Studies of Coat Protein-Mediated Resistance to Tobacco Mosaic Tobamovirus: Correlation between Assembly of Mutant Coat Proteins and Resistance

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Coat protein-mediated resistance (CP-MR) has been widely used to protect transgenic plants against virus diseases. To characterize the mechanisms of CP-MR to tobacco mosaic tobamovirus (TMV) we developed mutants of the coat protein that affected subunit-subunit interactions. Mutant CPs were expressed during TMV replication as well as in transgenic *Nicotiana tabacum* plants. The mutation T42→W increased protein aggregation and T28→W abolished aggregation and assembly, while the mutations T28→W plus T42→W and T89→W altered normal CP subunit-subunit interactions. The mutant T28W was unable to assemble virus-like particles (VLPs) during infection and in transgenic plants failed to aggregate; this protein conferred no protection against challenge of transgenic plants by TMV. The mutant T42W had strong CP subunit-subunit interactions and formed VLPs but not infectious virions. Transgenic lines with this protein exhibited stronger protection against TMV infection than transgenic plants that contained the wild-type (wt) CP. It is proposed that increased resistance conferred by the T42W mutant results from strong interaction between transgenic CP subunits and challenge virus CP subunits. CP carrying the mutation T89→W formed flexuous and unstable VLPs whereas the double mutant T28W:T42W formed open helical structures that accumulated as paracrystalline arrays. In transgenic plants, T89W and the double mutant CPs showed reduced ability to aggregate and provided lower protection against TMV infection than wt CP. A strong correlation between normal CP subunit-subunit interactions and CP-MR is observed, and a model for CP-MR involving interactions between the transgenic CP and the CP of the challenge virus as well as interference with virus movement is discussed.

The expression of nucleotide sequences derived from plant viruses in transgenic plants can induce protection against viral diseases (21), a concept referred to as pathogen-derived resistance (PDR) (35). Powell-Abel et al. (30) first reported that expression of the tobacco mosaic tobamovirus (TMV) coat protein (CP) gene in transgenic *Nicotiana tabacum* plants confers protection against challenge by TMV, a phenomenon termed CP-mediated resistance (CP-MR). Since that report, numerous other examples of CP-MR and PDR have been described (for reviews see references 12, 16, and 21). The molecular events that mediate PDR, including resistance provided by coat protein molecules, remain unclear, and it is likely that PDR can involve a variety of cellular and molecular mechanisms.

In the case of CP-MR against TMV, the accumulation of CP in the transgenic plants is indispensable for protection against TMV (29). Protection is largely (but not fully) overcome following inoculation with TMV RNA (27). Furthermore, CP-MR against TMV is somewhat specific, and there is a correlation between resistance and the degree of amino acid sequence similarity between the CP in the transgenic plants and the CP of the challenging virus (26).

Several hypotheses have been proposed to explain the mechanisms of CP-MR (12, 21). Data obtained with transgenic plants and protoplasts that express the TMV CP support a model whereby the transgenic CP prevents the uncoating of the challenge virus (28, 31, 39). The model suggests that either endogenous CP assembles to form virus-like particles (VLPs) that block the site for uncoating the challenge virus or CP inhibits virus disassembly by shifting the disassembly-assembly reaction in favor of assembly, thereby preventing virus infection in the inoculated cells (32). This hypothesis implies an eventual exchange of the CP subunits of the challenge virus with the subunits of the CP produced by the transgenic plant (7, 8).

To test these models we constructed single or double amino acid mutations in the CP. All the mutations altered normal CP subunit interactions. Mutants not capable of subunit-subunit interactions and mutants with reduced interactions did not confer protection against TMV infection. In contrast, the mutant with increased subunit-subunit interactions provided higher protection than that conferred by wild-type (wt) CP. Our results support the hypothesis that specific interactions between the transgenic CP and the CP of the challenge virus as well as an interference with virus movement are necessary components of CP-MR against TMV.

MATERIALS AND METHODS

Plasmid construction. The *Nco*I (filled in)-*Kpn*I TMV fragment (nucleotides 5459 to 6395) (15) containing the full-length CP gene was excised from the TMV cDNA clone U3/12-4 (18) and cloned into the bacterial plasmid pBluescript II KS+ (Stratagene) between the sites *Bam*HI (filled in) and *Kpn*I; the *Nco*I restriction site was maintained. The resulting clone, pKN2, was used in site-directed mutagenesis experiments using primers complementary to the sequence of TMV. The parameters for the PCR cycles were as follow: 94°C for 30 s, 45°C for 40 s, and 72°C for 45 s over the course of 25 cycles followed by 72°C for 10 min. By these reactions the threonine (Thr or T) residues at positions 28, 42, and

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89 were mutated to tryptophan (Trp or W). The amplified DNA fragments were then inserted in the homologous sites in the plasmid pKN2, and their sequences were confirmed by DNA sequence determination (36). The resulting plasmids are referred to as pT28W, pT42W, and pT89W, respectively. The fourth plasmid was constructed by combining the T28W and T42W mutants to produce pT28W:T42W.

Infectious TMV cDNA clones harboring the mutated CP genes were generated by transferring the *NcoI*-*KpnI* fragment from each of the mutants to the TMV cDNA plasmid U3/12-4. The resulting plasmids, which contained the full-length cDNA clones harboring the mutant CP genes, are referred to as pTMV-T28W, pTMV-T42W, pTMV-T89W, and pTMV-T28W:T42W.

To construct the intermediate plasmids that were used for plant transformation, the *DraI*-*KpnI* fragments containing the mutated CP genes (TMV nucleotides 5708 to 6395 [15]) from pT28W, pT42W, pT89W, and pT28W:T42W were cloned into the vector pMON316 (33), an expression cassette that placed each cDNA between the 35S promoter and the *tnos* 3' terminator. The resulting clones, pMonT28W, pMonT42W, pMonT89W, and pMonT28W:T42W, respectively, were used to produce transgenic plants.

Synthesis of in vitro transcripts and inoculation of plants. In vitro transcription reactions were used to produce full-length transcripts from the plasmids pTMV-T28W, pTMV-T42W, pTMV-T89W, pTMV-T28W:T42W, and U3/12-4, as described previously (18). One microgram of DNA previously linearized with *KpnI* was used in the transcription reaction. Leaves of 4-week-old *N. tabacum* Xanthi-NN and Xanthi-nn plants were first dusted with carborundum (Fisher Scientific) and then inoculated with one-fifth of the respective transcription reaction mixture.

Purification and analysis of VLPs from plants. TMV was purified from systemically infected leaves of *N. tabacum* Xanthi-nn plants as described previously (3). VLPs were purified as described previously (14). Infected leaves were homogenized in 0.5 M NaHPO₄ (pH 7)–0.1% (vol/vol) 2-mercaptoethanol and extracted once with *n*-butanol–chloroform (vol/vol) saturated with H₂O. The supernatant was then precipitated in the presence of 4% (wt/vol) polyethylene glycol (molecular weight of 8,000)–1% (wt/vol) NaCl, and the pellet was resuspended in 10 mM NaHPO₄, pH 7. The supernatant was again precipitated in presence of polyethylene glycol (molecular weight of 8,000) and NaCl, and the pelleted VLPs were resuspended in 10 mM NaHPO₄, pH 7.

Encapsidated RNA was purified from virions and VLPs by phenol extraction followed by phenol-chloroform extraction and ethanol precipitation. To determine whether viral RNA sequences were encapsidated in these VLPs, cDNA synthesis was performed with Superscript reverse transcriptase (Gibco-BRL) and different primers, and the products were used in a series of PCR amplification reactions. Primers for these reactions were selected so as to isolate sequences from the CP and the movement protein genes, including the origin of assembly sequence (TMV nucleotides 5420 to 5546).

Electron microscopy. Samples from the inoculated leaves were harvested 5 days postinoculation (dpi), macerated in 2% uranyl acetate, and applied to carbon-coated copper grids. Grids were dried and examined on a Phillips CM100 electron microscope at a magnification of $\times 39,000$.

Construction and screening of transgenic plants. *Agrobacterium tumefaciens* C58 (pGV2260), harboring the clone pMonT28W, pMonT42W, pMonT89W, or pMonT28W:T42W, was used to transform *N. tabacum* cultivars Xanthi-NN and Xanthi-nn by the leaf disc transformation protocol (19). The regenerated kanamycin-resistant plants (Kan^r) were screened for CP accumulation by a Western immunoblot assay, as described previously (34). Plant lines are referred to as nn- and nn-T28W, nn- and nn-T42W, nn- and nn-T89W, and nn- and nn-T28W:T42W for Xanthi-NN and Xanthi-nn, respectively.

A polyclonal rabbit antiserum generated against TMV-U1 was used as the first reactant, and alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G was used as the secondary antibody. Positive transformants were identified and maintained as individual lines. Seeds were collected from self-pollinated R1 plants, and homozygous plants were identified. Homozygous R2 plant seedlings were used for all studies.

Protection assays. Transgenic plant lines that express wt CP include *N. tabacum* Xanthi-NN line 748 (27) and Xanthi-nn line 3646 (30). All plants were grown in a greenhouse on the campus of the University of California, San Diego, without supplemental light and with temperatures ranging from 24 to 26°C during the day and 20 to 21°C during the night. A single leaf of each plant (4 to 6 leaf stage) was dusted with carborundum (320 GRIT; Fisher Scientific), purified TMV-U1 diluted in 20 mM NaHPO₄ (pH 7) was added, and the surface of the leaf was gently abraded. TMV at concentrations of 0.1, 1, 5, and 10 µg/ml was used as the inoculum. Relative levels of resistance to infection were determined by comparing the numbers of necrotic lesions on transgenic lines with the numbers of necrotic lesions on nontransgenic plants.

The relative level of systemic protection was tested by monitoring the appearance of disease symptoms and virus accumulation in the upper noninoculated leaves on Xanthi-nn. Virus accumulation was quantitated by an indirect sandwich ELISA (5, 6). The ELISA plates were coated with a polyclonal rabbit antiserum generated against TMV diluted 1/500 in 50 mM carbonate buffer (pH 6.4), after which the plate was blocked with 1% bovine serum albumin in Tris-buffered saline buffer. Leaf samples were ground in ELISA buffer (phosphate-buffered saline, 0.05% Tween 20, 0.2% ovalbumin, pH 7.0) and applied to the coated plate. Mouse antiserum generated against TMV (diluted 1/1,000) was used to

react with bound virus, and a goat anti-mouse antibody conjugated with horseradish peroxidase (diluted 1/1,000) was applied. Positive reactions were detected following addition of a solution containing 0.04% 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) and 0.01% H₂O₂ in a buffer containing 0.1 M citrate, pH 4. Optical densities of the reaction products at 405 nm were determined with a Bio-Rad multiplate reader.

Production and analysis of grafted plants. Grafted plants were generated with 6-week-old *N. tabacum* Xanthi-nn nontransgenic or transgenic plants. The junction of the slash graft was sealed with parafilm, and the plants were kept at 24°C and 100% relative humidity until the graft wounds healed. A leaf on the rootstock plant was inoculated with purified TMV-U1 as described above. Systemic infection and symptom development were scored daily on the scion.

Sucrose gradients. Three grams of leaves was frozen in liquid nitrogen and ground to a fine powder. The powder was suspended in 5 ml of 20 mM NaHPO₄ (pH 7) and filtered through eight layers of cheesecloth to remove cellular debris, and 3 ml of the filtrate was immediately loaded on 8 ml of a linear 5 to 40% (wt/vol) sucrose gradient. After 12 h of centrifugation in a Beckman rotor SW 41 at 155,000 $\times g$ at 4°C, fractions of 0.8 or 1 ml were collected from the top and aliquots of 10 µl were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot assay, as described above.

Purification of T28W mutant and wt CPs from plants. wt CP was isolated from purified TMV-U1 by the acetic acid degradation protocol (13). The mutated CP T28W was soluble in infected plants and was purified as follows. Leaves infected with TMV-T28W (TMV expressing T28W) were homogenized in 20 mM phosphate buffer, pH 7, to which 5% (wt/vol) diatomaceous earth was added to remove the chlorophyll. Following centrifugation at 10,000 $\times g$ for 10 min, the extraction procedure was repeated twice and the final supernatant was filtered through a 1-µm-pore-size filter and then loaded on an anionic exchange column (Poros HQ/m) with the BioCAD 700E perfusion chromatography workstation. A highly purified fraction (>99% purity) containing T28W mutant CP was recovered. Proteins were then concentrated and used for spectrofluorometer and circular dichroism (CD) analysis.

Physical structure analysis of mutated CP. (i) **Spectrofluorometry.** Tryptophan fluorescence emission spectrum measurements of the mutated and wt CP (at a concentration of 25 µg/ml of H₂O) were collected on the Hitachi F-2000 spectrofluorometer. The excitation wavelength was 295 nm to minimize interference with phenylalanine and tyrosine (9). The emission was recorded between 300 and 400 nm at room temperature in a 1-cm quartz cuvette. In each experiment the average of 10 spectral readings was determined.

(ii) **CD measurements.** CD spectra of wt and T28W CPs were collected on an AVIV 60DS spectropolarimeter between 185 and 260 nm in 0.2 nm wavelength steps with an average time of 4.0 s; the protein concentration was 50 µg/ml of H₂O. Multiple scans were obtained, and the baseline, corresponding to water, was subtracted from each scan.

RESULTS

Characteristics of the mutant proteins. The known atomic structure of the TMV CP (25) was used to select for mutation amino acids that are conserved among tobamoviruses and located at positions important for subunit interactions. In this study the Thr residues at positions 28, 42, and 89 were selected because their side chains are exposed on various surfaces of the CP molecule. Thr residues were mutated to Trp, which is predicted to increase the volume of the side chain by approximately 96%; if, as predicted, the side chains of the Thr residues are exposed on the surface between adjacent molecules or between adjacent turns of the helix, Trp in these positions would interfere with the normal quaternary structure of the protein. Thr at position 28 is located in the intermediate radius of the CP molecule and forms lateral contacts between adjacent CP subunits in the disc (2). Thr at position 42 is also located in the intermediate radius but is in a good position to make axial contacts between CP molecules that are vertically adjacent (Fig. 1). The Thr residue at position 89 is located in the small radial angle of the assembled disc and is highly conserved among tobamoviruses (2).

Mutant viruses were generated by replacing the CP gene in a TMV cDNA clone (U3/12-4) with sequences encoding the CP mutants T28W, T42W, and T89W and the double mutant T28W:T42W to give rise to pTMV-T28W, pTMV-T42W, pTMV-T89W, and pTMV-T28W:T42W, respectively. Infectious RNA transcripts were produced in vitro and inoculated to *N. tabacum* cv. Xanthi-nn and -NN plants. On *N. tabacum* Xanthi-NN, necrotic lesions similar to those induced by wt

FIG. 1. Computer-assisted graphic representation of three adjacent TMV CP molecules. The positions of the Thr28, Thr42, and Thr89 residues are indicated in panel A. Panel B displays CP molecules carrying the mutations Trp28, Trp42, and Trp89. The N terminus (N-term) and the C terminus (C-term) of the CP molecules are indicated and lie on the external surface of the assembled subunits.

TMV developed at about 3 to 4 dpi. However, none of the mutants produced systemic infection on *N. tabacum* Xanthi-*nn* plants, nor was evidence of systemic infection detected by ELISA (data not shown).

Electron-microscopic visualization of VLPs purified from inoculated leaves (Fig. 2) revealed that TMV-T42W assembles to form VLPs of variable length made up of stacked discs. No VLPs or aggregated forms of CP were detected in plants infected with TMV-T28W. However, the level of CP accumulation in leaves inoculated with TMV-T28W was similar to the CP level in leaves inoculated with wt TMV (data not shown).

In contrast, the double-mutant virus TMV-T28W:T42W formed particles with an open helical structure or paracrystalline arrays of CP (Fig. 2). The structures were similar to those produced by the PM2 mutant of TMV, which carries the mutation Thr28→Isoleucine (CP mutant T28I) (7, 37); however, preliminary analyses of the structures produced by PM2 and TMV-T28W:T42W show that the stacking conformations are not identical (unpublished data). The TMV-T89W mutant produced CP that assembled to form unstable and flexuous VLPs made up of fragmented stacks of multiple discs rather than helices (Fig. 2). Butler et al. (4) have shown that the discs of TMV CP subunits can be the dominant structures at pH 7, while at slightly lower pH, the helical form dominates.

A study was conducted to determine whether the VLPs contain viral RNA by attempting to produce cDNA from RNA purified from VLPs. For these studies oligonucleotides complementary to nucleotides 5782 to 5811 and 6238 to 6257 of TMV RNA (15) were used to attempt cDNA synthesis. Other primer combinations were then used in an attempt to amplify DNA fragments overlapping the CP and the movement protein genes by PCR. It should be noted that these primers would amplify sequences that contain the origin of assembly. The reactions produced a DNA fragment when TMV-RNA was used, but no amplification product was obtained in the case of RNA extracted from VLPs produced by the mutants TMV-T42W, TMV-T28W:T42W, and TMV-T89W (data not shown), demonstrating that the VLPs did not encapsidate viral RNA.

Moreover, experiments in which *N. tabacum* Xanthi-*nn* plants were inoculated with homogenates of infected Xanthi-*nn* leaves revealed that only about 0.5 to 1.6% of the level of wt TMV infectivity was recovered from leaves infected with mutant viruses. Treatment of the homogenates at 37°C for 1 h further reduced the infectivity to near zero, a result that supports the conclusion that the VLPs produced by the mutants did not contain TMV RNA.

Sucrose gradient experiments were performed to determine the degree of aggregation of the mutant CPs in infected leaves (Fig. 3A). In parallel, gradients of purified TMV, hemoglobin (4S), and catalase (11S) were similarly analyzed. TMV produced the expected distribution of aggregates; the majority of the material corresponding to full-length TMV was pelleted and not included in these studies. Virus carrying the mutation T28W produced CP aggregates of less than 4S, which confirms the absence of VLPs in electron-microscopic studies. TMV-T42W produced VLPs that were longer than those produced by wt TMV (Fig. 2) and in sucrose gradients produced a relatively large amount of aggregated proteins (>20S). Aggregates of the double mutant TMV-T28W:T42W were intermediate in sedimentation values, with most aggregates having a sedimentation value of up to about 11S. The TMV-T89W mutant yielded aggregates that were similar in distribution to those yielded by wt TMV; however, there were only small amounts of full-length virus particles in the case of TMV-T89W.

Similar experiments on extracts from transgenic plants that accumulate wt or mutant CPs were performed (Fig. 3B). The sedimentation of CP aggregates was very similar to those recorded from infected leaves. In both transgenic and virus-infected plants, the T28→W mutation prevented the formation of large aggregates whereas the T42→W mutation increased the formation of large aggregates.

CP protection in transgenic plants. Transgenic tobacco lines that expressed genes encoding the CP mutants T28W, T42W, T28W:T42W, and T89W were constructed. At least 10 independent transgenic lines for each mutant were selected, based

FIG. 2. Electron-microscopic analysis of assembled VLPs from *N. tabacum* plants infected with TMV-T42W, TMV-T28W:T42W, TMV-T89W, and TMV-U1. The samples were negatively stained and analyzed at a magnification of $\times 39,000$. The bar represents 100 nm.

on accumulation of CP to levels equivalent to those in transgenic line 3646 (contains wt CP; 0.03% [wt/wt] of total extractable proteins) and line 748 (contains wt CP; 0.04 to 0.05% [wt/wt] of total extractable proteins). Seeds from homozygous lines were selected and maintained.

The degree of protection against TMV infection was assayed and compared to that present in lines that contain wt CP. Three concentrations of virus inoculum were used: 0.1, 1, and 5 $\mu\text{g}/\text{ml}$. These concentrations produced means of about 80, 375, and 520 necrotic lesions per leaf, respectively, on nontransgenic *N. tabacum* Xanthi-NN plants. To simplify the discussion, we present the results of experiments with selected lines (as described above); the data were consistent amongst each of the 10 lines of each construct tested.

The percentages of Xanthi-nn plants that developed systemic disease symptoms with time after inoculation were scored, averaged, and compared with the percentages obtained with nontransgenic plants (0% protection) and line 3646. As shown in Fig. 4A the two plant lines containing CP T42W (nn-T42W lines) that were used in these studies (lines A and R) did not develop symptoms when inoculated with 0.1 μg of virus per ml, whereas line 3646 showed a delay in symptom appearance. The nn-T42W lines did not contain detectable virus in the systemic leaves at 40 dpi, based on ELISA (Fig. 4D). With inoculum concentrations of 1 and 5 $\mu\text{g}/\text{ml}$ (Fig. 4B and C) the nn-T42W lines exhibited better protection than line 3646, as reflected in symptom development (Fig. 4B and C) and accumulation of TMV CP and reflecting virus replication

(Fig. 4D). The transgenic lines containing T28W mutant CP (e.g., line E) developed disease symptoms to the same extent as did nontransgenic plants (Fig. 4A); symptoms appeared and developed normally and the virus accumulated to amounts similar to the amounts of virus in nontransgenic plants. Transgenic nn-T28W:T42W lines (e.g., line O) exhibited a relatively low degree of protection against infection (Fig. 4A), although the level of resistance was slightly greater than that of the nn-T28W lines. Transgenic nn-T89W plant lines (e.g., line E) exhibited about 50 to 75% of the level of protection exhibited by line 3646 (Fig. 4A).

Transgenic plants of the genotype Xanthi-NN which accumulated each of the mutant CPs were also developed, and 10 lines that accumulated CP levels equivalent to those in plant line 748 (which contains wt CP) were selected for further studies. Plants were inoculated with TMV at an inoculum concentration of 0.1 $\mu\text{g}/\text{ml}$, and the numbers of local necrotic lesions were determined, averaged, and compared with the number of lesions produced on nontransgenic plants (0% protection) and on line 748 plants (100% protection) (Table 1). Plant lines that contain T42W mutant CP exhibited levels of protection equivalent to that exhibited by line 748. In contrast, very low protection was conferred by nn-T28W lines and an intermediate degree of protection was conferred by lines nn-T89W and nn-T28W:T42W. These results are in agreement with the results obtained with genotype Xanthi-nn (Fig. 4).

A representative line of Xanthi-NN that contains the T42W mutant (i.e., line L) together with line 748 was further tested

FIG. 3. Sucrose gradient sedimentation of wt or mutated CP. Leaf homogenates were prepared from virus-inoculated plants (harvested at 5 dpi) (A) or transgenic plants (B) and analyzed on linear 5 to 40% sucrose gradients. Fractions were collected, and the proteins from an aliquot from each fraction were resolved on a SDS-12.5% polyacrylamide gel. Proteins were then transferred onto a nitrocellulose membrane and analyzed by Western blotting with polyclonal rabbit antiserum generated against the TMV-U1 virus as a first reactant and alkaline phosphatase conjugated to goat anti-rabbit immunoglobulin G as a secondary antibody. The numbers 1 to 11 in panel A and 1 to 14 in panel B represent fractions collected from the top of each gradient.

for their resistance to TMV inoculum levels of 1 and 10 $\mu\text{g}/\text{ml}$. Table 1 summarizes the results of these studies and shows that the degrees of protection in nn-T42W line L and line 748 are equivalent at all inoculum levels. This is taken as support of the conclusion that T42W and wt CP confer very high levels of protection against infection in these plants.

Studies of CP-MR to systemic infection in grafted plants. As the T42W mutant conferred greater levels of resistance to systemic infection with TMV (as well as with TMV RNA [data not shown]) than did wt CP, experiments were conducted to examine the effect of CP on systemic spread with grafted plants (Table 2). Nontransgenic Xanthi-nn plants, lines 3646 and nn-T42W, were used as rootstocks or scions. After the graft junctions healed, one leaf of the rootstock was inoculated with TMV at a concentration that would produce an average of 200 necrotic lesions on nontransgenic Xanthi-NN leaves. Symptom appearance and severity were scored daily.

Fifty percent of the nn\ nn grafted plants (the control plants used in the study; the symbol \ indicates a graft of the rootstock plant [left] and the scion plant [right], with nn designating Xanthi-nn) developed disease symptoms at about 9 dpi, and at 10 dpi all plants showed symptoms. The grafted plants nn\3646 and nn\T42W began to develop symptoms at 9 and 10 dpi, respectively, and at 13 dpi all plants showed symptoms. This demonstrates that plants of nn-T42W lines as well as line 3646 are more resistant to systemic infection under the conditions described above than are nontransgenic plants. Such resistance may reflect resistance during transport to and/or through the vascular system or during unloading from vascular tissues in the upper leaves.

A set of grafted plants was constructed with nn-T42W or 3646 plants as the rootstocks and nontransgenic Xanthi-nn plants as the scions, after which rootstocks were inoculated as described above. For the 3646\ nn grafted plants, the scions began developing symptoms at 11 dpi compared to 9 dpi for the nn\ nn grafted plants, and at 18 dpi all plants developed disease symptoms. In contrast, for the T42W\ nn grafted plants, 34% of the plants developed symptoms at 14 dpi and the remainder were free of infection through 30 dpi. This demonstrated that plants that express T42W mutant CP restrict spread of the infection from the inoculated rootstock to the scion (Table 2).

Effect of the T28→W mutation on the structure of the CP. Three of the four mutations made in the CP apparently did not disrupt the structure of the CP molecules as they assembled to form VLPs or smaller aggregates (Fig. 2 and 3). However, since the T28W mutant did not form VLPs or confer CP-MR and behaved as a soluble protein in plant extracts, we wanted to determine if its structure was disrupted by the mutation. We therefore performed CD spectroscopy experiments on T28W mutant CP purified from plants to greater than 99% purity. As shown in Fig. 5A the T28W mutant possesses a secondary structure substantially similar to that of wt CP. The spectrum shows negative peaks at about 220 and 208 nm and a positive peak at 192 nm, typical of α -helices (20), and demonstrates that the extent of the helical structure of the mutant CP is essentially the same as it is in wt CP.

X-ray fiber diffraction analysis of the structure of TMV (25) demonstrated that the side chain of Thr 28 is placed between two horizontally adjacent CP monomers. To determine whether the aromatic side chain of the Trp residue in the T28W mutant is exposed or buried in the tertiary structure of the CP, we measured the fluorescence emission spectra of the wt and T28W CPs with excitation at 295 nm. The results of these studies showed two interesting features. First, the position of the main peak at about 330 nm is not appreciably shifted in the mutant, which suggests that there is not a large change in the overall structure of CP T28W in comparison to wt CP. This result confirms the observations obtained by CD analysis. Second, the appearance of a new peak at 350 nm in the case of CP T28W supports the conclusion that the aromatic ring in the added Trp is exposed to solvent on the surface of the mutant CP molecule, which we propose is otherwise folded in the same manner as wt CP (Fig. 5B).

DISCUSSION

In this study we show that a mutant of the TMV CP that lacks the capacity to assemble to form VLPs or lesser aggregates does not confer CP-MR. We chose to mutate Thr residues to Trp because such a change would result in a significant increase in the volume of the side chain of the amino acid while not altering the charge of the residue. Amino acid Thr28 is located between two horizontally adjacent CP subunits in the helical array, where it is involved in lateral contacts between adjacent CP subunits (Fig. 1) (25). An increase in the volume of the amino acid side chain at this site is predicted to cause an increase in the radial angle between adjacent subunits. When these increases are summed over the helical turns of the virion, such a change is expected to produce unstable subunit-subunit interactions and prevent formation of VLPs. When Thr28 was changed to isoleucine (7), a mutation that increases the volume of the side chain by 44%, the result was the formation of CP aggregates and paracrystalline arrays that are similar but not identical to those produced by the mutant virus TMV-T28W: T42W (shown in Fig. 2). Furthermore, the T28I mutant con-

FIG. 4. Protection assay for TMV infection in transgenic *N. tabacum* Xanthi-nn plants expressing wt or mutated CP. (A) Results of the protection assay for the transgenic plants expressing the mutated CP genes, i.e., nn-T28W line E, nn-T42W lines A and R, nn-T2842W line O, and nn-T89W line E. The plants were inoculated with the TMV-U1 virus (0.1 µg/ml), and disease development was scored as the percentage of plants that exhibit symptoms on upper leaves. Ten plants were used in each sample. (B and C) Protection assays of transgenic plants expressing the wt CP or the mutated CP T42W, plant lines A and R, challenged with TMV-U1 at concentrations of 1 µg/ml (B) and 5 µg/ml (C). (D) Accumulation of TMV in the systemic leaves of transgenic plants that contain CP T42W, plant lines A and R, at 41 dpi. ELISA was performed on samples from 10 plants for each line, as described in Materials and Methods. The A_{405} s of the reaction products were averaged, and the reading from the control Xanthi-nn-infected plants was subtracted.

TABLE 1. CP-MR in transgenic *N. tabacum* Xanthi-NN that express wt or mutant CP

Expt and plant line	Inoculum ($\mu\text{g/ml}$) ^a	<i>n</i> ^b	Average no. of lesions ^c	% Protection ^d
Expt A				
Xanthi-NN	0.1	10	80 $\pm$ 36	0
NN-T28W B	0.1	5	73 $\pm$ 18	9
NN-T28W J	0.1	8	120 $\pm$ 27	0
NN-T28W I	0.1	8	110 $\pm$ 11	0
NN-T28W:T42W A	0.1	5	61 $\pm$ 21	24
NN-T28W:T42W L	0.1	8	48 $\pm$ 29	40
NN-T28W:T42W N	0.1	10	60 $\pm$ 15	25
NN-T89W H	0.1	6	21 $\pm$ 18	74
NN-T89W O	0.1	10	28 $\pm$ 22	65
NN-T89W W	0.1	10	31 $\pm$ 19	61
NN-T42W G	0.1	8	0.5 $\pm$ 0.3	99.4
NN-T42W L	0.1	12	0.3 $\pm$ 0.6	99.7
NN-T42W P	0.1	12	0.4 $\pm$ 0.8	99.5
748	0.1	12	0.16 $\pm$ 0.4	99.8
Expt B				
Xanthi-NN	1	12	376 $\pm$ 116	0
	10	11	697 $\pm$ 113	0
748	1	12	1.33 $\pm$ 1.6	99
	10	13	6.92 $\pm$ 2.9	99
NN-T42W L	1	12	6.5 $\pm$ 4.6	98
	10	13	28 $\pm$ 22	96

^a Concentrations of TMV inoculum used.^b Number of plants used per sample.^c Necrotic lesions were counted at 3 to 5 dpi for each plant and the counts were averaged. The numbers are means $\pm$ standard errors.^d The percentage of protection was determined as follows: % protection = 100 - (lesions on transgenic plant $\times$ 100 $\div$ lesions on nontransgenic NN plant).

ferred a level of CP-MR equivalent to that conferred by wt CP (7). It was predicted that in the T28W mutant the increase in volume of the side chain would be sufficiently great that aggregates of the CP would be unstable, as confirmed in this study. Experiments using CD and fluorometry predicted that the folding of the CP mutant T28W resembles that of wt CP (Fig. 5A and B) and that the side chain of Trp is most likely exposed on the surface of the folded CP molecule (Fig. 5B). This indicates that the inability of the CP T28W to aggregate is most likely due to steric hindrance caused by the presence of

the Trp side chain on the surface of the CP molecule. Unlike the T28I mutant, which can aggregate, the T28W mutant failed to protect against TMV infection.

In contrast to the CP T28W, the CP T42W exhibited an increased capacity for subunit-subunit interactions and aggregation (Fig. 2) compared with wt CP. Although unable to form virions, it confers a higher degree of protection in transgenic plants than does wt CP. For Xanthi-NN lines, the plants were free of systemic infection following inoculation with 0.1 μg of TMV per ml (Fig. 4D). At higher inoculum levels (1 and 5 $\mu\text{g/ml}$) the CP mutant T42W also confers greater resistance than wt CP.

A probable explanation for the increased aggregation of the T42W mutant compared with wt CP is that the Trp side chain interacts with hydrophobic amino acids of the adjacent CP subunits. Trp has been shown to play a key role in stabilizing interactions between a number of proteins. For example, the interactions of calmodulin with the calmodulin binding region of skeletal muscle myosin light chain kinase (23, 24) or with neuromodulin, a neuron-specific protein (1, 11), involve Trp residues. In each of these examples, tryptophan in one protein interacts with a hydrophobic pocket in the second protein. Similarly, in certain antibody-antigen complexes, Trp residues are involved in stabilizing the complex (10). A similar phenomenon may occur between two interacting CP T42W subunits, where the side chain of the Trp of the first subunit may be involved in a manner that increases interaction with a second CP subunit. This may result in more-stable subunit-subunit interactions, which are reflected in increased aggregation of the CP T42W mutant. This interpretation supports the hypothesis that strong subunit-subunit interactions between the transgenic CP T42W and the CP of the infecting virus result in strong CP-MR.

The double CP mutant T28W:T42W contains the mutant T28W, which disrupts protein aggregation, plus the T42W mutant, which increases CP aggregation. In infected plants the mutant CP aggregated to form paracrystalline arrays or an open helix (Fig. 2) and produced protein-protein interactions that were intermediate between those produced by T28W and T42W CPs, as judged by sedimentation in sucrose density gradients. As predicted, when expressed in transgenic plants this protein conferred low levels of protection against inoculation with TMV.

The mutant T89W produced a state of aggregation that is intermediate between those produced by wt and T28W:T42W CPs, as shown by sedimentation in sucrose gradients, and can assemble to form flexuous and relatively unstable VLPs (Fig. 2). Electron microscopy revealed that these VLPs may be formed by multiple stacked discs. This suggested that the T89W mutant interferes with the interactions of CP molecules to a lesser extent than the T28W and T28W:T42W CP mutants. In this case subunit-subunit interactions are not completely lost as they are in the case of the T28W mutant. As predicted, transgenic plants that accumulate the CP mutant T89W, when challenged with TMV, showed protection against TMV that is intermediate between that conferred by wt CP and the lack of protection conferred by T28W but higher than the protection conferred by the double mutant T28W:T42W.

The CP-MR studies with mutants T28W, T42W, T89W, and T28W:T42W demonstrated that the stronger the subunit interactions and CP aggregation, the greater the protection. These results support the hypothesis that an important component of CP-MR against TMV is a result of interactions between the transgenic CP and the CP of the infecting virus. We previously proposed (7, 31) that when the challenge virus undergoes disassembly in transgenic plants the CP in the host

TABLE 2. Systemic infection of grafted plants composed of nontransgenic plants and transgenic plants that express wt or mutated CP genes

Graft (rootstock/scion)	Genotype of grafted plants		% of plants with systemic symptoms on scion leaves at day ^a :							
	Rootstock	Scion	9	10	11	12	13	14	18	30
nn\ nn	Xanthi-nn	Xanthi-nn	50	100	100	100	100	100	100	
nn\ 3646	Xanthi-nn	3646	11	45	67	67	100			
nn\ T42W	Xanthi-nn	nn-T42W		11	45	89	100			
3646\ nn	3646	Xanthi-nn			16	34	34	34	100	
T42W\ nn	nn-T42W	Xanthi-nn						34	34	34

^a Days postinoculation.

FIG. 5. Physical analysis of wt and T28W CPs. (A) CD spectra of wt (grey line) and mutated T28W (dark line) CPs were collected at room temperature between 185 and 260 nm in 0.2-nm wavelength scan steps with an average time of 4.0 s. (B) Tryptophan fluorescence emission spectra of wt and mutated T28W CPs in H_2O . The excitation wavelength was 295 nm, and the emission was scanned from 300 to 400 nm in a 1-cm quartz cuvette at room temperature.

reencapsidates the RNA, thereby blocking ribosome binding and translation of viral RNA by driving the uncoating reaction toward assembly rather than disassembly. Reassembly requires that the CP be capable of assembly to produce VLPs and that the CP is sufficiently similar to the challenge virus CP to establish strong CP-virion interactions that reduce infection. In the present study, we show that CP molecules that are incapable of assembly as stable aggregates confer a level of resistance lower than that conferred by wt CP.

It is unclear what degree of similarity, either in amino acid sequence or in structure, between the transgenic CP and the CP of the challenge virus is required for CP-MR. Nejdat and Beachy (26) reported that CP-MR mediated by TMV CP is more effective against tobamoviruses with CP that has high amino acid identity with the TMV CP and less effective against those with more dissimilar CP. Here we show that a small change in the CP molecule that increases the volume of an amino acid results in either reduced interaction and no protection or increased interaction and greater protection.

CP-MR is not fully overcome by inoculation with viral RNA (27), and the resistance conferred by the T42W CP to inoculation with viral RNA is somewhat greater than that conferred by wt CP (data not shown). Nevertheless, the mechanism of protection conferred by the T42W CP may be similar to that conferred by wt CP; for example, both mutant and wt CPs are equivalent in protecting against initial infection (Table 1). To determine whether the T42W CP reduces systemic spread of infection, studies were conducted with grafted plants. In the case of the T42W//nn grafted plants (made up of nn-T42W as the rootstock and a nontransgenic plant as the scion), 66% of the plants did not develop systemic infections after inoculation with TMV; grafted plants of the type 3646//nn exhibited less resistance than T42W//nn, but were significantly more resistant than nn//nn plants (Table 2). In contrast, grafted plants of the

type nn//T42W and nn//3646 grafted plants became systemically infected after similar amounts of time, indicating that wt and mutated CP acted similarly in resistance to systemic infection when used as the scion. These results support the hypothesis that plants carrying the T42W mutant protein, and to some extent those with wt CP, restrict the infection from reaching phloem parenchyma and/or companion cells or from exiting them into sieve elements in the rootstock rather than prevent the virus from initiating infection in the scion. This phase of CP-MR is apparently in addition to that responsible for inhibition of virus disassembly.

This study demonstrated that the T42W CP inhibits systemic infection to a degree greater than does wt CP. Possible reasons for the increased activity include the following. (i) The T42W CP may interfere with encapsidation of viral RNA by wt CP produced during infection; because virus assembly is required for rapid systemic spread, the effect would be to reduce systemic infection. (ii) The incorporation of the T42W CP in virions may interfere with their normal disassembly after systemic spread, thereby preventing replication in newly infected cells. Indeed, Lu et al. (22) have shown that the CP mutant Glu50Gln modifies interactions of axial subunits when incorporated into TMV and prevents disassembly of TMV. (iii) T42W may interfere with local virus movement and thereby restrict systemic infection.

It is likely that the mechanisms of CP-MR are not the same for all viruses. For example, while the wt CP of TMV provides little resistance when plants are inoculated with TMV RNA (27), the CPs of potato virus X and alfalfa mosaic virus (AIMV) confer resistance to inoculation by viral RNA (12, 17, 38). In CP-MR against AIMV, plants inoculated with virions become infected but virus does not spread throughout the plant, demonstrating that protection occurs at a stage other than the initiation of infection (38). Recently, however, it was proposed that the AIMV CP may confer protection by interfering with an interaction between the challenging virus and a host component required for virus disassembly (40). It is clear that additional studies are needed to elucidate the multiple

mechanisms of CP-MR, and such studies are essential to the development of proteins that will have increased efficacy in conferring resistance.

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