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Coat protein-mediated resistance to TMV infection of *Nicotiana tabacum* involves multiple modes of interference by coat protein

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Abstract

Expression of tobacco mosaic virus (TMV) coat protein (CP) restricts virus disassembly and alters the accumulation of the movement protein (MP). To characterize the role of structure of transgenic CP in regulating virus disassembly and production of MP, we generated CPs with mutations at residues Glu50 and Asp77, located in the interface between juxtaposed CP subunits. In transgenic *Nicotiana tabacum* and BY-2 cells, three categories of coat protein-mediated resistance (CP-MR) levels were identified: wild-type CP-MR; elevated CP-MR; and no CP-MR. Mutant CPs that interfered with the accumulation of virus replication complexes conferred very high levels of protection to TMV, except by CP^{E50D} which provided no protection in the systemic host (Xanthi-nn) but high CP-MR in the local lesion host (Xanthi-NN). In transgenic BY-2 cells CP^{E50D} strongly reduced accumulation of MP:GFP. In general, there was a strong correlation between the capacity for CP to assemble to pseudovirions and CP-MR, while there was not strong correlation with packaging viral RNA and CP-MR. The data demonstrate that interference with one or more steps in virus infection and replication by wild type and mutant CP determine the degree of CP-MR.

Keywords

TMV; CP; tobacco; CP-MR; protein interaction; virus assembly; replication; MP

Introduction

The structural proteins of plant viruses have evolved to self-associate into complex macromolecules that are centrally involved in virus biology. The structural and biophysical properties of *tobacco mosaic virus* (TMV; type member of the genus *Tobamovirus*) coat protein (CP) have been widely used to study the role of macromolecular assembly in the biology of virus-host interactions, in particular in host resistance and disease development. The TMV

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genome encodes at least four proteins: the 126-kDa and 183-kDa replicase proteins, the 30-kDa cell-to-cell movement protein (MP), and the 17.5-kDa CP. The MP and CP are encoded by subgenomic RNAs that are co-terminal with the 3' end of virion RNA (Goelet et al., 1982). The CP can elicit specific host resistance in transgenic plants (coat protein mediate resistance; CP-MR). Other studies indicate that the three-dimensional structure of CP is critical to the control of these responses, either directly through specific structural motifs or indirectly via alterations in CP assembly and disassembly processes. [For more details see the review by Culver (2002)].

TMV particles are highly stable in vitro. Upon entry into a cell, however, disassembly of virions proceeds very rapidly via a process that involves virion destabilization that likely results from repulsion between carboxyl-carboxylate groups on amino acids (a.a.) at residues 50 (glutamic acid; Glu, E) and 77 (aspartic acid; Asp, D). These a.a. are located on the interface between adjacent and juxtaposed CP subunits. In the extracellular environment, the negatively charged carboxylate groups are stabilized by cations, such as Ca2+ ions or protons (Caspar and Namba, 1990). Structural studies of the virion revealed inter-subunit carboxylate interactions at three locations within the virus (Namba and Stubbs, 1986), including the interaction of Glu50 from one subunit with Asp77 of the subunit below (Figure 1).

Coat protein-mediated resistance (CP-MR) refers to the resistance of transgenic plants that produce CP to the virus from which the CP gene is derived (Powell-Abel et al., 1986). Despite extensive studies, the molecular mechanisms that govern CP-MR are not fully understood; furthermore, mechanisms of CP-MR are different for different viruses. In the case of CP-MR against TMV, the CP interferes with virus disassembly (reviewed by Bendahmane and Beachy, 1999). Bendahmane et al. (1997) showed that mutant CPs that lack the ability to aggregate failed to protect against TMV infection, whereas a CP with increased aggregation, eg., Thr42Trp (CP^{T42W}), provided higher levels of protection than did wild type (w.t.) CP. These results established a direct correlation between the capacity for CP self-assembly and CP-MR. These findings were later confirmed by Lu et al., (1998) using similar and also different mutant CPs expressed via a PVX transient expression vector. One of the mutants designed to remove repulsive inter-subunit carboxylate interaction between E50 and D77, namely mutant CPE50Q/D77N, was shown to stabilize CP–CP interactions and to provide significantly higher levels of CP-MR as compared to wild-type CP (Lu et al., 1998). Subsequently, Bendahmane et al. (2002) reported that w.t. CP can have a positive effect on the production of MP and, thereby, on virus replication and cell to cell movement. Other experiments showed that CP^{T42W} interferes with normal function of w.t. CP and has a negative effect on MP accumulation (Bendahmane et al, 2002) that reduces the formation of virus replication complexes (Asurmendi et al, 2004). This in turn restricts cell-cell spread of infection and increases the efficacy of CP-MR. A mutant of CP that did not assemble, CP^{T28W}, did not restrict production of VRCs (Virus Replication Complexes) or provide CP-MR.

In the present study, we applied knowledge of the atomic structure of TMV CP to generate mutant CPs that target the inter-subunit carboxyl-carboxylate interactions between Glu50 and Asp77 residues. The mutant CPs were then tested in virus infection and CP-MR assays. We show that CP-MR to TMV involves at least two independent mechanisms: (i) interference by transgenic CP with disassembly of challenge virus; and (ii) interference of transgenic CP with formation of replication complexes, thus interfering with virus movement. We propose that the degree of regulation of replication by aggregates of CP determines the relative strength of CP-MR that is conferred by w.t. and mutant CP.

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RESULTS

Effects of mutants of CP on virus biology

The known atomic structure of the TMV CP (Namba et al., 1989) was used to select for mutation amino acids (a.a.) that are important for CP subunit interactions. Interactions between carboxylate groups between Glu50 and Asp77 (Figure 1; located in RS and RR alpha-helices, respectively; Namba et al., 1989) result in repulsion of axially adjacent subunits. It was proposed that repulsion of these amino acids play an important role in TMV disassembly (Culver, et al., 1995; Lu et al., 1996). The removal or change of inter-subunit interactions is expected to enhance or reduce the stability of aggregates of CP molecules (depending upon the mutation) due to effects on the axially adjacent turns of the helix that forms as virus and virus-like particles (VLPs) are assembled. Ten amino acid substitutions were created to disrupt the specific carboxyl-carboxylate inter-CP subunit interactions between Glu50 and Asp77. The amino acids substitutions that created four types of mutations (see bellow) are expected to interfere with normal quaternary structure of CP subunits but not with its ternary structure.

Effects on virus assembly—Mutant CPs were examined for their ability to form VLPs following infection of tobacco leaves with virus constructs that contain mutant CP (Table 1). All virus mutants produced CP molecules indicating that mutations did not completely abolish virus replication or CP accumulation (data not shown). Electron microscopy studies revealed that all infections produced VLPs (Figure 2) indicating that the mutations did not affect self-assembly of the CP. However, most mutants failed to produce VLPs of the size of w.t. TMV (i.e., ~300 nm); this indicates that selected mutations affected virion or VLP stability.

Type 1 substitution mutations E50D and D77E were designed to alter hydrogen bonding patterns between side chains of adjacent a.a. while preserving the two negative charges in the axial inter-subunit interface, thus preserving electrostatic repulsion. However, because Glu has two -CH2 groups whereas Asp has one, mutant CP^{D77E} was expected to reduce the distance between side chain residues at 50 and 77, and increase the distance for the mutant CP^{E50D}. Both mutations were predicted to interfere with the packing and stability of VLPs. However, both mutations resulted in the formation of long VLPs, indicating that the protein has greater aggregation potential than w.t. CP, and was not limited to the length of viral RNA (Figure 2, panels G, H).

Type 2 substitution mutants E50Q or D77N were expected to remove the repulsive inter-subunit charges without altering side-chain hydrogen bonding. TMV-CP^{D77N} produced open disks and short stacks of disks in a non-helical structure, while TMV-CP^{E50Q} produced, as expected, long aggregates of CP (i.e., VLPs), (Figure 2, panels A,B).

Type 3 substitution mutants CP^{E50M} and CP^{D77A} are predicted to remove the repulsive inter-subunit charge and alter side-chain hydrogen bonding patterns. EM analysis of leaf extracts from plants infected with TMV-CP^{E50M} showed the presence of virion-like helical rods that are similar to those of the w.t. virus. However, particles formed by CP^{D77A} were flexuous and significantly shorter than those of w.t. TMV (Figure 2, panels I,J).

Type 4 substitution mutants E50R, E50K, D77R or D77K are predicted to alter the electrostatic repulsion site and induce inter-subunit salt-bridges that are expected to stabilize inter-subunit interactions and thus enhance the stability of the CP helical structure in virions and VLPs. Arg substitution mutants, TMV-CP^{E50R} and TMV-CP^{D77R}, as expected, produced very long VLPs (Figure 2, panels E,F), while the Lys mutants TMV-CP^{E50K} and TMV-CP^{D77K} produced VLPs of shorter length than the Arg mutants (Figure 2, panels C,D). The local structural changes introduced by differences in side chains of these basic amino acids may be responsible for differences in the aggregation of the mutant CPs. The side chain of Lys is hydrophobic, due to

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the four methylene groups and an amino group, whereas the Arg side chain consists of three hydrophobic methylene groups and the strongly basic gamma-guanido group. The gamma-guanido group of Arg and the amino group of Lys possess different ionization properties and positive charge distribution. VLPs formed by CP^{E50K} always co-purified with aggregates of material of unknown nature, presumably as a consequence of the mutation (Figure 2, panel C).

In order to determine whether mutant CPs form infectious virions we determined the ability of TMV carrying mutant CP to cause rapid systemic infection in *N. tabacum* Xanthi-nn. All mutant viruses, except TMV-CP^{E50D} and TMV-CP^{D77K}, systemically infected *N. tabacum* Xanthi-nn, at rates similar to, or slower than, w.t. TMV. (Table 1). Plants inoculated with TMV-CP^{E50K}, TMV-CP^{E50M}, TMV-CP^{D77E} or TMV-CP^{D77R} developed systemic symptoms by 8 to 10 dpi, similar to plants inoculated with w.t. TMV. Therefore, the particles formed by these mutant CPs are presumed to assemble with vRNA to provide systemic infection. Plants inoculated with TMV-CP^{E50R}, TMV-CP^{E50Q}, TMV-CP^{D77A} or TMV-CP^{D77N} developed symptoms after 25 dpi. Therefore, the infectious particles formed by these mutant CPs is presumed to be of low efficiency. Alternatively slow systemic spread may indicate lack of virion formation and nonvascular systemic spread. The delay in systemic infection may also be due to reduced cell-to-cell movement of viral RNA as observed with mutant CP^{T42W} (Bendahmane et al., 2002).

Effects of mutations on CP-MP in transgenic plants

Transgenic tobacco plants that express CP genes encoding mutations at E50 or D77 residues were developed. At least five independent transgenic lines were selected for each mutant based on accumulation of CP equivalent to accumulation of w.t. CP in *N. tabacum* Xanthi-nn line 3646 (Powell Abel et al., 1986), and N. tabacum Xanthi-NN line 748 (Nelson et al, 1987). CP-MR against TMV infection was assayed in homozygous transgenic lines and compared to CP-MR in lines 3646 and 748. TMV was applied at 0.1 μg/ml; this produced a mean of ~80 local lesions per leaf on non-transgenic *N. tabacum* Xanthi NN plants. We present the results of experiments with selected lines for each CP mutant; the data were consistent amongst the lines of each CP mutant tested.

CP-MR in Xanthi-nn transgenic lines

The percentage of Xanthi-nn plants that developed systemic disease symptoms were scored from 7 dpi, averaged, and compared with non-transgenic plants (0% protection) and plant line 3646. As shown in Figure 3, we observed three levels of protection.

1. Transgenic lines accumulating CP^{E50D} or CP^{D77K} (e.g., plant lines *e* and *j*, respectively) developed disease symptoms that were not different from non-transgenic plants. In these lines the onset and severity of disease symptoms and virus accumulation in upper systemically infected leaves were similar to non-transgenic plants (as analyzed in ELISA using anti-CP antibody; data not presented).
2. Transgenic lines accumulating CP^{E50K}, CP^{E50R} or CP^{D77N} (e.g., lines *a*, *b* and *c*, respectively) exhibited levels of CP-MR similar to that provided by w.t. CP in line 3646.
3. Plant lines accumulating CP^{E50Q} (line *a*), CP^{E50M} (line *d*), CP^{D77A} (line *a*), CP^{D77E} (line *h*) or CP^{D77R} (line *c*) did not develop symptoms when inoculated with 0.1 μg/ml of virus, whereas line 3646 showed a delay in symptom appearance. Lines nn-CP^{E50M}, nn-CP^{D77E} and nn-CP^{D77R} did not contain detectable virus in upper leaves at 23 dpi, based on ELISA. Lines nn-CP^{E50Q} and nn-CP^{D77A} showed very mild symptoms of infection and virus accumulation in the systemic leaves at 23 d.p.i., but provided higher protection to TMV infection than line 3646.

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CP-MR in Xanthi-NN transgenic lines—Transgenic Xanthi-NN plants which accumulated each of the mutant CPs were also developed, and five lines that accumulated CP levels equivalent to those in plant line 748 (contains w.t. CP) were selected for further study. Plants were inoculated with TMV at an inoculum concentration of 0.1 μg/ml, and the numbers of local necrotic lesions were determined, averaged, and compared with the number of lesions produced on non-transgenic plants (0% protection) and on line 748 plants (Table 1).

Little or no protection was exhibited in NN-CP^{D77K} lines and each plant line carrying this CP was equally as susceptible to infection as non-transgenic Xanthi-NN plants. This result was expected since CP^{D77K} did not provide protection in Xanthi-nn plants. Xanthi-NN plants expressing all other mutant CPs (except for CP^{E50D}) provided high levels of protection to TMV infection. However, there was not strong agreement between levels of protection in Xanthi-NN and Xanthi-nn transgenic lines. Based on these results, we grouped mutants into five classes of protection:

1. Mutants CP^{E50R} and CP^{D77N} provided protection equivalent to that provided by w.t. CP in Xanthi-nn lines, and provided high levels of protection in Xanthi-NN plants.
2. Mutants CP^{E50M}, CP^{E50Q}, CP^{D77A}, CP^{D77E} provided equally high levels of protection in Xanthi-nn and Xanthi-NN transgenic lines and levels of protection stronger than provided by w.t. CP. Some plant lines were equally as resistant as plant lines that contain CP^{T42W} (Bendahmane et al., 1997, 2002)
3. Mutant CP^{E50K} provided protection equivalent to that provided by w.t. CP in Xanthi-nn lines, but provided slightly lower protection in transgenic Xanthi-NN (90% to 96% compared to 99% protection in line 748).
4. Mutant CP^{D77R} provided stronger protection than w.t. CP in Xanthi-nn plants (equivalent to that in line nn-CP^{T42W}; Bendahmane et al., 1997) and lower protection than w.t. CP in Xanthi-NN.
5. Mutant CP^{E50D} provided no protection in Xanthi-nn plants but provided a high level of protection in Xanthi-NN.

CP-MR in transgenic BY-2 protoplasts—Earlier studies conducted in BY-2 protoplasts revealed that expression of CP^{T42W} reduced accumulation of MP and consequently reduced the formation of VRCs (virus replication complexes; Bendahmane et al, 2002; Asurmendi et al, 2004). To characterize virus replication in the mutant lines we followed the accumulation of replicase (Rep), MP and CP by ELISA following infection of protoplasts from BY-2 cells lines after inoculation with TMV RNA. The mutant CPs selected for this study represented different structural characteristics represented by the grouping described above. Lines BY-CP^{T42W} and BY-CP (w.t. CP) were included as controls and results are similar to those reported by Bendahmane et al, 2002). Protoplasts of cell lines BY-CP^{E50D}, BY-CP^{E50R}, BY-CP^{D77K}, and BY-CP^{D77R} were infected and aliquots were collected after 11, 16, 24 and 36 hpi: data are presented in Figure 4.

Cell lines BY-CP^{E50R} and BY-CP^{D77K} showed a similar pattern of accumulation of rep, MP and CP as the BY-CP line. In these lines the level of MP was significantly greater than in the non-transgenic line; similar results were observed with the level of replicase although the differences relative to the non-transgenic cell line were not as great as with MP. The amount of CP was greater in these cell lines when compared with the non-transgenic line in contrast with the results in BY-CP, which was not significantly different from the non-transgenic cell line.

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In contrast infection of protoplasts from BY-CP^{D77R} was similar to infection of cell line BY-CP^{T42W}. These two lines show a marked decrease of all three viral proteins when compared with the non-transgenic line, indicating reduction in virus replication (Bendahmane et al, 2002). The results observed in cell line BY-CP^{E50D} are more difficult to explain, as replication in this line is not as widely different than the non- transgenic line except at 36 hpi. It is worth noting that BY-CP^{E50D} is the most different line within the CP^{T42W} group with regard to accumulation of MP:GFP (see below). The accumulation of MP is indicative of significant virus replication but may indicate that regulation of MP production late in replication does not occur in presence of CP^{E50D}. This is also the only mutant that provides high level of CP-MR in Xanthi NN and low protection on the systemic host (Table 1).

Effect of mutant CPs on MP accumulation

We showed in earlier studies that CP^{T42W} interferes with cell to cell movement by directly affecting MP accumulation and/or function (Bendahmane et al., 2002). Glu50 is located on the RS alpha-helix of the CP, as is Thr42. Furthermore, ionic interactions between Glu50 and Asp77 occur in the same plane as does Thr42, i.e., in the axial interaction between two juxtaposed CP subunits. We therefore analyzed the effect of each mutation at Glu50 and 4 of the 5 mutations at Asp77 on MP:GFP fusion protein accumulation during infection to determine if changes in CP structure will affect accumulation of the fusion protein in infected protoplasts. Protoplasts from transgenic BY2 cells that express mutant or w.t. CP were infected with TMV-MP:GFP and the accumulation of MP:GFP was followed by fluorescence microscopy of each cell line at 10, 16, 24 and 36 hours post-infection (h.p.i). Since infection with TMV RNA overcomes CP-MR (Powell *et al*., 1986; Register and Beachy, 1988) we achieved a high proportion of infected protoplasts in these studies.

In non-transgenic BY2 cells infected with TMV-MP:GFP, the MP:GFP fusion protein accumulates in unique patterns at different times during the infection cycle (Heinlein et al., 1995). For the present study, the patterns of accumulation of MP:GFP can be grouped in three stages that reflect early, mid, and late stages of infection (Figure 5A). The results of the studies are presented in Figure 5B and, as shown, the progression of accumulation of MP:GFP follows three patterns into which each CP mutant can be placed.

The first group of mutant CPs comprises mutations at residue Glu50, e.g., CP^{E50K}, CP^{E50M} and CP^{E50R}. Expression of these mutant CPs have positive effects on MP:GFP accumulation that are similar to, but not identical with, results previously reported for w.t. CP (Bendahmane et al., 2002). In this group a high percentage of infected cells accumulate large fluorescent bodies that contain MP:GFP (such bodies are known to be virus replication complexes, VRCs; Kawakami et al, 2004) throughout the infection period 0-36 hpi. This result indicates that in these protoplasts VRCs continue to produce and accumulate MP:GFP or that the transition of MP:GFP to microtubules or degradation is negatively affected in these infections than in wild-type protoplasts. We conclude that MP:GFP continues to accumulate throughout infection in these cells; this is in agreement with data in Figure 4.

Group 2 includes cell lines producing CP mutants that do not have an effect on accumulation of MP:GFP; in these cell lines, MP-containing fluorescent structures accumulated as in infected non-transgenic BY2 protoplasts. In this group infection proceeds through the early and mid stages of infection in a now-familiar pattern (described by Bendahmane et al., 2002) and by late stages (36 hpi) the MP:GFP is associated with microtubules and relatively small fluorescent bodies. This group includes CP^{D77A}, CP^{D77E}, CP^{D77N} (all Asp77 mutants except CP^{D77R}). We conclude that these mutants do not affect the production or accumulation of flourescent MP:GFP-containing bodies in a positive or negative manner. Nevertheless, these CPs provide high levels of CP-MR in local lesion and systemic hosts (Table 1, and Figure 3).

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The third group of CP mutants includes CP^{E50D}, CP^{E50Q} and CP^{D77R}, each of which has a negative effect on MP:GFP accumulation similar to the effect of CP^{T42W} (Bendahmane et al., 2002). In protoplasts of cell lines that contain these mutants, MP:GFP accumulated in small bodies similar to those produced early in infection in non-transgenic protoplasts. Infections in these cells lines produced very few large VRCs and very few cells proceeded to mid- and late stages of infection. The data are in agreement with results of studies described in Fig. 4 showing that CP^{T42W}, CP^{E50D} and CP^{D77R} restrict production and/or accumulation of MP.

DISCUSSION

Earlier studies have shown that CP-MR against tobamoviruses is provided by accumulation of transgenic CP. Using mesophyll protoplasts from non-transgenic as well as from transgenic plants expressing w.t. CP, previous studies demonstrated inhibition of disassembly of TMV in cells expressing the CP (reviewed by Bendahmane and Beachy, 1999). We also showed a strong positive correlation between the ability of transgenic CP to self-aggregate to form VLPs and CP-MR (Bendahmane et al., 1997). Recently, we provided evidence that protection against CP-MR in this system is the cumulative effect of interference by transgenic CP with virus disassembly and accumulation of MP during virus infection. The latter effect is evident in a CP mutant CP^{T42W}, and reduces cell to cell virus movement of infection; Bendahmane et al., 2002).

To further characterize the mechanism of CP-MR we generated additional mutations in TMV CPs, and selected the site at the electrostatic repulsion site formed by Glu50 and Asp77 residues in adjacent subunits in the quaternary structure. Repulsion of subunits is apparently prevented by binding of divalent cations at this site (Namba and Stubbs, 1986), thus enabling assembly of virions and VLPs. Alterations in virion stability that take place in virus disassembly initiates infection and indicates a stability-switching mechanism that can respond to subtle changes in the surrounding environment. (Caspar 1963; Caspar and Namba, 1990). We generated mutants at each of these amino acid residues in order to interfere with Glu50–Asp77 interactions. Glu50 and Asp77 residues are located on the RS and RR helices within the CP three-dimensional structure, respectively (Figure 1). The objective was to remove this electrostatic repulsion site and increase the stability of interactions between axially adjacent CPs in the quaternary, but not the ternary structure of CP. As predicted, mutations affecting this site of inter-subunits interactions did affect the normal assembly of virions. Most mutant CPs formed long and/or flexuous VLPs, some of which are formed by stacked discs. (Figure 2).

We observed a variety of effects of the mutations on CP-MR in transgenic *N. tabacum* plants. CP^{D77K} did not provide resistance to TMV infection in transgenic Xanthi-NN or Xanthi-nn. This result was unanticipated since CP^{D77K} can assemble to VLPs. A possible explanation for the lack of CP-MR comes from the electron microscopy analysis of TMV- CP^{D77K} VLP, which shows that the CP^{D77K} is associated with unknown debris in plant extracts. This association may interfere with CP-MR by this mutant CP.

In the local lesion host Xanthi-NN, transgenic plants expressing CP^{E50D} provided 90% to 97% resistance to TMV infection. Resistance to infection per se (reflected in resistance in the local lesion host) was anticipated since CP^{E50D} produces VLPs. In contrast, this mutant conferred no CP-MR in the systemic host. The latter result was unexpected since CP^{E50D} reduced the production of MP and accumulation of MP:GFP in infected protoplasts (Figures 4, 5), conditions that would reduce cell-cell spread. However, a closer analysis of data in Figure 5 (Group 3) revealed that in cell line BY-CP^{E50D} there were higher numbers of large fluorescent bodies (VRCs) than in other cell lines in this group; this may increasing the likelihood of systemic infection. This result could explain the observation that resistance in these plant lines

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that contain CP^{E50D} gave high levels of CP-MR in some experiments and not in others. This is the only mutant in which variable results were observed.

CP^{D77N} provides protection similar to w.t. CP in Xanthi-nn (line 3646) and higher protection than w.t. CP protection in Xanthi-NN (line 748). The high stability of VLPs produced by TMV-CP^{D77N} indicates that CP^{D77N} interferes with virus disassembly and provides CP-MR equivalent to that provided by w.t. CP. In BY-CP^{D77N} cells the number of VRCs produced during mid-stage of infection is lower than in non-transgenic cells and significantly less than in BY-CP cells following infection with TMV-MP:GFP. Indeed, placing this mutant in either Group 1 or group 3 was not a clear decision. The negative effects of CP^{D77N} on VRC production may be at the origin of the high resistance in the local lesion host NN-CP^{D77N}.

Mutants CP^{E50Q}, CP^{D77R}, CP^{D77E} and CP^{D77A} provided very high levels of protection to TMV in transgenic Xanthi-nn as well as Xanthi-NN plants. Protection levels were similar to protection provided by CP^{T42W} (Bendahmane et al., 1997; 2002). Moreover, these mutant CPs reduced MP:GFP accumulation in infected transgenic BY2 cells. The data are in agreement with results of other mutants (above) and show that the cumulative effect of strong interference with virus disassembly and negative effects on MP accumulation in VRCs are necessary and responsible for high levels of CP-MR to TMV infection.

Mutant CP^{E50M} behaves like w.t. CP in all aspects studied here. Virions formed by CP^{E50M} were similar to those of w.t. TMV and systemic infection in non-transgenic plants was similar. , In transgenic protoplasts that express CP^{DE50M} the accumulation of MP:GFP following infection was similar to similar to protoplasts that produce w.t. CP. However, CP^{E50M} provided higher levels of CP-MR in Xanthi-nn and Xanth-NN plants than did w.t. CP. This indicates that CP^{E50M} may act through combinatorial action that involves interference with disassembly (as does w.t. CP) and a yet to be determined interference with infection by the challenge virus, thus representing another argument for the complexity of CP-MR.

These studies confirm that the role of TMV CP in regulating multiple steps in virus replication and in CP-MR is complex. Studies of mutant CPs such as those reported here are likely to form different conformational forms and intermediates in quaternary structure that are not characterized (see accompanying work by Asurmendi et al., 2007). Our data suggest that some of the transgenic conformers and/or intermediates of w.t. and mutants CPs impact one or more of the functions of CP to regulate virus infection, movement and replication.

Materials and Methods

TMV clones

TMV variants harboring the MP tagged with GFP (TMV-MP:GFP) or the CP tagged with GFP (TMV-CP:GFP) have been described (Heinlein et al., 1995; Bendahmane et al., 2002).

Mutant CPs and virus constructs

The TMV CP amino acids Glu50 and Asp77 were mutated to Asp, Gln, Arg, Lys or Met, and Asp77 was mutated to Glu, Asn, Arg, Lys or Ala using a PCR based site directed mutagenesis and cloned in the vector pKN2 as described (Bendahmane et al., 1997). The resulting intermediate plasmids are referred to as, pCP^{E50D}, pCP^{E50Q}, pCP^{E50R}, pCP^{E50K}, pCP^{E50M}, pCP^{D77E}, pCP^{D77N}, pCP^{D77R}, pCP^{D77K}, and pCP^{D77A}.

A DraI-KpnI fragment containing the mutated CP gene (TMV nucleotide 5708 to 6395; Goelet et al., 1982) from the above plasmids was ligated into an expression cassette that places each cDNA between the 35S promoter and the *nos* 3' terminator. The expression cassettes were then transferred into the vector pCGN1547 (McBride and Summerfelt, 1990). The resulting

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plasmids are referred to as pCG-E50D, pCG-E50K, pCG-E50M, pCG-E50Q, pCG-E50R, pCG-D77A, pCG-D77E, pCG-D77K, pCG-D77N or pCG-D77R.

cDNA clones harboring the mutant CPs from which infectious TMV RNA is produced were constructed by replacing the CP gene in the wild type TMV cDNA (U3/12-4; Holt and Beachy, 1991) with the mutant CP as described in Bendahmane et al. (1997). Plasmids thus produced are referred to as pTMV-CP^{E50D}, pTMV-CP^{E50Q}, pTMV-CP^{E50R}, pTMV-CP^{E50K}, pTMV-CP^{E50M}, pTMV-CP^{D77E}, pTMV-CP^{D77N}, pTMV-CP^{D77R}, pTMV-CP^{D77K}, and pTMV-CP^{D77A}.

Construction and analysis of transgenic plants

The plasmids pCG-E50D, pCG-E50K, pCG-E50M, pCG-E50Q, pCG-E50R, pCG-D77A, pCG-D77E, pCG-D77K, pCG-D77N or pCG-D77R were used to produce transgenic *N. tabacum* Xanthi-NN (a local lesion host of TMV) and Xanthi-nn plants (a systemic host of TMV) via *Agrobacterium tumefaciens* (Horsch et al., 1985). The regenerated kanamycin resistant plants (kan^r) were screened for CP accumulation using the polyclonal rabbit anti-TMV coat protein antibody in western immunoblot assays as described previously (Bendahmane et al., 1997). Plant lines are referred to as NN- or nn-CP^{E50D}, CP^{E50K}, CP^{E50M}, CP^{E50Q}, CP^{E50R}, CP^{D77A}, CP^{D77E}, CP^{D77K}, CP^{D77N}, CP^{D77R}, respectively. Plants that accumulated CP were self-pollinated and R1 plants and homozygous plants were identified and their R2 plant seedlings were used for all studies. Plant lines from amongst more than 15 lines containing each construct were selected for further study on the basis of accumulation of equivalent amounts of CP as determined by quantitative western immunoblot assays

Construction and analysis of transgenic BY2 cell lines

The plasmids pCG-E50D, pCG-E50K, pCG-E50M, pCG-E50Q, pCG-E50R, pCG-D77A, pCG-D77E, pCG-D77K, pCG-D77N or pCG-D77R were used to transform the tobacco BY2 cell line using *A. tumefaciens* mediated transformation. The regenerated kanamycin resistant cell lines (kan^r) were screened for CP accumulation using the polyclonal rabbit anti-TMV coat protein antibody in western immunoblot assay as above. Cell lines with high levels of CP were used for further studies. These lines are referred to as BY-CP^{E50D}, BY-CP^{E50K}, BY-CP^{E50M}, BY-CP^{E50Q}, BY-CP^{E50R}, BY-CP^{D77A}, BY-CP^{D77E}, BY-CP^{D77K}, BY-CP^{D77N} or BY-CP^{D77R}, respectively. Cell lines selected for further study contained equivalent amounts of mutant or wild type CP as determined by western immunoblot assays.

In vitro synthesis of viral RNA and plant and BY2 protoplast inoculation

In vitro transcribed full length viral RNAs were generated from w.t. and mutant TMV cDNA clones and used to inoculate plants as previously described (Bendahmane et al., 1997). Protoplasts were prepared from BY-2 suspension cell cultures, inoculated with *in vitro* transcribed viral RNA via electroporation, as described (Watanabe et al., 1987, Bendahmane et al., 2002). Infected plants and protoplasts were analyzed by fluorescence and laser scanning confocal microscopy as described (Bendahmane et al., 2002).

Purification and analysis of virus particles from infected plants

Wild type and mutant viruses were purified from infected plant leaves as described previously (Asselin and Zaitlin., 1978; Bendahmane et al., 1997).

Electron microscopy: Purified viruses at concentrations of 10 to 100 μg/ml were applied to carbon coated copper EM grids and then negatively stained with a 2% uranyl acetate solution for 2 min. Grids were allowed to dry prior examination on a Phillips CM100 electron microscope at 39,000 x.

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Assays for CP-MR

Transgenic *N. tabacum* Xanthi-NN and Xanthi-nn plant lines expressing the mutant CPs or wt CP [lines 748 (Nelson et al., 1987) and 3646 (Powell-Abel et al., 1986)] were grown in a greenhouse without supplemental light and with temperatures ranging from 24°C to 26°C during the day and 20°C to 21°C during the night. At 4 weeks after transplantating the plants were inoculated on a single leaf previously dusted with carborundum (320 GRIT, Fisher Scientific). TMV diluted to concentrations of 0.1 μg/ml in 20 mM $NaHPO_4$, pH7, was used as inoculum. On Xanthi-NN transgenic lines the relative levels of protection conferred by the different mutant CPs were scored by comparing the numbers of necrotic lesions on the transgenic lines versus non-transgenic plants. The relative degree of systemic protection was scored by monitoring the appearance of disease symptoms and virus accumulation in the upper non-inoculated leaves. Virus accumulation was quantified using an indirect sandwich ELISA as previously described (Bendahmane et al., 1997).

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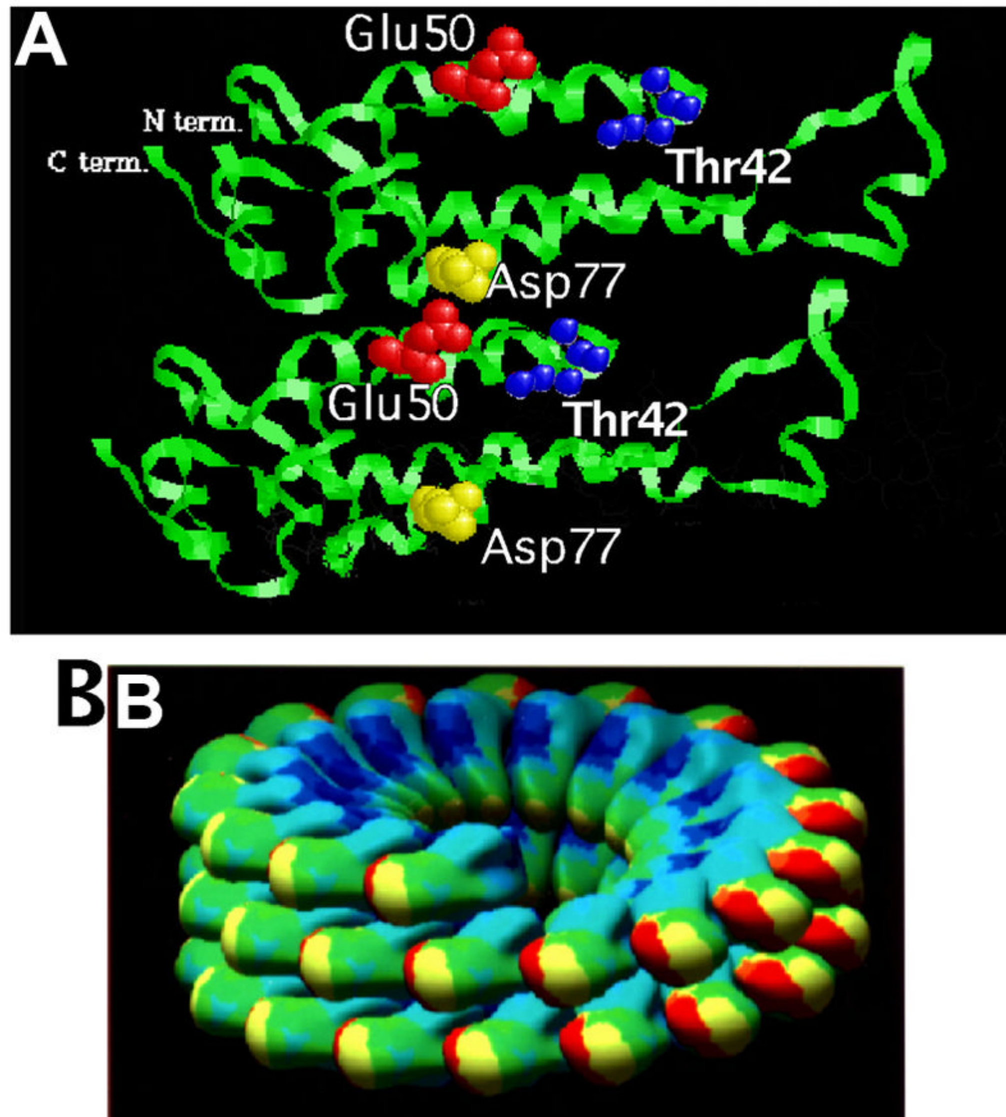

Figure 1.

(A) Ribbon representation of the alpha-carbon backbone of two CP subunits belonging to two superimposed helical turns of TMV virion. The N and C termini located at the outer radius if the virus are indicated. The position of the interacting carboxylate residues Glu50 and Asp77 and of the Thr42 residue is shown. (B) Computer graphic representation of two and half virion helical turns of CP subunits. The N-terminus and the C-terminus of the CP molecules lie on the external surface of the assembled subunits.

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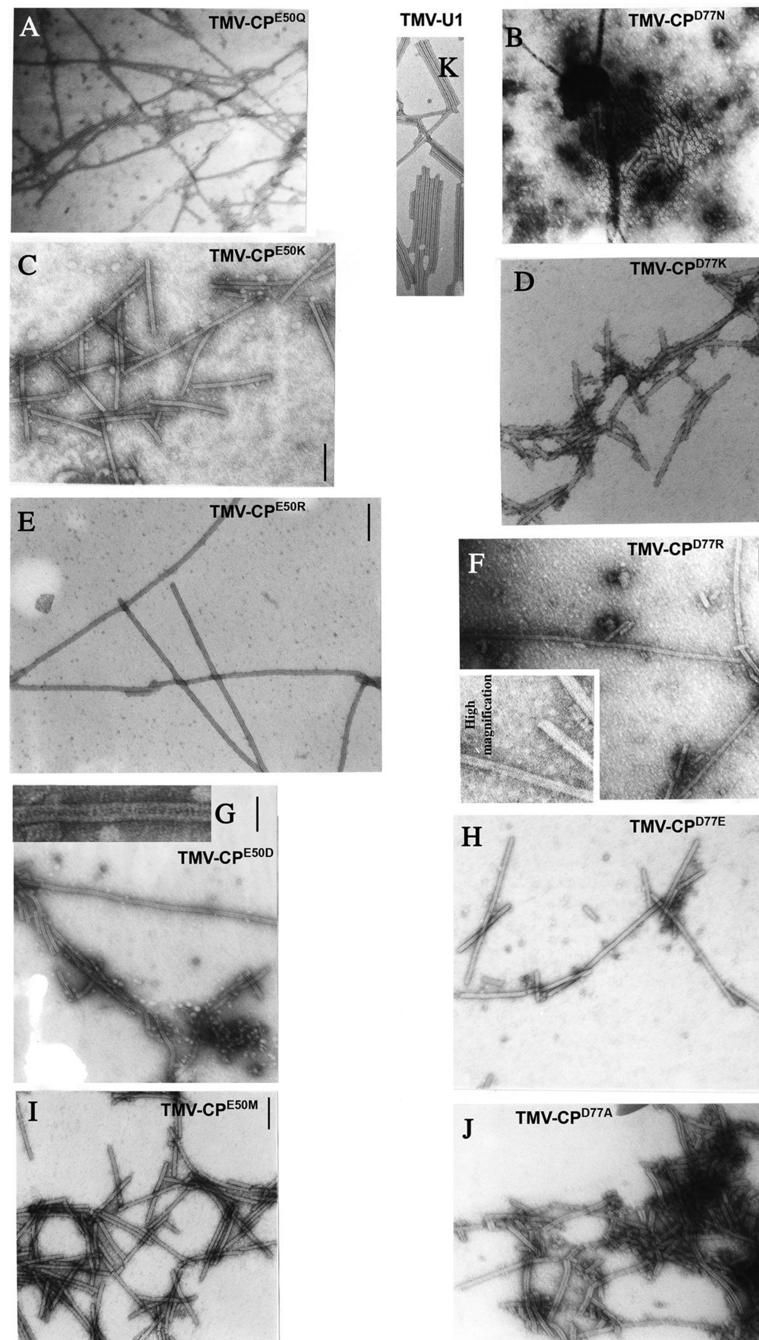

Figure 2.

Electron-microscopic analysis of assembled VLPs from *N. tabacum* plants infected with TMV mutated at amino acid E50 (TMV-CP^{E50Q}, TMV-CP^{E50K}, TMV-CP^{E50R}, TMVCP^{E50D}, TMV-CP^{E50M}) or at amino acid D77 (TMV-CP^{D77N}, TMV-CP^{D77K}, TMV-CP^{D77R}, TMV-CP^{D77E}, TMV-CP^{D77A}) or w.t. (TMV-U1). The samples were negatively stained and analyzed at a magnification of 39,000. The bar represents 100 nm.

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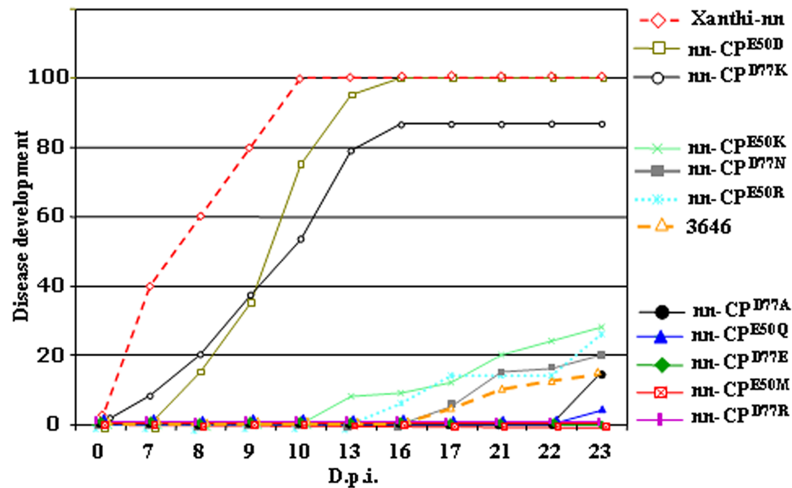

Figure 3. Resistance of transgenic plant lines that produce mutant CPs to systemic infection by TMV

Protection assay for TMV infection in transgenic *N. tabacum* Xanthi-nn plants expressing wt CP (line 3646) or CP carrying mutations at amino acids Asp77 or Glu50. The plants were inoculated with the TMV-U1 virus (0.1 mg/ml), and disease development was scored as the percentage of plants that exhibit symptoms on upper leaves and accumulation of TMV in the systemic leaves was measured by ELISA using anti-CP antibody. ELISA was performed on samples from 10 plants for each line, as described (Bendahmane et al., 1997).

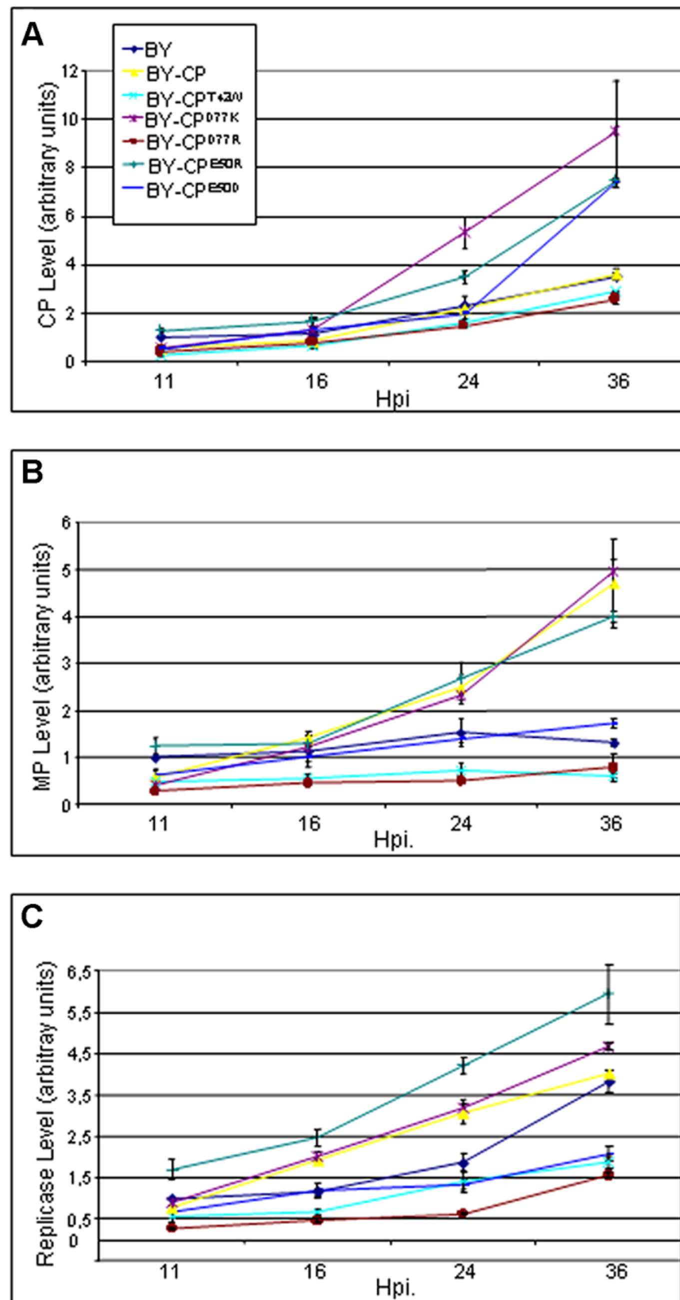

Figure 4. Accumulation of CP (A), MP (B) and replicase (C) proteins in BY-2 protoplasts non-transgenic or expressing wt CP or mutant CPs.

Protoplast were prepared from transgenic BY-2 cells expressing CP^{T42W}, CP^{D77k}, CP^{D77R}, CP^{E50R} or CP^{E50D}, and then infected with TMV RNA. Protoplast samples were harvested at 11, 16, 24, and 36 hpi. The effect of the transgenic CP (mutant or wt) on the accumulation of the CP, the MR and the replicase of the infecting virus was assessed using antibodies directed against each of these viral proteins in ELISA experiments as described (Bendahmane et al., 1997).

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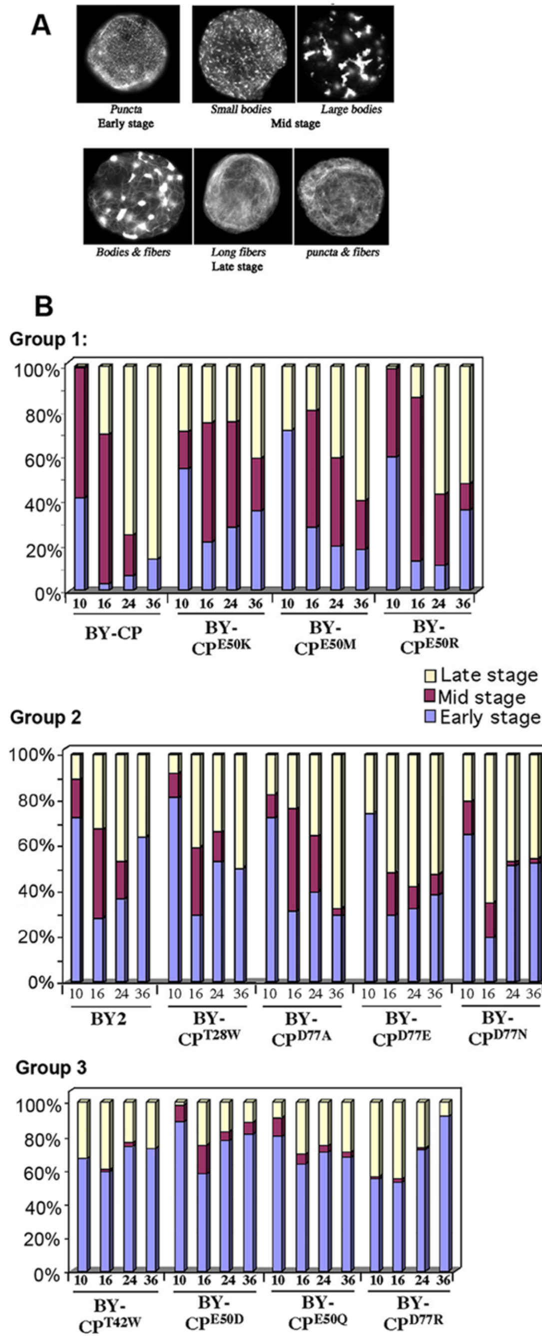

Figure 5. Effect of mutant CPs expressed in transgenic BY2 cells on MP:GFP following TMV-MP:GFP infection.

(A) Patterns of accumulation of MP:GFP in BY-2 protoplasts inoculated with TMV-MP:GFP as described in Heinlein et al. (1995). Protoplasts were inoculated with TMV-MP:GFP RNA and the accumulation of MP:GFP was analyzed by fluorescence microscopy. Pictures were taken at 12, 15, and 24 hpi corresponding to early, mid and late stages of infection by TMV, respectively. MP:GFP patterns found at each stage are presented. (B) Quantification of the presence of each of the six paterns (above) of MP:GFP accumulation during TMV-MP:GFP infection in protoplast expressing mutant CPs at 10, 16, 24 and 36 hpi. The presence of each pattern was normalized for the total number of cells counted per point and expressed as a

percent. The three groups of mutants CP with respect to their effect on MP accumulation are presented: group (1) CP mutants with postivie effects on MP accumulation similar to w.t. CP; group (2) CP mutants that lost the positive effect of w.t. CP on MP accumulation patterns; group (3) CP mutants that acquired negative effects on accumulation of MP.

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Table 1

Summary showing the effect of mutations in TMV CP at amino acids Thr28, Thr42, Glu50 and Asp77.

CP w.t.	[a]VLPs Virion	[b]Virus systemic movement in Xanthi-nn Yes	CP-MR against TMV in transgenic	
			[c]Xanthi NN (Local lesions) 99%	[d]Xanthi nn (Fig. 3) W.t. CP-MR
CP[E50K]	VLPs (long rods)	Yes[e]	90-96%	Equal to w.t. CP-MR
CP[E50R]	VLPs(long rods)	Delayed	99-99.4%	Equal to w.t. CP-MR
CP[D77N]	VLPs (short rods)	Delayed	99-100%	Equal to w.t. CP-MR
[f]CP[T42W]	Highly stable VLP	No	96-99%	Better than w.t. CP-MR
CP[D77R]	VLPs (long rods)	Yes	96-98%	Better than w.t. CP-MR
CP[E50M] CP[D77E]	VLPs (W.t.) VLPs (long rods)	Yes Yes	99.8-100% 98.6-99.4%	Better than w.t. CP-MR Better than w.t. CP-MR
CP[D77A] CP[E50Q]	VLPs VLPs (long rods)	Delayed Delayed	100% 96-99%	Better than w.t. CP-MR Better than w.t. CP-MR
[f]CP[T28W]	No ordered aggergates	No	0%	No protection
CP[D77K]	VLPs	No	0%	No protection
CP[E50D]	VLPs (long rods)	No	90-97%	No protection

[a]Effect of each mutation on virion assembly was assessed by introducing the mutation in TMV virus (see also Figure 2).

[b]Ability of TMV mutants to move systemically in Xanthi-nn. Virus systemic movement was measured by comparing the rate symptoms development on systemic leaves: Yes = Development of symptoms on the systemic leaves similar to that in wt TMV inoculated plants (about 8 to 10 days post-inoculation); No = inoculated plants never developed symptoms on the systemic leaves; Delayed = inoculated plants developed symptoms on the systemic leaves a least 25 to 30 days post-inoculation.

[c]CP mutated at one of the above amino acids were expressed in transgenic plants and their ability to provide CP-MR against TMV was measured in transgenic Xanthi-NN and in transgenic.

[d]CP mutated at one of the above amino acids were expressed in transgenic plants and their ability to provide CP-MR against TMV was measured in transgenic Xanthi-nn (Figure 3).

[e]Symptoms produced by TMV-CP[E50K] on systemic leaves of infected *N. tabacum* Xanthi-nn plants consisted in leaf curling and local lesions formation.

[f]Bendahmane et al. (1997)

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