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Mutant Coat Protein of Tobacco Mosaic Virus Induces Acute Chlorosis in Expanded and Developing Tobacco Leaves

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The production of a mutated coat protein of tobacco mosaic virus caused acute (bright yellow) chlorosis in both expanded and developing tobacco leaves. In systemically infected tobacco leaves, synthesis of coat protein was not necessary for induction of the classic light green/dark green mosaic symptoms. However, the appearance of a bright yellow mosaic in leaves systemically infected with the mutant virus was due to synthesis of the mutant coat protein. This symptom resulted from the addition of bright yellow chlorosis, associated with the synthesis of mutant coat protein, to the normal light green/dark green mosaic symptoms that were caused by some viral product other than the coat protein. Electron microscopy of tissue from the yellowed areas in systemically infected leaves showed that normal chloroplast development was disrupted. These chlorotic areas contained only immature chloroplasts. Protein aggregates similar to the coat protein bodies previously seen in expanded leaves inoculated with chlorosis-causing coat protein mutants were also observed in acute chlorotic tissue of systemically infected leaves. These findings show that the combination of bright yellow chlorosis and normal mosaic symptoms on nascent leaves result from two separate virus-host interactions.

One of the most frequent plant responses to viral infection is chlorosis. This abnormal leaf tissue is a result of the disruption of cellular homeostasis which, in turn, disrupts chloroplast development, maintenance, and/or function. Viral-induced chlorosis can occur in both developing leaves and fully expanded leaves. Chlorosis in developing leaves is often displayed as a mosaic or mottled pattern of light green or yellow tissue, with abnormal chloroplasts, and dark green areas, with normal chloroplasts. In contrast, chlorosis in expanded leaves often begins as yellow spots at the infection sites, and then spreads uniformly as the virus spreads throughout the leaf. Chlorosis in infected developing leaves results from the abnormal development of chloroplasts in new leaves (Matthews 1981), whereas expanded leaves have fully developed chloroplasts at the time of infection.

It is not clear whether chlorosis on developing leaves and on fully expanded leaves is the result of similar or separate virus-host interactions. Several naturally occurring tobacco mosaic virus (TMV) mutants that cause chlorosis on directly inoculated, expanded leaves have been isolated. These include the *flavum* strain ([Melchers 1940], a spontaneous coat protein mutant [Aach 1958]) and PV 223, a yellow mosaic strain of TMV isolated from *Nicotiana glauca* R. Graham growing on the island of Grand Canary (McKinney 1929). Additionally, deletions in the coat protein open reading frame (ORF) result in TMV mutants that induce chlorotic responses in inoculated leaves similar to those induced by the naturally occurring mutants described above (Dawson *et al.* 1988). It has previously been observed that a correlation exists between the accumulation of unassembled TMV coat protein and the appearance of yellowing in tobacco leaves (von Sengbusch 1965; Jockusch and Jockusch 1968; Lindbeck *et al.* 1991). Chlorosis results from the degradation of fully developed chloroplasts in the leaves (Lindbeck *et al.* 1991).

The induction of classic light green/dark green mosaic symptoms in developing leaves has been associated with the accumulation of coat protein in chloroplasts (Reinero and Beachy 1986, 1989; Hodgson *et al.* 1989). However, recent genetic evidence has shown that coat protein is not responsible for the development of mosaic symptoms in *N. sylvestris* Spegs. & Comes (Culver and Dawson 1989). Mutants of TMV that fail to produce coat protein induce normal mosaic symptoms. Also, transgenic plants expressing TMV coat protein (Abel *et al.* 1986; Culver and Dawson 1991) do not develop mosaic symptoms. Further, mutations in the TMV genome that cause attenuation of mosaic symptoms have been mapped to the open reading frame encoding the 126/183-kDa replicase proteins (Nishiguchi *et al.* 1985; Holt *et al.* 1990), suggesting that the function of these proteins is correlated with the appearance of mosaic symptoms.

In this paper, we examine the role of a chlorosis-inducing coat protein mutant (TMV cp 35-5) in the development of symptoms in nascent and fully expanded tobacco leaves. We present genetic evidence that synthesis of mutant coat protein caused the appearance of bright yellow symptoms in inoculated expanded leaves. We also show that synthesis of this mutant coat protein induces bright yellow mosaic symptoms in developing leaves. The yellow mosaic symptoms associated with a systemic infection by mutant cp 35-5 are the result of an additive process: the intense yellow chlorosis, directly correlated with the presence of the mutant coat protein polypeptide, added to the normal mosaic symptoms induced by TMV that is not caused by the coat protein. Together, these findings indicate that the development of a bright yellow chlorosis on fully expanded leaves and normal mosaics in developing leaves are the result of two separate virus-host interactions.

MATERIALS AND METHODS

Virus and plants. TMV [-CP] (Culver and Dawson 1989) is a mutant of wild-type TMV (TMV 204; Dawson *et al.* 1986) in which the coat protein ORF start codon is altered from AUG to AGA (Culver and Dawson 1989). TMV cp 35-5 (Dawson *et al.* 1988) is a mutant of wild-type TMV with a deletion of nucleotides 5841–6055 (nucleotide numbering is according to Goelet *et al.* 1982) in the viral genome, resulting in deletion of amino acids 43–114 of the coat protein polypeptide. TMV nc 35-5, a mutant with the coat protein start codon changed to AGA, was created by substituting nucleotides 999–5781 from pTMV [-CP] into pTMV cp 35-5. Mutants were maintained as cDNA clones in pBR322 from which infectious *in vitro* transcripts can be produced (Dawson *et al.* 1988).

Virus was propagated in *N. tabacum* L. 'Xanthi' grown in a greenhouse and maintained in plant growth chambers at 25° C, with a 12-hr photoperiod and a photon flux density of 245 $\mu E \cdot m^{-2} \cdot s^{-1}$ at leaf height, after inoculation, unless stated otherwise.

Protein extraction, SDS-PAGE, and western blotting. Proteins were extracted from leaves 7 days after inoculation using the method of Godefroy-Colburn *et al.* (1986) as modified by Lehto *et al.* (1990). The proteins were subjected to western immunoblot analysis to detect TMV-encoded 126-kDa protein and 17.5-kDa coat protein using rabbit antisera to these proteins, as described by Lehto *et al.* (1990).

RNA isolation and sequencing. Total RNA was extracted from leaves 7 days after inoculation using the guanidine hydrochloride method of Logemann *et al.* (1987). After ethanol precipitation, the RNA pellet was washed three times with 70% ethanol to remove any remaining guanidine hydrochloride, dried, and resuspended in sterile water. Viral RNA was sequenced using the method of Zimmern and Kaesberg (1978).

Electron microscopy of systemically infected leaf tissue. Leaves systemically infected with wild-type TMV, cp 35-5, and nc 35-5 were sampled 7 days after the appearance of symptoms. The tissue was fixed, embedded, sectioned, and stained as previously described (Lindbeck *et al.* 1991). Immunolocalization of TMV coat protein was carried out as previously described (Lindbeck *et al.* 1991). Sections were viewed in a Philips EM 400 transmission electron microscope.

RESULTS

Chlorosis in inoculated, expanded tobacco leaves was caused by mutant coat protein synthesis. The TMV coat protein mutant TMV cp 35-5 produced copious amounts of the altered coat protein that was correlated with acute yellowing symptoms in inoculated leaves (Dawson *et al.* 1988; Lindbeck *et al.* 1991). To examine whether the production of mutant coat protein was directly responsible for chlorosis and chloroplast disorganization of expanded tobacco leaves infected with this mutant, the start codon of the coat protein ORF in pTMV cp 35-5 was removed, and the symptoms induced by the resulting mutant (TMV nc 35-5) were compared with the symptoms induced by TMV cp 35-5. Approximately 4 days after inoculation of the Xanthi tobacco plants with infectious RNA transcripts from pTMV cp35-5, chlorotic spots were visible in the inoculated leaves (Fig. 1A). These spots expanded radially with time to cover the leaf. In contrast, leaves inoculated with transcripts from pTMV nc35-5 exhibited no symptoms 7 days after inoculation (Fig. 1B), or later. The infectivity of RNA transcripts of both mutants was confirmed by their ability to produce local lesions on Xanthi-nc tobacco 2 days after inoculation. To confirm that nc 35-5 retained the altered start codon, total RNA was extracted from inoculated Xanthi tobacco leaves 7 days after inoculation, and the viral RNA coat protein gene was sequenced. Wild-type RNA and TMV cp 35-5 RNA extracted from inoculated leaves were used as controls. TMV nc 35-5 retained the AGA mutation of TMV [-CP] (data not shown), and no other variations were seen in the coat protein gene in a 64-base region downstream from the altered start codon.

Both mutants replicated efficiently in Xanthi tobacco leaves as demonstrated by western immunoblot analysis of proteins extracted from inoculated leaves using antisera to TMV 126-kDa replicase protein and coat protein. The replicase protein was present in leaves inoculated with both TMV cp 35-5 and TMV nc 35-5 at similar concentrations (Fig. 2A, lanes 1 and 2). However, coat protein was present only in leaves inoculated with TMV cp 35-5 (Fig. 2B, lanes 1 and 2). These results demonstrate that mutant coat protein synthesis was required for the induction of chlorotic symptoms on directly inoculated, expanded leaves. (The weaker reaction of coat protein antiserum with the TMV cp 35-5 coat protein band compared to wild-type coat protein on western blots is caused by reduced affinity for the TMV cp 35-5 polypeptide because of the reduced number of epitopes in the deleted coat protein [Dawson *et al.* 1988]).

Mosaic symptoms in developing tobacco leaves were not correlated with coat protein accumulation. It has previously been shown (Culver and Dawson 1989) that mosaic symptoms identical to those induced by wild-type TMV developed when the coat protein-less mutant TMV [-CP] moved into and infected apical cells of *N. sylvestris* plants. The mosaic symptoms produced by TMV [-CP] were slow to develop because it took 9–12 wk for the mutant to infect the upper leaves. TMV [-CP], which does not form virions, moves efficiently from cell to cell but inefficiently over long distances. The time required for mosaic symptoms to develop in nascent leaves of *N. sylvestris* after inoculation of an expanded leaf may reflect the time required for the virus to move the distance from the site of infection in the inoculated leaf to the apex via cell-to-cell transport. Of the numerous plant species infected with this mutant, mosaic symptoms occurred only in *N. sylvestris*, which may be due to the relatively short distance between the inoculated leaf and the shoot apex.

To examine whether TMV mutants incapable of producing coat protein would induce mosaic symptoms in other plants if the apex became infected, and whether this occurs with different coat protein-deficient mutants, Xanthi tobacco plants were inoculated with wild-type TMV (TMV 204) and two different mutants that were altered to be unable to produce coat protein (TMV [-CP] and TMV nc 35-5). In contrast to *N. sylvestris*, Xanthi tobacco shoots

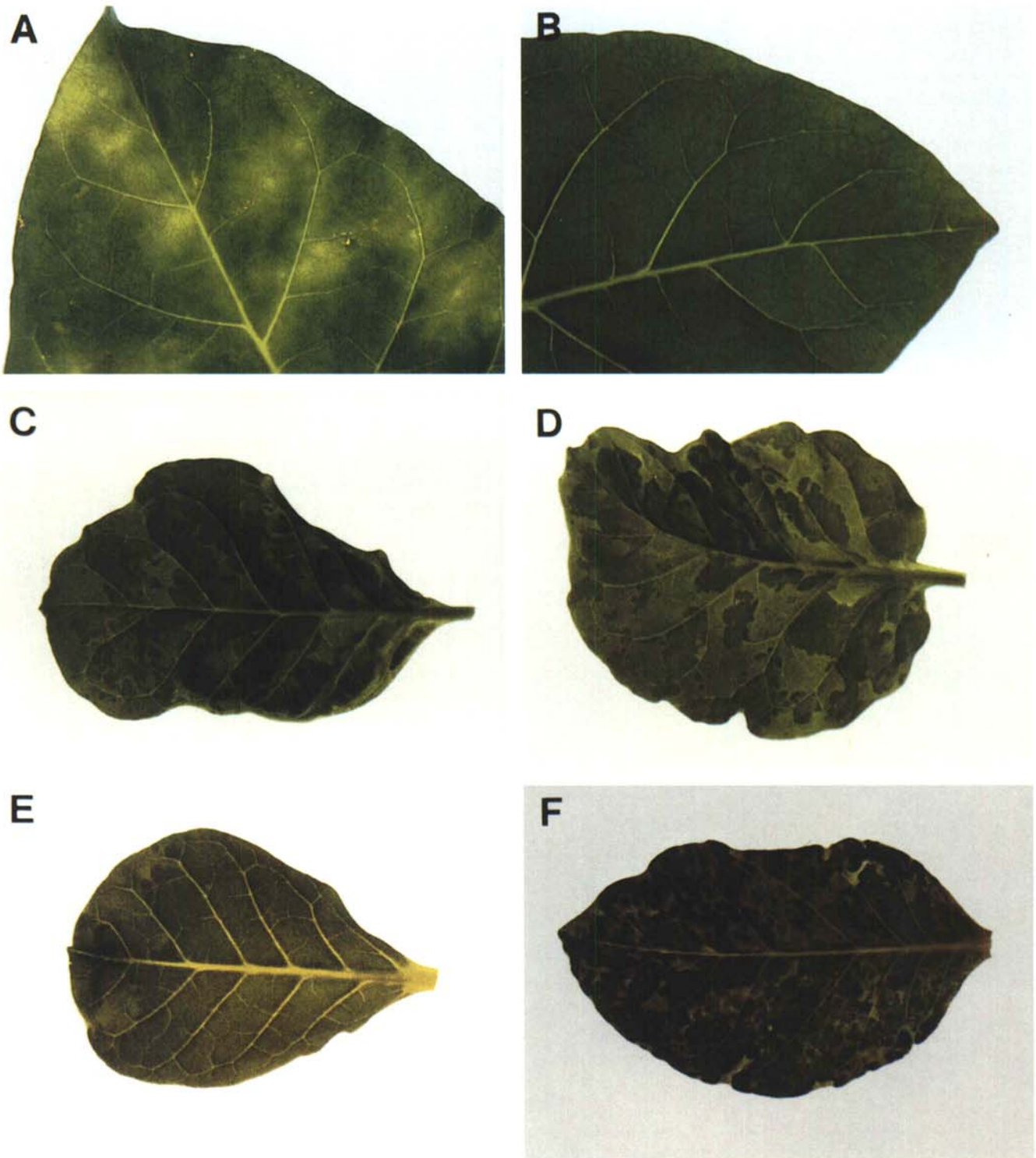

Fig. 1. Symptomatology of wild-type tobacco mosaic virus (TMV) and coat protein mutants on inoculated and/or systemically infected tobacco (*Nicotiana tabacum* 'Xanthi') leaves. **A,** Inoculated leaf infected with TMV cp 35-5; **B,** inoculated leaf infected with TMV nc 35-5; **C,** noninoculated leaf systemically infected with wild-type TMV showing mosaic symptoms; **D,** noninoculated leaf systemically infected with TMV [-CP] showing mosaic symptoms; **E,** noninoculated leaf systemically infected with TMV nc 35-5 showing mosaic symptoms; and, **F,** noninoculated leaf systemically infected with TMV cp 35-5 showing bright yellow mosaic symptoms.

grew so quickly that TMV [-CP] and TMV nc 35-5 mutants did not move into the shoot apex and systemic infection did not occur. To overcome this problem, two inoculation protocols were devised. First, transcripts of pTMV [-CP] and pTMV nc35-5 were inoculated directly on to the apices using a sterile toothpick wrapped with a small amount of cotton wool. The plants were then placed in a low-light growth room (photon flux density = 25 $\mu E \cdot m^{-2} \cdot s^{-1}$). Second, transcripts of pTMV [-CP] and pTMV nc35-5 were injected into the base of a leaf petiole and the plants placed in the low-light growth room. After 1 wk the leaf was removed by cutting the petiole above the injection site, and the stipules were allowed to develop into leaves in the low-light growth room.

Approximately 2 wk after inoculation of the apex with either TMV [-CP] or TMV nc 35-5, light green/dark green mosaic symptoms were observed on the emerging leaves (Fig. 1). The mosaic symptoms on the apically infected, emerging leaves of TMV [-CP]-inoculated (Fig. 1D) and TMV nc 35-5-inoculated (Fig. 1E) tobacco plants appeared that were identical to those obtained with wild-type TMV (Fig. 1C). The leaves that developed from the stipules developed mosaic symptoms only at the base of the leaves (data not shown), indicative of the slow movement of the viral mutants.

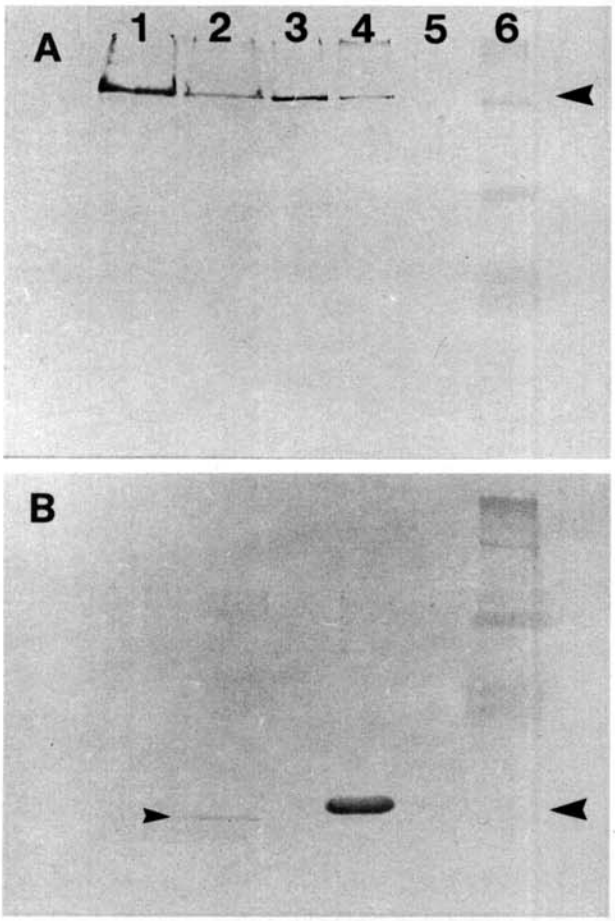

Fig. 2. Western blot analysis of tobacco mosaic virus (TMV) 126-kDa replicase protein and coat protein in infected tobacco leaves. Immunoblots of proteins extracted from leaves inoculated with TMV nc 35-5 (lane 1) or TMV cp 35-5 (lane 2), and from noninoculated leaves systemically infected with TMV [-CP] (lane 3) or wild-type TMV (lane 4) were incubated with **A,** rabbit anti-126-kDa antiserum, or **B,** rabbit anti-coat protein antiserum, followed by goat anti-rabbit IgG-alkaline phosphatase conjugate. Lane 5 contains proteins from a mock-inoculated leaf; lane 6 contains prestained molecular weight markers used as a guide for the rate of migration of the proteins (cannot be used to determine molecular weight) in the 8% acrylamide gel. The large arrowhead in A indicates the position of the 126-kDa protein band. The large arrowhead in B indicates the position of the wild-type coat protein band. The small arrowhead indicates the position of the TMV cp 35-5 coat protein band.

To confirm that the mosaic symptoms resulted from the coat protein-less mutants, noninoculated leaves with mosaic symptoms from tobacco plants inoculated with TMV [-CP], TMV nc 35-5, or wild-type TMV were ground in liquid nitrogen and resuspended in water. After sitting at room temperature for 1 hr, infectivity was assayed on Xanthi-nc tobacco. Local lesions appeared only on those plants inoculated with extract from wild-type TMV-infected leaves. The viral genomes of free RNA TMV mutants like TMV [-CP] and TMV nc 35-5 are rapidly degraded in leaf extracts at room temperature, whereas wild-type TMV particles are resistant to RNA degradation. The absence of lesions on the Xanthi-nc tobacco plants inoculated with TMV [-CP] and TMV nc 35-5 extracts demonstrated the lack of stable virions in the TMV [-CP]-infected or TMV nc 35-5-infected leaves and showed that the mosaic symptoms were not caused by a wild-type TMV infection caused by reversion or contamination.

Proteins were extracted from mosaic leaves infected with mutant TMV [-CP] or wild-type TMV as described above and subjected to western immunoblot analysis using antisera to TMV 126-kDa replicase protein and coat protein. The results (Fig. 2) showed that while TMV [-CP] and wild-type TMV produced similar levels of replicase proteins in mosaic leaves (Fig. 2A, lanes 3 and 4), coat protein was present only in mosaic leaves infected with wild-type TMV (Fig. 2B, lanes 3 and 4). Similar results were obtained with mosaic leaves infected with TMV nc 35-5 (data not shown). These results indicate that, as in *N. sylvestris*, coat protein is not required for the appearance of mosaic symptoms in systemically infected *N. tabacum* leaves and that mutations within the coat protein gene had no effect on the induction of mosaic symptoms.

Acute chlorotic symptoms in developing leaves can result from coat protein accumulation. In contrast to systemic infections of wild-type TMV and the mutants TMV [-CP] and nc 35-5, which resulted in a mosaic pattern of light green and dark green areas, systemic infections of cp 35-5 on *N. sylvestris* and *N. tabacum* resulted in a mosaic pattern of bright yellow areas mixed with light green and dark green areas. To determine if this bright yellow mosaic results from the presence of the mutant coat protein, we inoculated Xanthi tobacco with infectious transcripts of pTMV cp 35-5 and pTMV nc 35-5 on expanded leaves and maintained the plants in a low-light growth room (photon flux density = 25 $\mu E \cdot m^{-2} \cdot s^{-1}$). Two weeks after inoculation, the stems of the infected plants were severed immediately above the inoculated leaves. Mosaic symptoms were observed on the emerging leaves approximately 2 wk after severing the stems. The mosaic pattern observed on leaves systemically infected with nc 35-5 (Fig. 1E) was similar to the mosaic seen on leaves systemically infected with wild-type TMV

and TMV [-CP] (compare Fig. 1E with 1C and 1D). The mosaic pattern on the leaves systemically infected with cp 35-5 consisted of dark green symptomless areas alternating with bright yellow areas across the leaf (Fig. 1F). Proteins were extracted from cp 35-5 and nc 35-5 systemically infected leaves and were analyzed by western immunoblot analysis using antisera to TMV 126-kDa replicase and coat proteins. The results showed that, while both viruses replicated in the infected leaves, a coat protein-related polypeptide was present only in leaves infected with cp 35-5 (data not shown). The development of intense yellow areas of the mosaic caused by cp 35-5 was caused by the synthesis of mutant coat protein in systemically infected leaves.

Electron microscopy of leaf tissue systemically infected with cp 35-5. In contrast to the plastids of the light green areas of a wild-type TMV-induced mosaic, which developed into essentially normal chloroplasts (Fig. 3A), the plastids in the yellow areas of a cp 35-5 mosaic showed little development past the proplastid stage (Fig. 3B). Plastids in the adjacent symptomless green tissue were ultrastructurally similar to a chloroplast from healthy tissue (compare Fig. 3C with 3D). We have previously shown that cp 35-5 causes significant degradation of developed chloroplasts in expanded, inoculated leaves (Lindbeck *et al.* 1991). In systemically infected tissue, mutant cp 35-5 prevented normal chloroplast development. We observed that the most common plastid modifications in light green tissue from mosaic leaves were the accumulation of numerous large osmophilic lipid bodies and large starch granules in affected chloroplasts (see Fig. 3A). The thylakoid system of these chloroplasts was apparently normal, although in some chloroplasts portions of the stromal thylakoid system were elongated to accommodate the large starch granules. A number of the chloroplasts exhibited a displaced thylakoid system similar to that observed by Esau and co-workers (see Fig. 113, in Esau 1968).

We have previously identified discrete protein aggregations within the cytoplasm of leaves inoculated with chlorosis-inducing coat protein deletion mutants (Lindbeck *et al.* 1991). Immunocytochemical localization of TMV coat

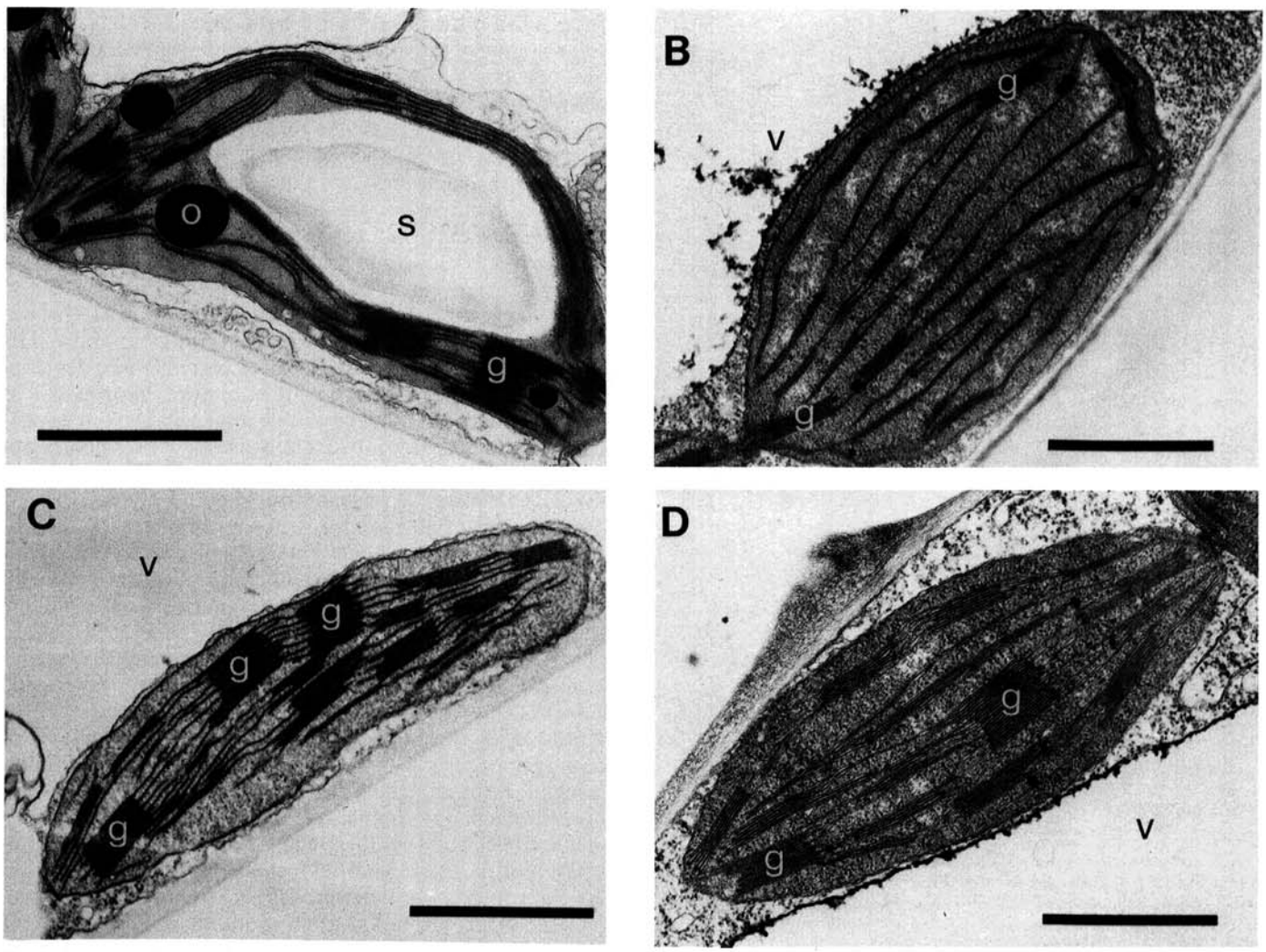

Fig. 3. Electron micrographs of tissue from noninoculated tobacco leaves systemically infected with wild-type tobacco mosaic virus (TMV) or mutant cp 35-5. **A,** A chloroplast from a light green area from a wild-type-induced mosaic; **B,** an immature chloroplast from a yellow area from a TMV cp 35-5-induced mosaic; **C,** a chloroplast from symptomless green tissue from a TMV cp 35-5-induced bright yellow mosaic; and, **D,** a chloroplast from a healthy leaf. Abbreviations are as follows: g = grana; o = osmophilic body; s = starch; v = vacuole. Bars = 1 μm.

protein in these tissues showed that these aggregates contained coat protein. Similar protein aggregates occurred in the bright yellow areas of mosaic symptoms caused by a systemic cp 35-5 infection (Fig. 4A). Immunolocalization of TMV coat protein in this tissue demonstrated that these protein aggregates also contained coat protein (Fig. 4B). These aggregates were not present in the symptomless green tissue adjacent to the bright yellow areas. We also determined the number of gold particles associated with the immature chloroplasts in yellow areas of cp 35-5-induced mosaics in thin sections probed with preimmune and anti-coat protein antisera. The results showed that similar numbers of gold particles per square micrometer are associated with the chloroplasts (preimmune: 29.93 ± 1.43; anti-coat protein: 26.31 ± 1.70; mean ± SE), demonstrating that the mutant coat protein did not accumulate in chloroplasts in the yellow areas of mosaics and further support our hypothesis that coat protein affects chloroplasts from outside the organelle.

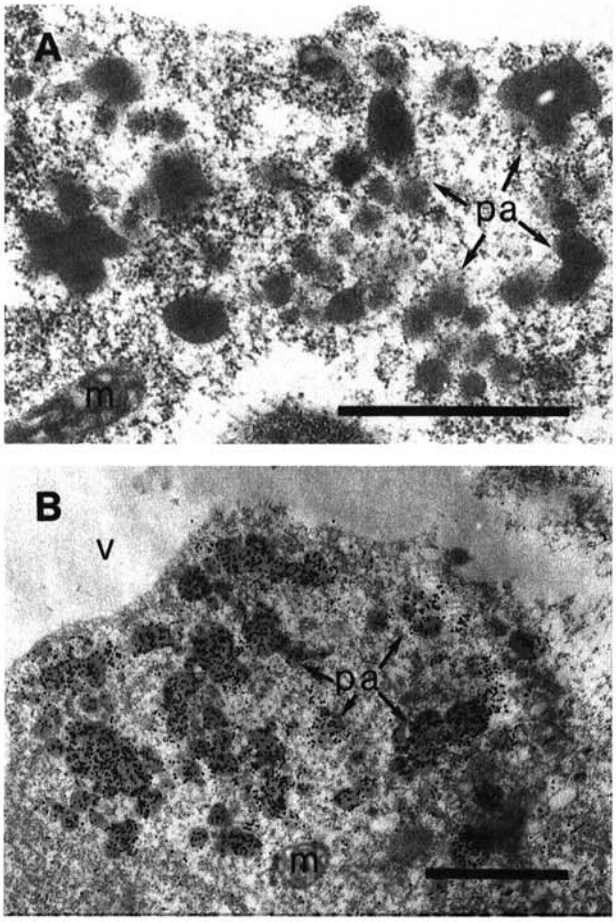

Fig. 4. Electron micrographs of tissue from a yellow area of a noninoculated tobacco leaf systemically infected with mutant cp 35-5. **A,** Micrograph of the discrete dark-staining protein aggregates observed in the yellow areas of TMV cp 35-5-induced bright yellow mosaics; **B,** micrograph of the dark-staining protein aggregates labeled with rabbit anti-TMV coat protein antiserum and protein A-gold probe demonstrating that these aggregates contain TMV coat protein. Abbreviations are as follows: m = mitochondria; pa = protein aggregates; v = vacuole. Bars = 1 μm.

DISCUSSION

The results presented here demonstrate that the development of disease symptoms in TMV-infected tobacco plants can result from two different virus-host interactions. Bright yellow chlorotic symptoms in inoculated expanded leaves and in systemically infected developing leaves were directly correlated with the accumulation of nonassembled mutant coat protein polypeptides. In contrast, coat protein was not the cause of the normal light green/dark green mosaic symptoms in infected developing leaves. However, while not directly causing mosaic symptoms, the mutant coat protein modified the symptoms produced. The symptoms resulting from a systemic cp 35-5 infection were the result of two types of interactions occurring in the developing infected leaves: The intense yellowing associated with cp 35-5 coat protein polypeptide was superimposed on the mosaic symptoms normally associated with systemic TMV infections.

A number of studies have examined ultrastructural changes caused by mosaic-inducing plant viruses (see Esau 1968, for review). In all cases, changes were observed in chloroplast ultrastructure in light green or light green-yellow tissue of the mosaic. Arnott *et al.* (1969) documented three general types of abnormal plastid, often existing in mixed populations, in TMV-infected tomato plants. Electron microscopy of chlorotic tissue from a leaf systemically infected with cp 35-5 showed abnormal plastid development. Ultrastructurally, the plastids were neither proplastids, the precursors of chloroplasts in meristematic tissue, nor fully developed chloroplasts. Plastids of this type, which contain little chlorophyll, are normally associated with newly emerged leaves (Thomson and Whatley 1980) and would normally develop into mature chloroplasts. This observation suggests that the normal development of proplastids into chloroplasts was prevented by cp 35-5 in developing leaf tissue. The presence of immature chloroplasts in this tissue accounts for the yellow appearance.

The induction of yellow areas by the coat protein mutant cp 35-5 in both inoculated and systemically infected leaves suggests that similar mechanisms may be involved in the development of acute chlorotic symptoms in these leaves. The development of acute chlorosis in nascent leaves involves the prevention of normal chloroplast development, whereas the development of acute chlorosis on expanded leaves involves the degradation of fully developed chloroplasts. We previously hypothesized that the simplest mechanism for the development of acute chlorosis in expanded leaves is that the mutant coat protein interferes with the synthesis and/or translocation of cytoplasmically produced chloroplast proteins (Lindbeck *et al.* 1991). A similar mechanism in nascent leaves could explain the disruption of chloroplast development. Interference with the synthesis of cytoplasmically synthesized chloroplast proteins or prevention of the translocation of these proteins into developing chloroplasts would effectively prevent any further development of proplastids into chloroplasts as these proteins are essential for normal chloroplast development (see Dyer 1984, for review).

Although mutant cp 35-5 is an artificially created mutant, it causes symptoms in plants that are similar to those caused

by several naturally occurring mutants, for example the *flavum* TMV strain and PV 223, and *in vitro* created point mutants (J. N. Culver and W. O. Dawson, unpublished). The naturally occurring mutants are characterized by the appearance of a yellow mosaic in systemically infected leaves and apparently exist as a component of the wild-type virus population in the field. McKinney (1929) observed that "One of the outstanding characteristics of the viruses of yellow mosaic is their ability to produce a general chlorosis on old foliage." The results presented with cp 35-5 demonstrate how a mutation in one viral gene product can greatly modify symptoms and suggests that a similar mechanism may explain why naturally occurring TMV variants induce yellow symptoms.

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