraldehyde, and embedded in Lowicryl HM20 (Polysciences Inc., Warrington, PA), or they were postfixed with OsO_4 and embedded in Araldite 502 (Polysciences Inc.) as previously described (17). Ultrathin sections were cut transversely or parallel to the leaf veins, stained in uranyl acetate and lead citrate, and viewed in a Zeiss 109 electron microscope at 80 kV. Three independent samples, each including two rolled leaves, were embedded for this study and 30–100 sections per sample were examined with an electron microscope.

Immunoelectron microscopy. Leaf tissue sections were stained by the two-step method of Lin and Langenberg (17). However,

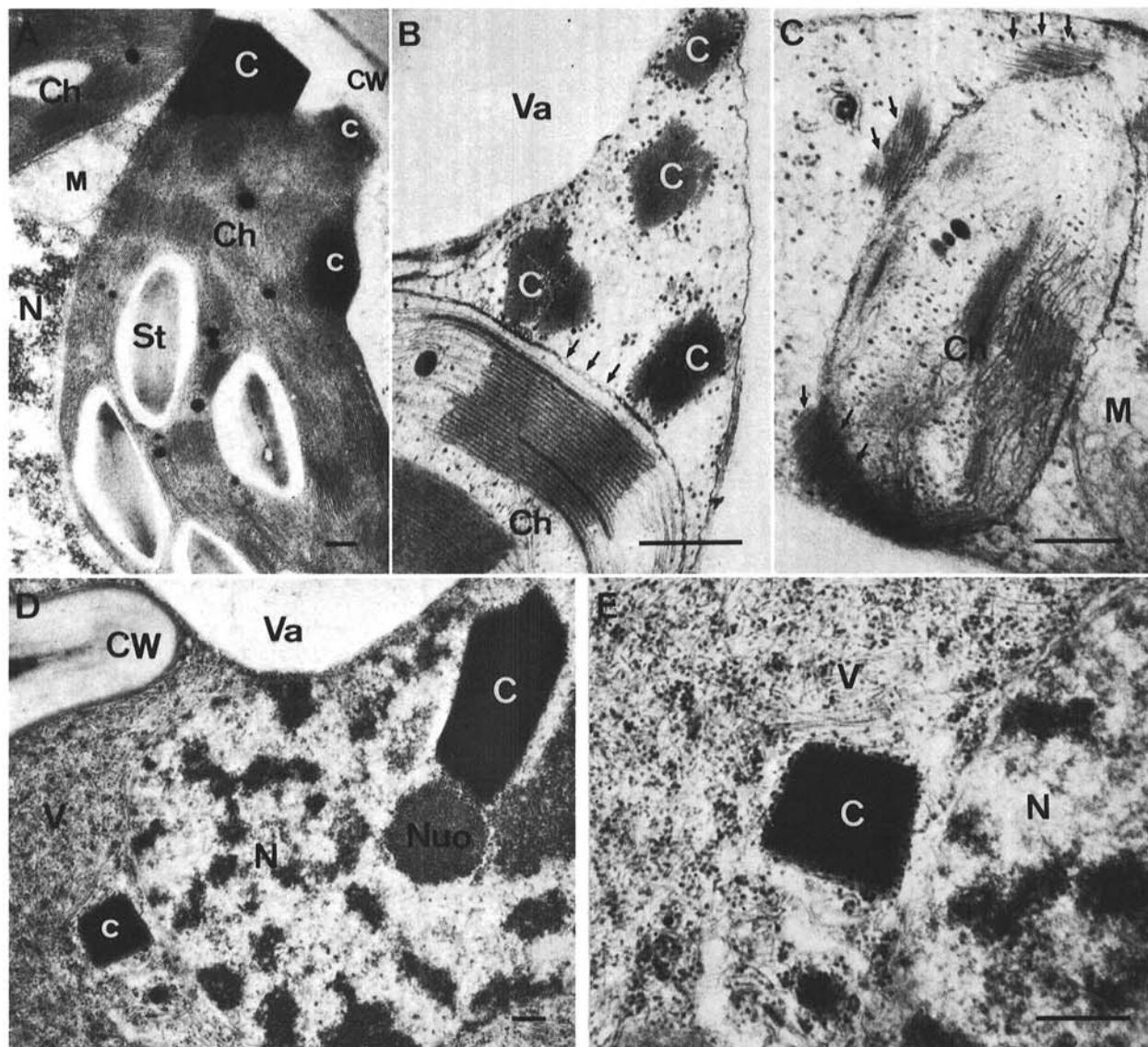


Fig. 1. Thin sections of glutaraldehyde-fixed, osmic tetroxide-postfixed, and Araldite 502-embedded BoMV-infected green bamboo leaves. Parenchyma cells are shown in A–E. A, electron-dense crystalline bodies (C) with no clear internal structure are present inside the chloroplasts in the cell at the earliest stage of infection. No BoMV virions are visible. B, part of a cell that is at a slightly later stage of infection than the one depicted in A. Electron-dense bodies with crystalloid structure can be seen in the cytoplasm and ribosome-like beads can be seen on their surface. Note that the conformation of the chloroplast membrane is abnormal (arrows). C, bundles of BoMV virions (arrows) closely associated with the chloroplast (Ch) membrane at the second stage of infection. D, at a late stage of infection, BoMV virions (V) are scattered only in the cytoplasm, whereas the electron-dense crystalline bodies (C) are seen in the cytoplasm as well as in the nucleus (N). E, an enlargement of part of D, showing the association of BoMV with electron-dense crystalline bodies (C) in the cytoplasm. Cy = cytoplasm, CW = cell wall, M = mitochondrion, Nuo = nucleolus, St = starch grain, V = virus aggregate, Va = vacuole. All bars represent 300 nm.

for osmium tetroxide-postfixed and Araldite-embedded tissues, sections were treated with saturated potassium periodate for 1 h before immunostaining (1).

RESULTS

Electron microscopy. Electron microscopic examination of BoMV-infected young bamboo leaves revealed high concentrations of BoMV virions in most infected cells. They were observed in the epidermis, mesophyll, as well as vascular bundles. Very

few or no virions of BoMV were present in some cells adjacent to the heavily infected cells. Thus, a study of disease progress could be conducted using cells at several stages in the disease progression. We propose three stages or levels of infection, distinguishable by observable cytopathogenic changes as subsequently described.

Compared to the apparently healthy normal cells, the first cytological change observed in the infected cells was the appearance of unusual electron-dense bodies inside the chloroplasts (Fig. 1A). The bodies, which varied in size (0.1–2.7 μm) and

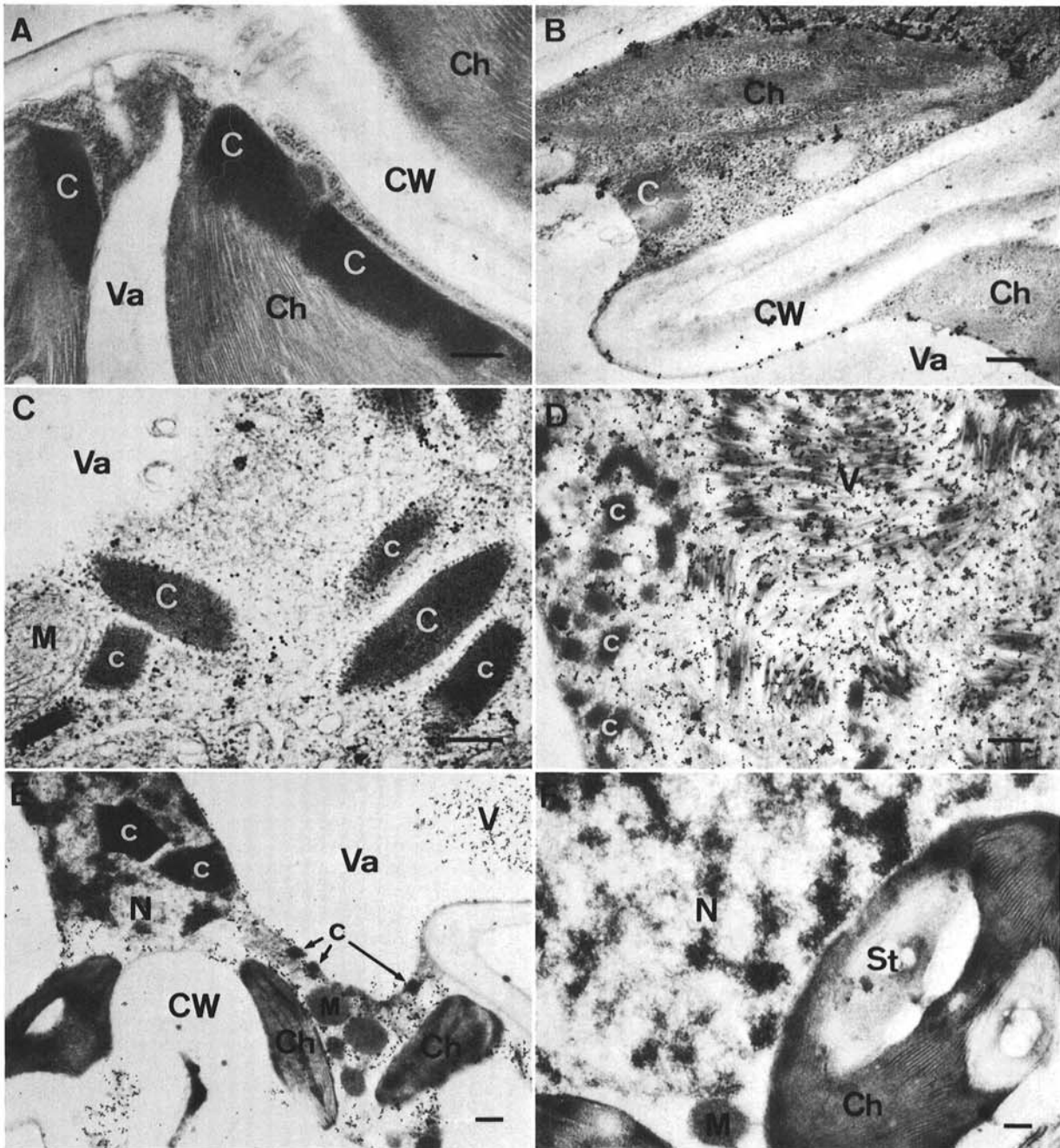


Fig. 2. Thin sections of green bamboo leaves embedded in Araldite 502 (except that of Fig. 2E, which was embedded in Lowicryl HM20) and stained with dilute anti-BoMV serum, followed by gold-labeled goat anti-rabbit IgG. Parenchyma cells are shown in A–F. **A**, similar stage of infection as illustrated in Figure 1A. No BoMV virions or antigen could be detected by gold-IgG complexes. **B**, similar stage of infection as illustrated in Figure 1B. BoMV virions (arrows) with gold labels are associated with chloroplast and plasma membranes, but viral antigen is not detected within the chloroplast (Ch). **C**, no visible virions could be seen in the neighboring cell to the one in Figure 1B, but specific gold labels are present in the cytoplasm and are associated with electron-dense crystalline bodies (C). **D**, large virus aggregates (V) bearing gold labels are present in the cytoplasm, however, the electron-dense crystalline bodies (C) are not labeled at this late stage of infection. **E**, virus aggregates with gold labels are present in the cytoplasm and vacuole (Va), but they are not detected in any other organelles or compartments, even though electron-dense crystalline bodies (C) can be found in the nucleus (N) at the late stage of infection. **F**, thin section of a healthy control green bamboo leaf in which neither the virus particles nor the electron-dense crystalline bodies are present. Very few gold particles can be detected due to background staining. Cy = cytoplasm, CW = cell wall, M = mitochondrion, Nuo = nucleolus, St = starch grain. All bars represent 300 nm.

shape, usually had one side attached to the chloroplast membrane. No detailed fine structure of the electron-dense matrix could be ascertained in the early stages of infection. In other cells presumed to be at a later stage, the boundary between the electron-dense bodies and the chloroplast membrane was observable, and the crystalloid structures that gradually developed inside the electron dense mass became visible. No BoMV virions were observed at this stage. Thus, formation of the electron-dense crystalline bodies (EDCBs) inside the chloroplasts served as infection initiation markers.

During the second stage of infection, the EDCBs were observed in greater concentrations free in the cytoplasm rather than being attached to the chloroplasts. Moreover, ribosomelike beads appeared on the surface of EDCBs (Fig. 1B), and an unusual conformation of chloroplast membranes was noted (Fig. 1B, arrows). When the EDCBs were abundant in the cytoplasm, BoMV virions first appeared as small aggregates in parallel arrays associated with the chloroplasts (Fig. 1C). The alignment of the BoMV virion's long axis to the chloroplast's outer membrane varied from aggregate to aggregate, sometimes being parallel or at right angles to the membrane and at other times having no uniform orientation. No association between the virion aggregates and other organelles of the infected cells was observed. The BoMV virions gradually became dissociated from the chloroplast membrane and were randomly dispersed in the cytoplasm or, more often, were associated with the EDCBs in the cytoplasm (similar to Fig. 1D).

At a late stage of infection, BoMV virions were present in either aggregate form or dispersed in the cytoplasm of most infected cells. Less frequently, BoMV virion aggregates could be observed in the vacuoles. As Kitajima et al (9) described earlier, the EDCBs commonly occurred in the cytoplasm or in the nucleus of the cells in which virions accumulated (Fig. 1D). Very often, the cytoplasmic EDCBs were intimately associated with BoMV virions (Fig. 1E).

The cytological changes induced by BoMV infection as described above were not observed in either uninfected cells of diseased plants or the cells of healthy control bamboo leaves.

Immunoelectron microscopy. To detect the distribution of BoMV antigen at several stages of infection in infected cells and determine the serological relationship of EDCBs to the capsid protein, ultrathin sections of BoMV-infected leaf tissues were first treated with dilute anti-BoMV-CP serum followed by gold-labeled goat anti-rabbit IgG.

In cells at the earliest stage of infection, no BoMV virions or antigen were detected using gold-IgG complexes (Fig. 2A). Although the unusual electron-dense bodies appeared at this stage, they were not labeled. This indicates that EDCB antigenic determinants are not identical to those of BoMV.

At a later stage, after BoMV virions appeared, most of the gold labels were associated with the BoMV virions that were attached to the chloroplasts (Fig. 2B). Some gold labels were associated with the BoMV virions or BoMV antigens that were attached to the plasma membrane (Fig. 2B). However, no specific gold labels were found within the chloroplasts (Fig. 2B). The EDCBs with ribosomelike beads on their surfaces were not labeled with gold-IgG complexes at this stage, either. In some cells that contained a large quantity of EDCBs, but in which few virions were observed, a slight immunogold labeling of the cytoplasm did occur (Fig. 2C).

At a late stage of infection, when the cells contained a large amount of BoMV virions, the gold-IgG complexes specifically labeled the BoMV virions, whether they occurred in the cytoplasm (Fig. 2D) or in the vacuoles (Fig. 2E). No gold labels were bound to the EDCBs, although the EDCBs were closely associated with the BoMV in the cytoplasm (Fig. 2D,E). No gold labels were found in nuclei or in any other organelles of infected cells (Fig. 2E).

When the sections were treated with dilute normal rabbit serum, followed by the gold-IgG complexes, there was no specific labeling in infected cells (data not shown). Healthy control cells treated with anti-BoMV-CP serum only showed minimum background labeling (Fig. 2F).

DISCUSSION

One of the cytopathological characteristics of all known potexviruses is that they induce the formation of cytoplasmic inclusions that consist of virion aggregates. These inclusions can be banded bodies, dense bodies, paracrystals, or spindle-shaped bodies (4,5). BoMV, which we propose as a member of the potexvirus group, also has the characteristic in the formation of virion aggregates in the cytoplasm. However, BoMV is unique in that it induces virus-specific EDCBs in both the cytoplasm and the nucleus of infected cells that are serologically unrelated to the BoMV capsid protein. In tobacco leaf cells infected with potato virus X (PVX), specific laminated inclusions, antigenically unrelated to PVX, are also induced in the cytoplasm and occasionally in the nucleus (5,23). However, they are structurally and morphologically different from the EDCBs with no known functions.

In many respects, the cytological changes observed during the early stages of BoMV infection are quite different from those observed with other potexvirus infection (e.g., the initial appearance of EDCBs in the chloroplasts and the subsequent appearance of BoMV virions at the surface of the chloroplasts). However, the association of virions with chloroplasts has been recorded for some other unrelated viruses, particularly the turnip yellow mosaic virus of the tymovirus group (6,21) and the barley stripe mosaic virus of the hordevirus group (3,16,18). Membranous vesicles were usually found at the periphery of proplastids or chloroplasts in the cells at the early stage of infection. However, no such vesicles were ever found in the present study, although the chloroplasts of BoMV-infected cells at certain stages of infection had an abnormal conformation.

Immunological localization of BoMV capsid protein revealed that at an early stage of infection the virions or their antigens in the cytoplasm were attached to the chloroplasts or plasma membrane of the cell. At a late stage of infection, labeled BoMV virions were found mostly in the cytoplasm and occasionally in the vacuoles (Fig. 2D,E), an observation consistent with that of Kitajima et al (9). Although BoMV virions were observed in close association with chloroplasts, BoMV virions or their antigens were not detected within chloroplasts, nuclei, or any other organelles at any stage of infection (Fig. 2B,E). This characteristic is shared with most potexviruses, except narcissus mosaic virus whose aggregates have been found within the nuclei (24). Furthermore, it was noted that it was mostly the BoMV virions that became labeled with immunogold. A few gold particles were also deposited alongside the plasma membrane and the EDCBs, indicating the possible presence of free viral capsid protein in these sites within the infected cells.

The EDCBs have been found in all tested host plants, including four species of bamboo, barley (not shown), *C. amaranticolor* and *G. globosa* (9). Because they could serve as infection initiation markers and were accumulated at high concentrations in the infected cells, the detection of EDCBs provides a convenient means for disease diagnosis.

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