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Coat Protein-Related Polypeptides from *in Vitro* Tobacco Mosaic Virus Coat Protein Mutants Do Not Accumulate in the Chloroplasts of Directly Inoculated Leaves

A. G. C. Lindbeck,[1,2] W. O. Dawson,[1] and W. W. Thomson[2]

Departments of [1]Plant Pathology and [2]Botany and Plant Sciences, University of California, Riverside 92521 U.S.A.
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Chloroplasts from leaves directly inoculated with a series of coat protein deletion mutants of tobacco mosaic virus were examined by electron microscopy. Wild-type tobacco mosaic virus did not cause any ultrastructural changes in fully developed chloroplasts. However, specific mutations in the coat protein gene caused chlorosis on fully expanded leaves by causing significant degradation of the chloroplasts in these tissues. Mutants that caused weak or transient yellowing had no observable effect on the chloroplasts in these tissues. Immunocytochemical localization of the coat protein-related polypeptides from the deletion mutants indicated that the polypeptides do not accumulate in the chloroplasts. The polypeptides accumulate in discrete dark-staining bodies in the cytoplasm of infected cells, which we have termed "coat protein bodies." The coat protein bodies were often associated with X-bodies. These data suggest that the altered coat protein which was on the outside of the chloroplasts caused the degradation of the chloroplasts in infected cells. Coat protein probably subverts the chloroplasts by interfering with chloroplast protein synthesis and transport in the cytoplasm. A number of possible mechanisms are suggested.

Additional keywords: *Nicotiana tabacum*.

Plants respond to viral infections in a number of different ways, often leading to reduced growth or death (Matthews 1981). A major type of disease symptom is chlorosis that results in less photosynthesis and growth. In tobacco plants systemically infected with tobacco mosaic virus (TMV), these symptoms can range from the classic dark and light green mosaic to mild chlorosis, heavy chlorosis, necrosis, or virtually no symptoms at all. The development of chlorotic symptoms usually results from the alteration of chloroplast structure or function. This alteration is related to the development of chloroplasts in cells at the time of infection. For example, in developing leaves the typical symptom induced by TMV is the dark and light green mosaic. This type of symptom has been shown to result from the lack of development of normal chloroplasts in virus-infected cells (Matthews 1981). However, on directly inoculated leaves this strain of TMV (U1) is virtually symptomless. Mutants of this virus that cause a variety of symptoms on directly inoculated leaves (McKinney 1935; Dawson *et al.* 1988) can be isolated. The mechanism by which these symptoms are induced is not understood.

In developing leaves, it has been suggested that viral coat protein plays a role in the development of chlorotic symptoms. *In vivo* evidence from systemically infected tobacco (Reinero and Beachy 1986) suggests that coat proteins from TMV strains U1, PV230, and PV39 accumulate in the chloroplasts and that there is a correlation between the level of accumulation of coat protein and the severity of the symptoms. It has been reported (Reinero and Beachy 1989; Hodgson *et al.* 1989) that the accumulation of coat protein in tobacco and spinach inhibits photosystem II activity and appears to be associated with photosystem II. However, immunogold labeling of thin sections of tobacco leaves systemically infected with U1 suggests that very little coat protein accumulates in chloroplasts (Hills *et al.* 1987). Culver and Dawson (1989) have reported the appearance of identical mosaic-type symptoms in *Nicotiana sylvestris* Speg. and Comes inoculated with wild-type TMV and a strain of TMV with the start codon for the coat protein gene altered so that no coat protein was synthesized. This suggests that in developing leaves the coat protein is not absolutely required for the virus-induced, abnormal development of chloroplasts.

In contrast, in leaves with fully developed chloroplasts, the U1 strain of TMV replicates to high levels causing no visible changes to the chloroplasts and is virtually symptomless on directly inoculated leaves. However, different strains of TMV and spontaneous mutants of TMV U1 cause visible chlorotic symptoms on directly inoculated leaves (McKinney 1935). Dawson *et al.* (1988) have produced a series of TMV mutants with insertions and/or deletions in the coat protein gene that produce a variety of symptoms on leaves with fully developed chloroplasts. The different mutants induced three types of symptoms in Xanthi tobacco: no symptoms, yellowing, and necrosis. Most mutants produced coat protein-related polypeptides *in vivo*, although the size and amount of protein produced in inoculated leaves varied among the different mutants. Most mutants that produced no or low levels of coat protein did not induce chlorotic symptoms. Those mutants that retained the native carboxyl terminus of the coat protein tended to produce more coat protein and induced chlorotic symptoms in the areas of the leaf infected by virus. Two of the mutants caused necrosis. These results suggest that,

Address correspondence to W. W. Thomson: Department of Botany and Plant Sciences, University of California, Riverside, CA 92521 U.S.A.

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while the wild-type coat protein gene does not induce chlorotic symptoms in directly inoculated leaves, specific mutations in the coat protein gene affect chloroplast structure or function.

In this study, we examined the alterations of ultrastructure of fully developed chloroplasts in virus-infected areas and attempted to localize coat protein-related polypeptides from these coat protein mutants in infected cells. Our results show that, while several of the coat protein mutants cause significant degradation of the chloroplasts in infected cells, very little coat protein-related polypeptide accumulated within the chloroplasts of plants infected with these mutants. Coat protein-related polypeptides from mutants that did not cause chloroplast degradation also do not accumulate in chloroplasts. Instead, the coat protein appears to accumulate in discrete bodies mostly found in X-bodies.

MATERIALS AND METHODS

Virus and plants. Wild-type TMV (TMV204; Dawson *et al.* 1986) and a series of coat protein mutants derived from the wild-type virus (Dawson *et al.* 1988) that give rise to symptoms in the inoculated leaves were used in this study (see Table 1). Tobacco (*N. tabacum* L.) cultivar Xanthi was grown in a greenhouse. Before inoculation the plants were transferred to the dark for 48 hr. After inoculation with the viruses, the plants were maintained in plant growth chambers at 25° C with a 12-hr photoperiod with a photon flux density of 245 $\mu E \cdot m^{-2} \cdot s^{-1}$ at leaf height. Tissue samples were taken from the inoculated leaves 7 days after the onset of visible symptoms (11–12 days after inoculation).

Antibodies. Polyclonal antibodies to TMV coat protein had been previously prepared (Dawson *et al.* 1988). The antibodies were cross-absorbed with Xanthi tobacco before use to remove antibodies that bound to host proteins. One to 2 g of healthy tissue was ground in a sterile mortar with a pestle in liquid nitrogen, and 10 ml of phosphate-buffered saline (PBS; 0.15 M NaCl, 0.01 M KPO_4, pH 7.2) was added. Sodium chloride was added to the leaf extract to a final concentration of 0.5 g per 100 ml. Ten volumes of leaf extract was added to the antibody solution, and the mixture was incubated for 2 hr at 37° C. The solution was centrifuged for 30 min at 20,000 × *g* at 4° C, and the supernatant was stored at −20° C for 2–3 hr. After thawing, the solution was centrifuged as above. The supernatant was termed the first dilution of the antibody.

Table 1. Summary of coat protein mutants[a]

Mutant	Nucleotides deleted from coat protein gene[b]	Amino acids deleted from coat protein	Symptoms on inoculated leaves
TMV204	0	0	None
cp +2	+6[c]	+2	Yellow spots
cp 4	6020−6153	102−147	Yellowing
cp 10	6043−6084	110−158	Necrosis
cp 27	5949−6079	79−122	Necrosis
cp 28	5874−6191	54−158	Yellow spots
cp 35	5841−6219	43−158	None
cp 35-5	5841−6055	43−114	Yellow areas

[a] Data taken from Dawson *et al.* 1988.
[b] Nucleotide numbering as described by Goelet *et al.* 1982.
[c] Number of nucleotides added at position 6056.

Protein A-gold probe. Colloidal gold particles (11.5-nm-diameter) were made using the method of Slot and Geuze (1985). The size of the gold particles was checked with a Philips EM400 transmission electron microscope.

The amount of protein A required to stabilize the gold sol was determined by titration as described by Roth and Binder (1978). One hundred microliters of serial dilutions of protein A was added to 500 μl of the gold sol with agitation. After 1 min at room temperature, 100 μl of 10% NaCl was added with agitation. The tube containing the minimum amount of protein A necessary to stabilize the gold was determined as the one containing the least protein whose sol did not change from red to blue after the addition of NaCl. Sufficient protein A was added to the gold sol to exceed the stabilization point by 10%. After 1 min, 300 μl of 0.1% bovine serum albumin (BSA; fraction V, RIA grade, Sigma, St Louis, MO) was added to ensure that the gold particles were maximally stabilized. The stabilized gold was stored at 4° C in a light-tight container.

Before use, an aliquot of the protein A-gold was centrifuged for 10 min in an Eppendorf 5414 bench microcentrifuge at room temperature. The gold pellet was resuspended in 0.25 volumes of PBS + BSA (PBS containing 0.1% BSA).

Tissue preparation. Leaf disks, approximately 2–3 mm in diameter, were fixed in 50% strength Karnovsky's fixative (Karnovsky 1965), postfixed in 1% osmium tetroxide in 0.05 M phosphate buffer (pH 7), dehydrated in acetone, and embedded in Spurr's resin (Spurr 1969). After four changes of resin over 2 days, the resin was polymerized overnight (approximately 16 hr) at 70° C.

Immunocytochemical staining. Thin sections of tissue were mounted on 300-mesh nickel grids. All grids were incubated at room temperature by floating the grids, section side down, on 30-μl drops of the appropriate solution.

The sections were chemically modified before antibody labeling using the method of Craig and Goodchild (1984). The grids were incubated for 10 min in saturated sodium metaperiodate, washed for 4 × 1 min in double-filtered distilled water, and incubated for 10 min in 0.1 N HCl. After four washes in double-filtered distilled water as described above, the grids were incubated for 5 min in PBS + BSA and transferred to antibody diluted 1:10 (final dilution 1:100) in PBS + BSA for 1 hr. The appropriate dilution of antibody was determined by dot blot assay with the protein A-gold probe. Dilutions of the antibody were applied to a nitrocellulose membrane, incubated for 5 min in PBS + BSA, and then incubated for 1 hr with the protein A-gold probe. The membrane was then processed as was done for western blots. The lowest dilution of antibody that resulted in no increase in stain intensity was used for immunolocalization.

Sections incubated with antibody were washed for 5 × 1 min in PBS + BSA and then incubated for 1 hr with the protein A-gold probe. After washing for 1 min in PBS + BSA, the grids were fixed for 3 min in 1% glutaraldehyde in PBS and washed for 5 × 1 min in double-filtered distilled water. The sections were then stained for 7 min in saturated uranyl acetate in 50% ethanol and for 7 min in Reynolds' lead citrate (Reynolds 1963). The sections were viewed in

a Philips EM400 transmission electron microscope.

Sections treated identically as given above but with incubation with PBS + BSA, preimmune serum, or anti-coat protein antibodies preabsorbed with purified wild-type coat protein in place of the primary antibody and tissue from healthy, uninfected plants were used as controls to determine the specificity of the labeling.

RESULTS

Ultrastructure of altered chloroplasts. We examined the effects of the coat protein deletion mutants on fully developed chloroplasts. Chloroplasts from healthy plants and from leaves directly inoculated with wild-type TMV were examined as controls. Chloroplasts from leaves infected with wild-type TMV (Fig. 1B) were ultrastructurally similar to chloroplasts from healthy leaves (Fig. 1A). Occasionally virion inclusions could be seen in chloroplasts from leaves infected with wild-type TMV (Fig. 1B), but these inclusions were rare and most chloroplasts did not contain virion inclusions. Virions were also observed in the cytoplasm and the vacuole. An examination of tissue from the necrotic and chlorotic areas on leaves directly inoculated with the coat protein mutants showed that the chloroplasts in these areas exhibited significant structural alterations.

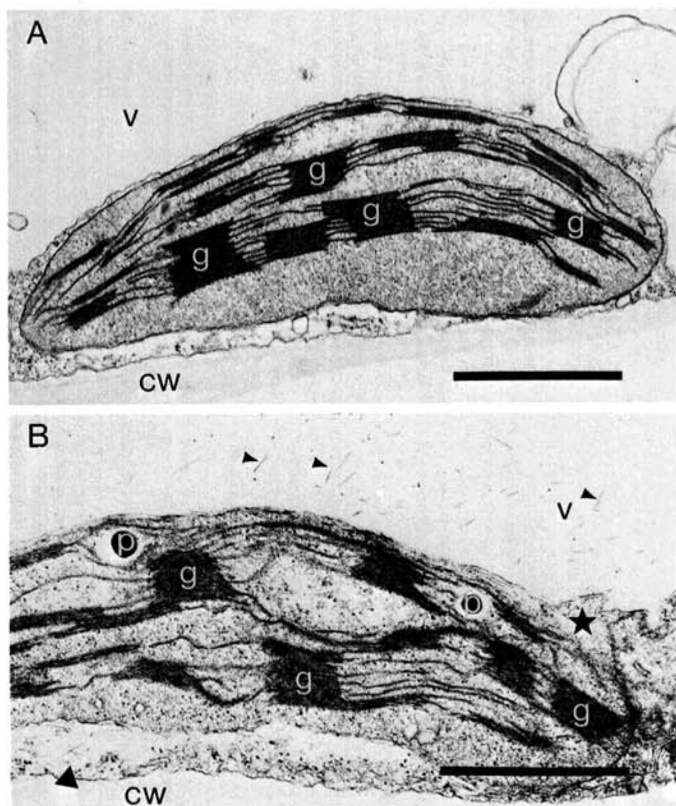

Fig. 1. Electron micrographs of chloroplasts from a leaf of a healthy plant and a leaf directly inoculated with the U1 strain of tobacco mosaic virus (TMV). (Bars = 1 μm) **A**, Healthy. **B**, TMV U1. The chloroplasts appear to be structurally similar. Note the presence of a small virion inclusion in the chloroplast from the leaf infected with TMV U1 (star). Virions can also be seen in the cytoplasm (large arrowhead) and the vacuole (small arrowheads). Other abbreviations are as follows: cw, cell wall; g, grana; p, plastoglobuli; and v, vacuole.

Chloroplasts from the chlorotic areas of leaves inoculated with mutants that cause mild to strong yellowing (cp 4 and cp 35-5) contained vesicle-like structures (Fig. 2A). In some chloroplasts these structures appeared to be transverse sections of tubules. Stereopair electron micrographs of affected tissue showed that these structures were tubules which extend into the stroma and appear to form an interconnected complex within the stroma. Similar structures were also observed in chloroplasts in tissue from the inner half (later stage of infection) of the thin chlorotic band of cells that surrounds the necrotic lesion caused by cp 10 and the chlorotic area that precedes the necrotic lesion caused by cp 27. In a number of the plastids examined, the tubules appeared to run in a circular fashion at 90° to the longitudinal axis of the plastids. In most of the plastids examined, apparently structurally normal photosynthetic membranes were also present.

The tubules appeared to be derived from rearrangements of the photosynthetic membranes in affected chloroplasts. Chloroplasts from tissue inoculated with cp 4 contain grana that appeared to be unstacking (inset Fig. 2B), forming convex membrane structures, 4 days after inoculation. In

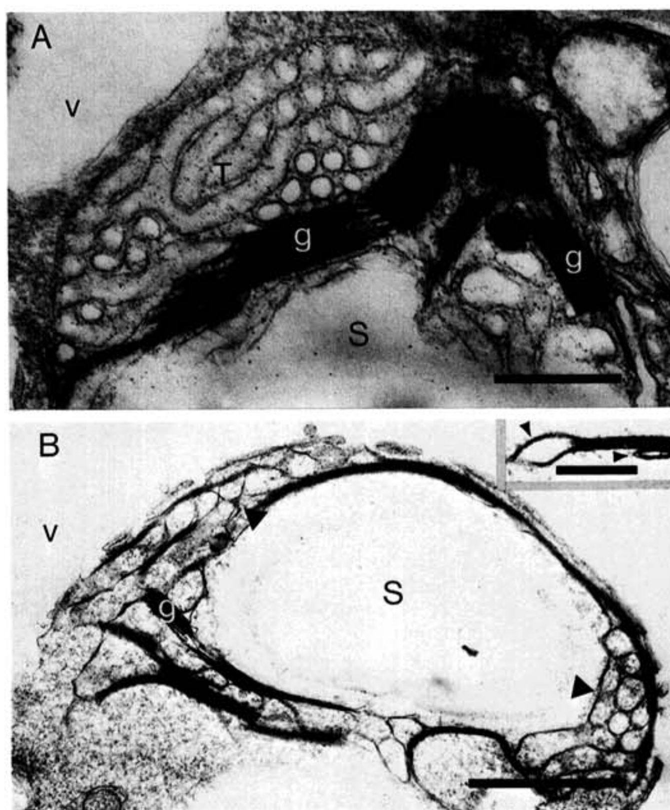

Fig. 2. Electron micrographs of chloroplasts from chlorotic leaf areas resulting from the replication of the coat protein mutants cp 4 and cp 35-5. **A**, Micrograph of a tubular complex (T) in a chloroplast from the center or the chlorotic area caused by cp 35-5 (bar = 0.5 μm). **B**, Micrograph of the putative precursors (large arrowheads) of the tubules in a chloroplast from the chlorotic area caused by cp 4 (bar = 1 μm). Note the presence of normal photosynthetic membranes in both of these chloroplasts. The inset shows convex membrane structures (small arrowheads) in a chloroplast from a leaf infected with cp 4 that appear to be formed by the unstacking of a granum (bar = 0.25 μm). Other abbreviations are as follows: g, grana; S, starch granule; and v, vacuole.

some chloroplasts the photosynthetic membranes appeared to be completely unstacked and arranged in circular structures (Fig. 2B). At 7 days after inoculation, tubules had formed in these chloroplasts.

Chloroplasts from chlorotic areas of leaves inoculated with mutants that cause weak yellowing (cp +2, cp 28, and cp 35) appeared to be structurally normal (Fig. 3A, 3B, and 3C). Chloroplasts from necrotic tissue from leaves inoculated with the mutants that cause necrosis (cp 10 and cp 27) were degraded to the point where no internal structure was visible. Chloroplasts from tissue located in the outer half (early stage of infection) of the chlorotic bands surrounding the necrotic lesions caused by cp 10 and cp 27 appeared to be structurally normal.

Localization of mutant coat proteins. To determine if the altered coat proteins accumulated in chloroplasts from leaves directly inoculated with coat protein mutants, we have attempted to localize mutant coat proteins in infected cells. We were particularly interested in whether the coat protein of the mutants that degrade chloroplasts enters the chloroplasts.

Coat protein from wild-type TMV accumulated in virion inclusions in the cytoplasm and vacuoles of infected cells. Most chloroplasts of infected cells did not contain detectable amounts of coat protein, even when the chloroplasts were adjacent to large virion inclusions (Fig. 4A). In rare chloroplasts, accumulations of gold particles were found, but only associated with virion inclusions (Fig. 4A). However, these inclusions were found in only two of the approximately 60 chloroplasts examined. Gold particles were also found associated with virions in X-bodies (Fig. 4B) in the cytoplasm of infected cells. These results are consistent with those reported previously (Hills *et al.* 1987).

Cells from chlorotic tissue from expanded leaves inoculated with coat protein mutants that cause yellowing on directly inoculated leaves contained dark-staining bodies in the cytoplasm (Fig. 5A). The mutant coat protein was localized to these structures. These "coat protein bodies" were often associated with X-bodies in the cytoplasm of infected cells. In some mutants, where X-bodies do not appear to be present, the coat protein bodies were in the cytoplasm of the cell. In leaves infected with one of these mutants (cp 10), coat protein bodies could be found in the green symptomless tissue immediately adjacent to the infection sites (Fig. 5B), suggesting that the accumulation of coat protein in discrete bodies in symptomless tissue may precede the appearance of symptoms in infected leaves.

Gold particles were also observed in the vacuoles of infected cells. In the case of cells infected with wild-type TMV, this label was often associated with single virions in the vacuole. However, some of the label in the vacuoles of these cells and all the label in the vacuoles of cells infected

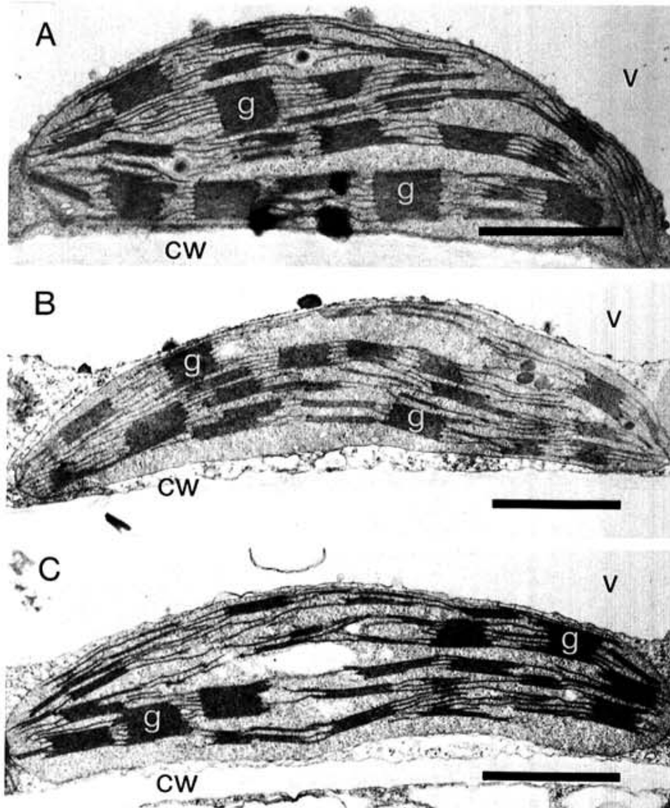

Fig. 3. Electron micrographs of chloroplasts from leaves inoculated with coat protein mutants that cause transitory or no yellowing (bars = 1 μm). **A**, cp +2; **B**, cp 28; and **C**, cp 35. Abbreviations are as follows: cw, cell wall; g, grana; and v, vacuole.

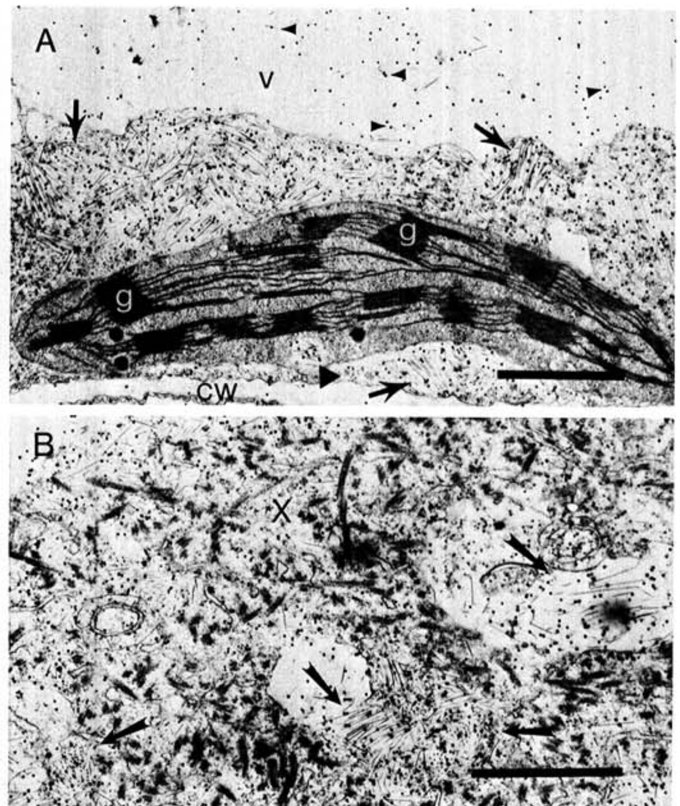

Fig. 4. Electron micrographs of tissue from leaves inoculated with the U1 strain of tobacco mosaic virus and labeled with anti-coat protein antibody and the protein A-gold probe (bars = 1 μm). **A**, Micrograph of a chloroplast surrounded by virions. Gold particles are associated with the virion inclusion in the chloroplast (large arrowhead). Label is also associated with virions in the cytoplasm (arrows) and individual virions (small arrowheads) in the vacuole (v). Very little label can be seen in the chloroplast (cw, cell wall; g, grana). **B**, Micrograph of an X-body (X) from a leaf inoculated with the U1 strain of tobacco mosaic virus. Gold particles are associated with virions in the X-body (arrows).

with the coat protein mutants (which cannot form virions) are presumably associated with free coat protein.

It has been shown (Dietzgen and Zaitlin 1986) that TMV coat protein and the large subunit of ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBPCase) have an immunological cross-reactivity and some amino acid sequence homology. To determine if some of the label seen in chloroplasts from infected tissue is due to nonspecific binding of antibody to chloroplast components, we quantified the number of gold particles per square micrometer of chloroplast surface area in sections from healthy tissue and tissue infected with wild-type TMV that were treated with anti-coat protein antibodies, antibodies preabsorbed with coat protein, and preimmune serum (Table 2). We chose tissue infected with wild-type TMV because the anti-coat protein antibody has a greater affinity for wild-type coat protein than for the mutant polypeptides for which it has varying levels of affinity (see Fig. 2B, Dawson *et al.* 1988). The data show that approximately 60% of the label in chloroplasts from tissue infected with wild-type TMV could be due to binding of antibody to other chloroplast components, possibly RuBPCase. The data also show that some coat protein can enter the chloroplasts. However, it should be noted that the chloroplasts from this tissue show no ultrastructural alterations and the leaves remain symptomless.

These data suggest two possibilities for the action of altered coat protein in altering chloroplasts in infected cells: 1) the altered coat proteins are biologically active at very low concentrations, or 2) the low levels of coat protein in the chloroplasts are not biologically significant, and the altered coat proteins exert their influence from outside the chloroplasts.

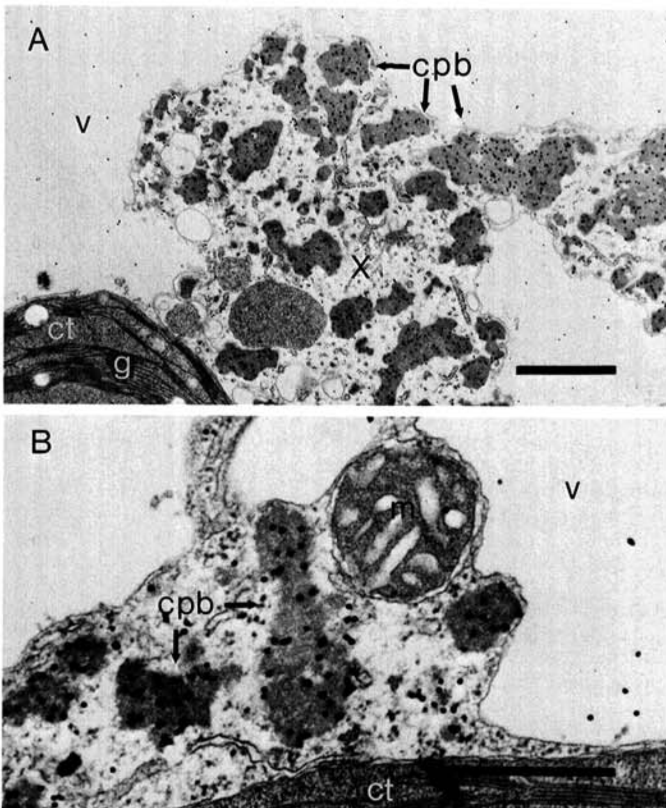

Fig. 5. Electron micrographs of tissue from leaves inoculated with the coat protein mutant cp 10 and labeled with anti-coat protein antibody and the protein A-gold probe. **A**, Micrograph of tissue from the outer half (early stage of infection) of the thin chlorotic band adjacent to the necrotic area caused by cp 10. Discrete dark-staining bodies, labeled with gold particles, can be seen embedded in an X-body (X). We have called these bodies coat protein bodies (cpb) (bar = 1 μm). **B**, Micrograph of symptomless green tissue adjacent to the infection site on leaves inoculated with cp 10. Coat protein bodies are present and are labeled with gold particles (bar = 0.5 μm). Other abbreviations are as follows: ct, chloroplast; g, grana; m, mitochondrion; and v, vacuole.

DISCUSSION

TMV replicates in the cytoplasm and most of the virions are found in the cytoplasm. Although there have been reports of virus particles within chloroplasts, there is little evidence to suggest that the chloroplast is the site of virus replication. Different strains of TMV differ greatly in the amount of virions found in chloroplasts. Chloroplasts from tissue infected with the U5 strain of TMV often contain many virions, while chloroplasts from tissue infected with the U1 strain of TMV contain few, if any, virions (Shalla 1969). However, there is little difference in the symptoms caused by these strains (Siegel and Wildman 1954).

It has been suggested that coat protein plays a role in the development of disease symptoms in chloroplasts. There can be an association of TMV with chloroplasts. In infections with some TMV strains, pseudovirions (Shalla 1969; Shalla *et al.* 1975) consisting of viral coat protein encapsulating chloroplast RNA (Rochon and Siegel 1984) have been observed in chloroplasts. More recently, both TMV coat protein (Reinero and Beachy 1986, 1989) and TMV RNA (Schoelz and Zaitlin 1989) have been isolated from Percoll-purified chloroplasts. In chloroplasts isolated from spinach infected with TMV (Hodgson *et al.* 1989), it has been shown that coat protein is associated with photosystem II complexes in the thylakoids. Further, a ubiquitinated coat protein has been found accumulated in chloroplasts (Dunigan *et al.* 1988). Also, TMV coat protein and the large subunit of RuBPCase have an immunological cross-reactivity and some amino acid sequence homology (Dietzgen and Zaitlin 1986). Our data suggest that some of the gold particles observed in chloroplasts in our study, and the study of Hills *et al.* (1987), are due to antibody binding to RuBPCase in the chloroplasts.

The appearance of chlorotic symptoms on tobacco plants infected with TMV involves two separate systems. The appearance of chlorosis associated with mosaic symptoms on systemically infected leaves involves the prevention of

Table 2. Number of gold particles observed in chloroplasts of healthy tissue and tissue infected by wild-type tobacco mosaic virus[a]

Tissue type	Anti-cp[b] antibody	Anti-cp+[c] antibody	Preimmune serum
Control	24.44 ± 3.82[d]	3.10 ± 0.41	4.88 ± 1.32
Wild type	38.68 ± 8.7	4.43 ± 0.76	5.78 ± 1.64

[a] The number of gold particles per square micrometer of chloroplast surface area.

[b] Coat protein.

[c] Serum was preabsorbed with coat protein.

[d] Mean ± the standard deviation.

normal chloroplast development in expanding leaves by TMV. Although coat protein has been implicated in this process (Reinero and Beachy 1986, 1989) and has been found associated with photosystem II in chloroplasts from systemically infected leaves (Hodgson *et al.* 1989), it has also been shown (Culver and Dawson 1989) that coat protein is not absolutely required for the development of mosaic symptoms. In contrast, the appearance of chlorotic symptoms on directly inoculated leaves involves the degradation of fully developed chloroplasts in fully expanded leaves. Although TMV U1 coat protein does not induce chlorotic symptoms and chloroplast alterations in inoculated leaves, other tobamoviruses do induce chlorotic symptoms. In this study, we have shown that specific mutations in the coat protein gene of TMV U1 induce degradation of fully developed chloroplasts in inoculated leaves. The observation that the mutant coat proteins accumulate outside the chloroplasts rather than inside the chloroplasts suggests that coat protein exerts its influence from outside the chloroplast. We believe that while altered coat proteins can enter chloroplasts in infected cells, they exert their effects through subversion of the cytoplasmic machinery for protein synthesis and transport.

A number of important chloroplast proteins are encoded in the nucleus and synthesized on cytoplasmic ribosomes (for review, see Dyer 1984). The simplest way in which coat protein could interfere with normal chloroplast function is to prevent these proteins from entering the chloroplasts. We have shown that a viral protein accumulates in the cytoplasm of infected cells, and a second viral protein, the 126-kDa protein necessary for virus replication (Ishikawa *et al.* 1986), has been shown to accumulate in X-bodies (Hills *et al.* 1987; Wijdeveld *et al.* 1989). Dark-staining bodies have previously been observed (Kolehmainen *et al.* 1965) in leaves inoculated with the *flavum* strain (Melchers 1940), a spontaneous coat protein mutant (Aach 1958). These dark-staining bodies are similar to those we have observed, although they were not identified as containing coat protein. It is possible that these accumulations also contain other proteins including chloroplast proteins which could result in chloroplast disruption.

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