Reduced Photosystem II Activity and Accumulation of Viral Coat Protein in Chloroplasts of Leaves Infected with Tobacco Mosaic Virus

Antonio Reinerö and Roger N. Beachy

Plant Biology Program, Department of Biology, Washington University, St. Louis, Missouri 63130

ABSTRACT

We previously reported (A. Reinerö, RN Beachy 1986 Plant Mol Biol 6:291-301) that coat protein (CP) of tobacco mosaic virus (TMV) accumulates in chloroplasts of systemically infected leaves. To determine the significance of such interaction we examined electron transport rates in chloroplasts containing different levels of TMV-CP. Tobacco (Nicotiana tabacum L.) plants were infected with either a TMV strain inducing chlorosis or with a strain inducing mild symptoms, and both the accumulation pattern of TMV-CP inside chloroplasts as well as the rates of photosynthetic electron transport were followed. The CP of the TMV strain inducing chlorosis was detected inside chloroplasts 3 days after infection, and thereafter accumulated at a rapid rate, first in the stroma and then in the thylakoid membranes. On the other hand, the CP of the TMV strain that caused only mild symptoms accumulated in chloroplasts to lower levels and little CP was associated with the thylakoids. In vivo and in vitro measurements of electron transport revealed that photosystem II activity was inhibited in plants infected with the aggressive TMV strain while no reduction was observed in plants infected with the mild strain. The capacity of chloroplasts to synthesize proteins was equivalent in organelles isolated from healthy and virus-infected leaves. The possibility that a large accumulation of TMV-CP inside chloroplasts may affect photosynthesis in virus-infected plants by inhibiting photosystem II activity is discussed.

Plant disease resulting from virus infection is thought to be caused by metabolic changes in particular sets of plant cells during the establishment and replication of the virus (3, 9, 29). However, little is known about the specific biochemical and molecular processes affected in those cells. This is in part due to the fact that responses to virus infection can vary dramatically when different viruses or even closely related virus strains infect a given host. For instance, the response of tobacco plants to systemic infection by TMV can range from absence of disease symptoms to severe leaf distortion and chlorosis, depending on the TMV strain. Several studies have shown that, in general, cellular responses to virus infection are independent of the amount of virus produced in a cell (15, 29). Thus, the involvement of specific viral gene products with disease development has been proposed, although no conclusive evidence has been presented of such a role for any of the viral products (see Ref. 26 for a recent review).

Many studies have attempted to link effects on chloroplasts with symptom development in virus-infected plants (reviewed in Ref. 3), and although virus-like particles have been described in chloroplasts (22, 23), no correlation with disease has yet been established. In a previous report (18), we showed that TMV-CP molecules produced during infections by different TMV strains could be detected inside chloroplasts isolated from infected tobacco plants. In that study, the levels of TMV-CP inside the organelle seemed to be correlated with the severity of the chlorotic symptoms; lower amounts of TMV-CP were detected in organelles of plants not showing disease symptoms. However, at the time of the first report it had not been determined whether TMV-CP accumulation began before disease symptoms were observed, or whether chloroplast function was affected.

We report here the inhibition of PSII activity in chloroplasts accumulating large levels of TMV-CP, in both stroma and thylakoid membranes, in early stages of infection.

MATERIALS AND METHODS

Materials

Tobacco (Nicotiana tabacum cv Xanthi) plants, a systemic host of TMV, were grown in growth chambers with a photoperiod of 14 h and temperatures of 24°C (day) and 18°C (night). The light intensity at plant level was 20 W m⁻². The plants were fed daily, throughout the experiments, with a commercial N, P, K fertilizer ("Peters 15-16-17", W. R. Grace). Two strains of TMV, obtained from the American Type Culture Collection (ATCC), Rockville, MD, were used to inoculate tobacco seedlings. TMV-PV230, an aggressive

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2 Present address: Department of Biology, University of California, 405 Hilgard Ave., Los Angeles, CA 90024.

3 Abbreviations: TMV, tobacco mosaic virus; CP, coat protein; d.a.i., days after inoculation; MV, methylviologen; DBMIB, dibromothymoquinone; PQ, plastoquinone; DPC, 1,5-diphenyloxcarbazide; DHQ, durohydroquinone; Fₐ, initial level of Chl fluorescence after dark adaptation; Fₚ, maximal level of fluorescence achieved at Fₚ; Fₘ, variable fluorescence defined as Fₚ = Fₚ - Fₐ; Qₐ, primary electron acceptor of PSII.
strain which induces a pale-white/green chlorosis or, the 'masked' strain, PV42, which induces either very mild mosaic or no visible symptoms. Two expanded leaves of 3 to 4 leaf seedlings were inoculated with a suspension containing 50 μg TMV/mL. Analyses were carried out with expanding, first two systemically infected leaves located immediately above the inoculated leaves at different d.a.i. Symptoms of vein clearing were observed on plants infected with TMV-PV230 by 4 to 5 d.a.i. and well defined chlorotic symptoms were observed by 7 d.a.i.

**Chloroplast Isolation**

Intact chloroplasts were isolated from the 60/80% interface of a Percoll step gradient and treated with thermolysin as described (18). The intactness of chloroplasts was examined according to Lilley et al. (8) by the ferricyanide reduction method and by phase contrast microscopy. Envelope-free chloroplasts (thylakoid membranes) used in electron transport studies were isolated from the 21/45% interface of a Percoll gradient (1) and washed as described for intact chloroplasts. Stromal and thylakoid proteins were extracted from equivalent numbers of protease-treated intact chloroplasts (determined by Chl analysis [18]) as described by Roscoe and Ellis (20) except that 5.6 mM EDTA was included in the lysis medium to prevent degradation of the chloroplast proteins by residual thermolysin molecules.

**Protein Synthesis by Isolated Chloroplasts**

Conditions for *in organello* protein synthesis were those described by Nison and Jagendorf (14). Intact chloroplasts were resuspended in 350 mM sorbitol, 0.96 mM DTT, 50 mM Hepes-KOH (pH 8.0), 200 mM MgCl₂, 10 mM NaPO₄, and incubated in the presence of 26.7 μCi/mL (120 μCi/nmol) ³H-leucine and 200 mM each of the other common amino acids. The reactions were carried out for 45 min at 27°C, under a light intensity of 90 W m⁻².

**Electrophoresis, Protein Blotting, and Fluorography**

Electrophoresis of protein samples in 12.5% w/v SDS-PAGE was performed according to Laemmli (7). Immuno-blotting experiments were carried out as described (18). Fluorography of gels was performed according to Jen and Thach (6).

**In Vivo Chlorophyll Fluorescence Induction Kinetics**

The procedure for fluorescence induction and decay measurements were essentially as described by Miles (10). Leaf segments from TMV-infected plants were held in a hinged mask to expose a 4 × 10 mm area of the upper leaf surface. After 1 to 2 min of dark adaptation, leaf segments were exposed to an actinic light (≈300 W m⁻²) filtered through a blue filter (440 nm). The kinetics of fluorescence induction was measured at an angle of 45° relative to the leaf surface through a red-sensitive photomultiplier and recorded on a chart recorder. Three to five different, randomly selected sections of a systemically infected leaf were assayed for each time point. Analyses of fluorescence curves were done according to Miles (10), Miranda et al. (11), and Papageorgiou (16).

**Electron Transport Measurements**

Photosynthetic electron transport was estimated polarographically, using a membrane-covered Clark-type oxygen electrode, as O₂ uptake resulting from the aerobic reoxidation of MV or DBMIB. Reactions were run in a thermostatically controlled vessel at 25°C, illuminated by red actinic light at 280 W m⁻². The assay medium contained 100 mM sucrose, 40 mM Tricine-NaOH (pH 8.3), 3 mM MgCl₂, 1 mM ADP, 5 mM Na₂HPO₄, and envelope-free chloroplasts equivalent to 50 μg Chl/mL. Whole chain electron transport was measured in the presence of 0.1 mM MV as electron acceptor. PSII activity was determined in the presence of 15 μM DBMIB as electron acceptor; electron transport reactions with DBMIB as electron acceptor, although slow, reflect pure PSII activity because electron transfer between PSII and PSI is completely blocked by the DBMIB itself (4). Electron donation to the oxidizing side of PSII was measured in the presence of 0.5 mM DPC as electron donor and 0.1 mM MV as electron acceptor. Water oxidation was blocked by treating the thylakoid membranes with 0.8 mg Tris-HCl (pH 8.0) for 15 min (2). Electron flow between PQ and the PSI electron acceptor X was assayed using 0.5 mM DHQ as electron donor, in the presence of 5 μM DCMU, 200 units of superoxide dismutase and 0.1 mM MV. All electron transport reactions were run consecutively and repeated three times for each experiment using thylakoids obtained from a single chloroplast isolation procedure. A total of five experiments were carried out to obtain average rates of electron flow.

**RESULTS**

**Time-Course of TMV-CP Accumulation in Chloroplasts**

In a previous report (18) we showed that TMV-CP could be detected inside protease-treated intact chloroplasts isolated from TMV-infected tobacco plants. To further characterize the interaction between TMV-CP and chloroplasts, we examined the pattern of accumulation of TMV-CP from two TMV strains in chloroplasts of systemically infected tobacco leaves at different d.a.i. The two TMV strains, PV230 and the 'masked' strain PV42, replicate to similar levels in infected leaves (18, 19). However, PV230 induces chlorosis while PV42 causes no clearly visible disease symptoms, thereby making it possible to differentiate general cellular changes related to the infection process from changes specifically related to the expression of disease symptoms. The first two leaves systemically infected with TMV-PV230 show mild vein clearing by 4 to 5 d.a.i and clear chlorotic symptoms at 7 d.a.i.

Chloroplasts were isolated at 3, 5, 7, and 12 d.a.i. from the first two systemically infected leaves as well as from comparable leaves of mock-inoculated plants. Chloroplast suspensions containing 80 to 90% intact chloroplasts (as determined by the ferricyanide reduction method and by phase contrast microscopy [8]) were treated with thermolysin as described (18) to remove proteins adsorbed to the chloroplast envelope. Chloroplast proteins were then extracted and examined for the presence of TMV-CP by immunoblotting techniques using antibodies raised against purified CP from TMV-UV, as described earlier (18). The coat proteins of both TMV-PV230 and of TMV-PV42 immunoreacted with equal efficiency to the antibodies (not shown).
Figure 1. TMV-CP levels inside chloroplasts at different times after inoculation. Plastid proteins were extracted from equivalent numbers of chloroplasts (on a Chl basis) isolated from leaves infected with TMV-PV42 (A) or with TMV-PV230 (B) at the indicated d.a.i. Proteins were subjected to 12.5% w/v SDS-PAGE and CP was detected by immunoblotting techniques using antibodies raised against purified TMV-CP. Five µg of protein were loaded on each lane.

Figure 2. Accumulation of TMV-CP in stroma (A) and thylakoids (B) of chloroplasts isolated from leaves infected with TMV-PV230 (control) or TMV-PV24 (at different d.a.i.). Stromal and thylakoid proteins were extracted from equivalent numbers of chloroplasts (on a Chl basis) and treated as described in Figure 1. X-ray films on which bands were not overexposed were scanned with a Joyce-Loebl Chromscan 3 densitometer. Integration of peak areas in (A) and (B) is given as relative equivalent units.

In Vivo Chl Fluorescence Studies in Infected and Noninfected Leaves

Photosynthesis in virus infected leaves was first examined by carrying out in vivo studies of Chl fluorescence. Measurements of fluorescence induction kinetics is a technique that has been widely used to identify mutants with a genetically impaired photosynthetic apparatus (10, 16). Figure 3 shows Chl fluorescence induction curves from systemically infected leaves (first two leaves above those inoculated) as well as from comparable leaves of mock-inoculated plants, at different d.a.i. It can be seen that control plants and plants infected with TMV-PV42 for 7 or 12 d (Fig. 3, A–C) did not present significant differences in either F₀ (initial level of Chl fluorescence after dark adaptation; shown in Figure 3 by horizontal arrows) or in variable fluorescence (Fᵥ). Fᵥ is defined as Fₕ – F₀ (16) where Fₕ is the maximal fluorescence achieved at P (Fig. 3A) when the primary acceptor of PSI, a quinone called Q₀, is largely reduced. Similar results were observed with leaves of plants infected with TMV-PV42 for less than 7 d (not shown).

Plants infected with TMV-PV230 showed, on the contrary, high F₀ values and a progressive reduction in Fᵥ, from 3 to 7 d.a.i. (Fig. 3, D–E). Fluorescence curves in Figure 3, C and E, have been normalized to the Fᵥ value of control (Fig. 3A) to show more clearly the effect of TMV infection on Fᵥ. Changes in Fᵥ (normalized on Fₕ) throughout the infection process are quantified in Table 1. It can be seen that reduction in Fₕ/Fᵥ in leaves infected with TMV-PV230 is first detected between the third and fourth d.a.i., declining to about 4% of control by 12 d.a.i. Infection with TMV-PV42 did not affect Fᵥ (Table 1). Altered kinetics of in vivo fluorescence induction usually reflect altered rates of electron transport through PSII and/or PSI. To confirm such alterations(s), an in vitro analysis of electron transport in these chloroplasts was undertaken.

Electron Transport in Isolated Chloroplasts

Assays were carried out to measure electron transport reactions in thylakoid membranes isolated from TMV-infected leaves at 4 d.a.i. This time was chosen because leaves infected with TMV-PV230 are still green, but they have begun to show vein clearing which was used as an indication that the infec-
tion process had become established. Furthermore, in vivo Chl fluorescence analysis showed a 34% decline in PSII activity in TMV-PV230-infected plants (as determined by loss in $F_o$) after 4 d of infection (Table 1). This result allowed us to clearly differentiate electron transport rates between control (mock-inoculated) and TMV-infected samples. Infection with strain PV230, but not with the 'mock' strain, caused a reduction in the rate of whole chain electron transport ($H_2O \rightarrow MV$; Table 2). The reduction occurred prior to the site of electron donation by DHQ (see $H_2O \rightarrow DBMIB$ and DHQ $\rightarrow MV$; Table 2) thus implicating PSII as the site where perturbation of electron transport occurred. PSII activity was partially restored by an artificial donor (DPC) suggesting that the damage might be, in part, affecting the oxidizing side of PSII, the O$_2$-evolving system. Since the decline in whole chain electron transport (24% of control, $H_2O \rightarrow MV$; Table 2) closely paralleled the decline in PSII activity (27%, $H_2O \rightarrow DBMIB$; Table 2) the inhibition of the latter is probably responsible for the overall decrease.

### In Organello Protein Synthesis

Numerous studies have suggested that the synthesis of plastid-encoded polypeptides is greatly depressed in virus infected leaves (5, 12, 27). Since five of the eight polypeptides forming PSII are chloroplast-encoded (13), a reduction in chloroplast protein synthesis could explain a decline in electron flow through PSII (24). Thus, an analysis of the capacity of chloroplasts to synthesize proteins was carried out in plastids isolated from leaves infected with TMV-PV230. The analysis was done 4 d.a.i., the time at which we first detected a decline in electron transport. Our studies detected neither a reduction in total amino acid incorporation in these chloro-

### Table 1. In Vivo Chlorophyll Fluorescence in Control and TMV-Infected Leaves

<table>
<thead>
<tr>
<th>d.a.i.</th>
<th>Fluorescence</th>
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<tbody>
<tr>
<td></td>
<td>$F_o/F_n$,*</td>
</tr>
<tr>
<td>Control</td>
<td>1.29</td>
</tr>
<tr>
<td>TMV-PV230</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>1.17</td>
</tr>
<tr>
<td>4</td>
<td>0.85</td>
</tr>
<tr>
<td>7</td>
<td>0.06</td>
</tr>
<tr>
<td>12</td>
<td>0.05</td>
</tr>
<tr>
<td>TMV-PV42</td>
<td>7</td>
</tr>
<tr>
<td>12</td>
<td>1.26</td>
</tr>
</tbody>
</table>

* $F_o/F_n$ = normalized variable fluorescence ($F_v$).

### Table 2. Electron Transport in Thylakoid Membranes of Chloroplasts Isolated from Healthy and TMV-Infected Plants

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Electron Transport Rates*</th>
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<tbody>
<tr>
<td></td>
<td>TMV-PV230 infected$^a$</td>
</tr>
<tr>
<td></td>
<td>% control</td>
</tr>
<tr>
<td>$H_2O \rightarrow MV$</td>
<td>76 ± 3$^*$</td>
</tr>
<tr>
<td>$H_2O \rightarrow DBMIB$</td>
<td>73 ± 14$^*$</td>
</tr>
<tr>
<td>DPC $\rightarrow MV$</td>
<td>86 ± 3$^*$</td>
</tr>
<tr>
<td>DHQ $\rightarrow MV$</td>
<td>103 ± 9</td>
</tr>
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* Activity measurements (mean of 5 experiments) are expressed as % of control rates ± SD. Control rates for TMV-PV230 and for TMV-PV42 experiments, respectively (in μeq mg Chl$^{-1}$ h$^{-1}$): 625 and 635 in $H_2O \rightarrow MV$; 175 and 121 in $H_2O \rightarrow DBMIB$; 334 and 294 in DPC $\rightarrow MV$; 727 and 703 in DHQ $\rightarrow MV$.

$^a$ Four days after inoculation.

$^*$, $^{**}$ Significantly different from control, using original data, at $P = 0.001$ and $P = 0.01$, respectively, according to the paired $t$ test.
plasts nor an alteration on the pattern of protein synthesis (Fig. 4). Protein synthesis carried out in absence of light or in the presence of inhibitors of protein synthesis such as cycloheximide (10 mg/mL) or chloramphenicol (100 mg/mL) confirmed that the labeled proteins were synthesized in the chloroplasts and not in the cytoplasm (not shown).

DISCUSSION

Disease in virus-infected plants is considered to be initiated by interactions between a product of virus replication and one or more cellular components (29). To date, however, no consensus has been reached regarding the identification of the viral product(s) responsible for disease symptoms and the initial biochemical or molecular processes that are affected by the infection (9, 26, 29). In most cases, the lack of a consensus can be attributed to differences in the time following inoculation when analyses were done, and in the basis used for comparison (3). A more appropriate approach to identify the primary biochemical process affected by virus infection should include examining cellular functions before disease symptoms develop. Another approach is to use virus strains or mutants which do not induce disease symptoms. In the present study two closely related TMV strains were used; TMV-PV230, an aggressive strain which induces severe chlorosis around 7 d.a.i. in systemically infected tobacco (cv Xanthi) leaves, and TMV-PV42 or masked strain which does not induce clear disease symptoms in this host. These two strains replicate to produce equivalent levels of virus after infection (18, 19). Furthermore, the CP molecules of these strains are antigenically related to each other and have identical mol wt and isoelectrical points (18). However, as previously described, the CP of TMV-PV230 and of TMV-PV42 accumulate to different levels in chloroplasts isolated from systemically infected leaves (18).

In an attempt to establish a relationship between the accumulation of CP in chloroplasts with alterations in photosynthesis, which could explain the appearance of chlorosis in TMV-PV230 infections but not in the TMV-PV42 infections, we followed the accumulation of CP of both virus strains inside chloroplasts of systemically infected tobacco leaves. The results shown in Figures 1 and 2 revealed that while CP of both strains are present inside chloroplasts 3 d.a.i., the levels of CP of TMV-PV230 were two-fold the levels of CP of the masked strain. Thereafter, the difference increased to about 10 times as the CP of TMV-PV230 accumulated at a rapid rate in both the stroma and in the thylakoid membranes whereas the CP of TMV-PV42 accumulated to relatively low levels, especially in the thylakoids.

The analysis of photosynthetic rates in infected leaves revealed that electron flow through PSII was greatly reduced in chloroplasts containing large levels of TMV-CP, i.e. those isolated from TMV-PV230 infections. The analysis of in vivo Chl fluorescence showed increasing levels of Fv and decreasing values of Fm, compared to controls, in plants infected with TMV-PV230 but not in plants infected with TMV-PV42 (Fig. 3; Table 1). These alterations suggest that a biochemical lesion develops on the reducing side of PSII (16) in chloroplasts of TMV-PV230-infected leaves as the infection progresses. The results of in vitro electron transport measurements support the above interpretation (Table II) and suggest that the oxidizing side of PSII might also be affected (DPC → MV; Table 2). If so, this would indicate that the in vivo fluorescence curves represent the kinetics and intensity of Chl fluorescence emitted from two pools of PSII complexes: one comprising PSII that have only the reducing side altered and another in which both sides of PSII are affected. This difference among PSII complexes is possible given the asynchronous nature of virus infections in leaves (3). Studies using infected protoplasts where synchronous infections can be obtained (21) could be carried out to clarify this issue. As expected on the basis of the fluorescence studies, thylakoid membranes from TMV-PV42-infected leaves showed normal rates of electron flow through the entire electron transport chain (Table II).

Zaitlin and Jagendorf (28) reported alterations in photosynthetic electron flow in TMV-infected tobacco plants which, it was suggested, were caused by low levels of intracellular nitrogen as a consequence of virus multiplication. Our results, using expanding, systemically infected leaves from plants maintained under high nitrogen conditions (see “Materials and Methods”) and using two virus strains that replicate to similar levels (18, 19), indicate that the inhibition in electron transport through PSII must be caused by a factor other than low levels of intracellular nitrogen.

PSII is a highly organized chlorophyll:protein complex spanning the thylakoid membranes and is made up of at least five integral plastid-encoded polypeptides plus three extrinsic nuclear-encoded polypeptides (13). Hence, alterations in

Figure 4. Autoradiograph of polypeptides synthesized in chloroplasts isolated from healthy (lane 1) and TMV-PV230-infected (lane 2) leaves. 3H-Labeled polypeptides were separated by 12.5% w/v SDS-PAGE and visualized by fluorography. The position of mol wt markers (in kD) are indicated on the left margin. The position of known chloroplast polypeptides are marked in the right margin with arrows indicating: (a) 70 kD, PSII Chl a protein (PSI); (b) 60 kD, CF1-α-subunit; (c) 55 kD, LS RuBisCo; (d) 51 kD, PSII RC; (e) 49 kD, PSII RC; (f) 39 kD, Cyt f apoprotein; (g) 33 kD, precursor of 32 kD Q protein.
either Chl content and/or polypeptides forming PS II could 
adversely affect electron flow through PS II. We previously 
showed that Chl content per chloroplast as well as Chl a/b 
ratios are not affected by TMV infection (18, 19). In this 
study, the in vitro electron transport studies (Table II) 
suggested that the three extrinsic polypeptides are present 
in thylakoid membranes of infected leaves. Likewise, in orga-
nello protein synthesis showed that the synthesis of plastid-
encoded polypeptides is unaffected by virus infection when 
PS II is inhibited by about 30% (Fig. 4; Tables 1 and 2). Thus, 
seems unlikely that altered protein synthesis in chloroplasts 
could be responsible for the decline in PS II activity. Other 
possibilities are that alterations in the normal assembly of 
PS II (25) and/or accelerated turnover of PS II polypeptides 
(24) occur during TMV-CP230 infections.

The accumulation of CP in stroma and thylakoid mem-
blanes in TMV-CP230 infections at times when PS II activity 
gradually declining seems to indicate a relationship be-
tween these two events. One possibility is that the CP accu-
mulation inside chloroplasts is the result of chloroplast alterations 
caused by reduced electron flow through PS II. However, 
seems unlikely since CP is detected inside chloroplasts at 
times when electron flow through PS II is still 90% of control 
(Figs. 1 and 2; Table 1). We suggest instead that PS II activity 
may be inhibited by the large accumulation of CP of TMV-
CP230 in both stroma and in thylakoid membranes, a 
phenomenon not detected in symptomless infected leaves. High 
levels of CP inside chloroplasts could affect PS II by, for 
instance, becoming bound to PS II polypeptides thus mak-
ing them nonfunctional. Preliminary evidence has shown 
that chloroplasts from TMV-CP230-infected leaves contain 
lower levels (compared to control plants) on the 44 kD PS II 
reaction center polyepitide and present a new 63 kD poly-
eptide which immunoreact to both, 44 kD and TMV-CP 
antibodies (19).

Whether the accumulation of TMV-CP in chloroplasts and 
the inhibition of electron transport through PS II are actually 
involved in causing chlorotic symptoms in infected tobacco 
leaves remains unclear. Studies with other TMV strains or 
mutants that produce a variety of disease symptoms and the 
analysis of transgenic tobacco plants which express the TMV-
CP gene (17) should help to address this question.

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