Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.

Article No. jmbi.1999.2860 available online at http://www.idealibrary.com on IDEAL® J. Mol. Biol. (1999) 290, 9–20

JMB

Display of Epitopes on the Surface of Tobacco Mosaic Virus: Impact of Charge and Isoelectric Point of the Epitope on Virus-Host Interactions

Mohammed Bendahmane, Moses Koo, Erik Karrer and Roger N. Beachy*

Department of Cell Biology
Division of Plant Biology
The Scripps Research Institute
10550 North Torrey Pines Road, la Jolla, CA 92037, USA

**Corresponding author*

The biophysical properties of the tobacco mosaic tobamovirus (TMV) coat protein (CP) make it possible to display foreign peptides on the surface of TMV. The immunogenic epitopes G5-24 from the rabies virus (RV) glycoprotein, and 5B19 from murine hepatitis virus (MHV) S-glycoprotein were successfully displayed on the surface of TMV, and viruses accumulated to high levels in infected leaves of *Nicotiana tabacum* Xanthi-nn. The peptide RB19, which contains an arginine residue plus the 5B19 epitope fused to the CP (TMV-RB19), resulted in the induction of necrotic local lesions on inoculated leaves of *N. tabacum* Xanthi-nn and cell death of infected BY2 protoplasts. RNA dot blot assays confirmed that expression of the acidic and basic pathogenesis-related PR2 genes were induced in infected Xanthi-nn leaf tissue. TMV that carried epitope 31D from the RV nucleoprotein did not accumulate in inoculated tobacco leaves. Analysis of hybrid CPs predicted that the isoelectric points (pI):charge value was 5.31: − 2 for wild-type CP, 5.64: − 1 for CP-RB19, and 9.14: + 2 for CP-31D. When acidic amino acids were inserted in CP-RB19 and CP-31D to bring their pI:charge to near that of wild-type CP, the resulting viruses TMV-RB19E and TMV-4D:31D infected *N. tabacum* Xanthi-nn plants and BY2 protoplasts without causing cell death. These data show the importance of the pI of the epitope and its effects on the hybrid CP pI:charge value for successful epitope display as well as the lack of tolerance to positively charged epitopes on the surface of TMV.

© 1999 Academic Press

Keywords: plant virus; TMV; epitope display; virus-host interactions

Introduction

A variety of plant and animal viruses have been studied for their potential use as carrier and presenter of foreign peptides as linear or conformational antigens, and some have been tested as potential animal vaccines (Haynes *et al.*, 1986; Hamamoto *et al.*, 1993; Usha *et al.*, 1993; Jagadish *et al.*, 1993; Fitchen *et al.*, 1995; Sugiyama *et al.*, 1995; Turpen *et al.*, 1995; Beachy *et al.*, 1996; Lomonossoff & Johnson, 1996; Burke *et al.*, 1988; Dedieu *et al.*, 1992). Plant viruses are of special interest as carriers of immunogenic peptides and for use as potential vaccines, in part because they are not infectious in humans and other animals. Compared to most animal viruses, the structure of most plant viruses is very simple, and are often formed with a single coat protein (CP) type. Most plant viruses are not enveloped, and many accumulate to very high levels in their host. For these reasons, viruses such as tobacco mosaic tobamovirus (TMV), cowpea mosaic comovirus (CPMV) and johnsongrass mosaic potyvirus (JGMV) have been investigated for their potential use as epitope presenters with different degrees of

Present addresses: M. Bendahmane and R. N. Beachy, The Donald Danforth Plant Science Center, 7425 Forsyth Blvd., Box 1098, St. Louis, MO 63105, USA; M. Koo, Plant Molecular Biology and Biotechnology Research Center, Department of Biochemistry, Gyeongsang National University, Chinju 660-701, Korea.

Abbreviations used: CP, coat protein; TMV, tobacco mosaic virus; CPMV, cowpea mosaic comovirus; RV, rabies virus; w.t., wild-type; d.p.i., days post-inoculation; h.p.i., hours post-inoculation; MHV, murine hepatitis virus; HR, hypersensitive response.

E-mail address of the corresponding author: rnbeachy@danforthcenter.org

0022-2836/99/260009–12 $30.00/0 © 1999 Academic Press

success (Haynes *et al*., 1986; Hamamoto *et al*., 1993; Usha *et al*., 1993; Jagadish *et al*., 1993; Fitchen *et al*., 1995; Sugiyama *et al*., 1995; Turpen *et al*., 1995; Beachy *et al*., 1996; Lomonossoff & Johnson, 1996).

TMV is composed of a single type of CP of 17.5 kDa that encapsidates the 6.4 kb viral genome of positive-sense RNA into rod-shaped virus particles of about 300 nm × 18 nm. TMV possesses characteristics which make it an attractive particle on which to display peptides including epitopes: (i) the structure of the TMV CP (Figure 1(d)) is known at 2.9 Å resolution (Namba *et al*., 1989), making it possible to apply strategies for rational design of foreign sequences on the surface of virus particles. (ii) TMV CP self assembles, and assembled CP and virions are stable at optimal conditions (Durham *et al*., 1971). (iii) TMV accumulates to high levels in many host plants. Levels as high as 10 mg of virus per gram of fresh leaf material can be obtained by simple purification procedures.

Haynes *et al*. (1986) were the first to take advantage of the self-assembly properties of the TMV CP and expressed a peptide from VP1 of poliovirus 3 as a fusion with TMV CP in *Escherichia coli*. Rats that were immunized with the hybrid CP carrying the poliovirus epitope produced neutralizing antibodies specific to the poliovirus VP1 peptide.

In some cases, epitopes expressed on the surface of plant viruses interfere with the normal virus infection cycle. Such results were observed with TMV and CPMV where insertion of certain foreign

Sequences inserted

(a) TMV CP-MHV hybrids: 900 909 913

TMV-5B19 L L G C I G S T C A

TMV-RB19 R L L G C I G S T C A

TMV-RB19E R L L G C I G S T C A E

TMV-5B19L P L L G C I G S T C A E D G N

(b) TMV-RV hybrids: 404 418

TMV-31D A V Y T R I M M N-G G R L K R

TMV-2D:31D D D A V Y T R I M M N G G R L K R

TMV-4D:31D D D G D D A V Y T R I M M N G G R L K R

253 275

TMV-G5.24 P P D Q L V N L H D F R S D E I E H K V V E E

(c) Vector: pCPs/p

W T S154 G155 P A T OPA

TGG ACT AGT XXXXXX GGT CCT GCA ACT TGA

ACC TGA TCA YYYYYY CCT GGA CGT TGA ACT

SpeI PpuM1

NcoI KpnI CP

TMV-U1 cDNA clone (U3/12-4) NcoI

Replicase CP KpnI MP

(d) TMV coat protein Gly155 Assembly Ser154 Peptide

Figure 1. Virus hybrids and amino acid sequence of the peptides used in this study. (a) Peptides from the MHV S-glycoprotein. Numbers above the arrows represent amino acids in S-glycoprotein (Buchmeier *et al*., 1984; Luytjes *et al*., 1989). (b) Peptides from rabies virus glycoprotein and nucleoprotein. The amino acid coordinates in the nucleoprotein (for the peptide 31D) and in the glycoprotein (for the peptide G5-24) are indicated (Dietzschold *et al*., 1990). Amino acid residues inserted to neutralize the positive charge of the peptides are underlined. (c) Site of insertion of the peptides in TMV CP. The amino acid coordinates for w.t. CP are indicated (Goelet *et al*., 1982). The map of the vector pCPs/p developed in this study is shown. (d) Computer-assisted graphic representation of TMV CP structure. The position of the insertion of the different peptides at the C terminus of the CP is indicated.

sequences resulted in interference with viral assembly, loss of systemic infection, and/or induction of a necrotic response in the systemic host (Takamatsu *et al*., 1990; Porta *et al*., 1994; Beachy *et al*., 1996). Such effects limit the use of viruses for production of peptides, including their use as vaccines.

Here we used TMV as a model to investigate why some CP hybrids induce cell death or failed to accumulate in infected leaves. Our studies demonstrate the importance of the isoelectric point (pI) of the peptide and the pI:charge value of the hybrid CPs on cell death and virus infectivity. Such effects can be prevented by lowering the pI:charge value of the hybrid CP to a value close to that of wild-type (w.t.) TMV CP.

Results

Hybrid viruses and pCPs/p vector

Chimeric CP molecules were produced by inserting nucleotide sequences encoding different peptides between TMV nucleotides 6176 and 6177 or between nucleotides 6188 and 6189 (Goelet *et al*., 1982), which placed the peptides between amino acid residues Ser154 and Gly155 (as described by Fitchen *et al*., 1995) or after Thr158 (last amino acid of the CP). The chimeras were created using either PCR-based site-directed mutagenesis, or direct cloning in the vector pCPs/p (Figure 1(c)). The vector pCPs/p was constructed by inserting the restriction sites *Spe*I and *Ppu*MI in the CP gene sequence, making it possible to insert peptide sequences between amino acid residues Ser154 and Gly155 in the CP reading frame.

Analysis of TMV-rabies virus hybrids

RNA transcripts produced from cDNA clone TMV-G5.24, which contains the coding sequence of the peptide G5-24 from the rabies virus (RV) glycoprotein (amino acid residues 253 to 275; Dietzschold *et al*., 1990) fused to the TMV CP, produced normal necrotic local lesions on *Nicotiana tabacum* Xanthi-NN plants three to four days post-inoculation (d.p.i.). On inoculated *N. tabacum* Xanthi-nn, systemic infection with TMV-G5.24 was delayed by about 10 to 15 days compared with infection by w.t. TMV. Similar delays in systemic infection have been observed with certain assembly defective mutants of TMV CP (Dawson *et al*., 1988; Culver *et al*., 1995). Symptoms produced by TMV-G5.24 were more mild than those produced by w.t. TMV, and consisted of large yellow patches rather than light green-dark green symptoms produced by w.t. TMV.

Virus particles could be purified from the systemically infected leaves with a yield of 1 to 3 mg per gram of leaves. Total protein extracts from infected leaves and protein isolated from purified virus were separated by SDS-PAGE. As shown in Figure 2(a) and (b), CP-G5.24 migrated at the expected position for a protein with a molecular mass of about 20.3 kDa. Monoclonal antibody

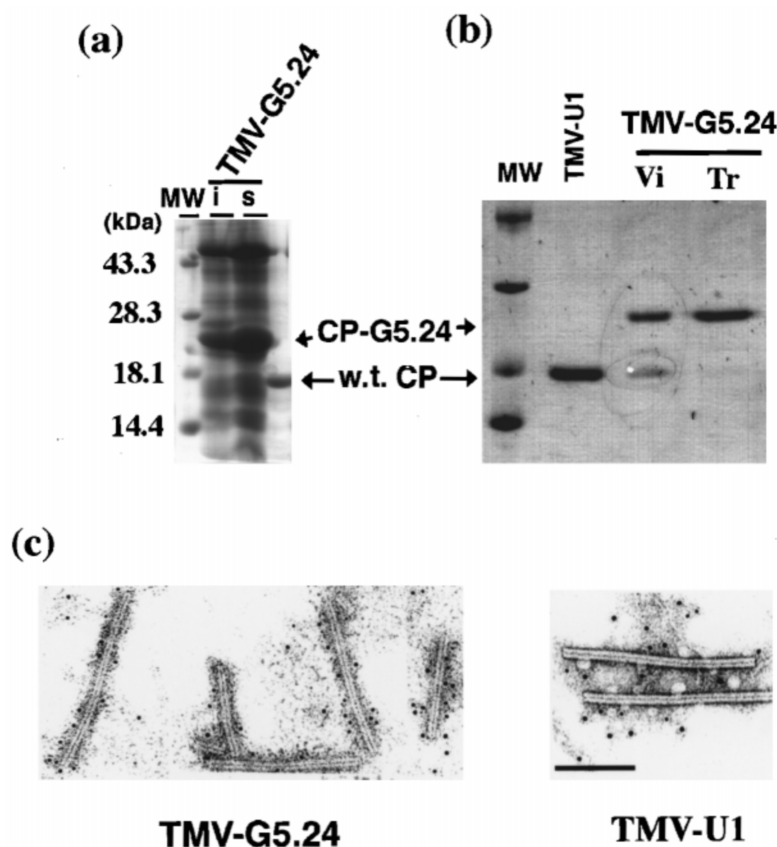

Figure 2. (a) Coomassie blue-stained gel showing the accumulation of hybrid CP-G5.24 in *N. tabacum* Xanthi-nn plants infected with TMV-G5.24. i, inoculated leaf; s, systemically infected leaf; w.t. CP, wild-type TMV coat protein; Mw, protein molecular mass standards, in kDa. (b) Coomassie blue-stained gel showing the patterns of the hybrid CP-G5.24 in virus purified from plant inoculated with *in vitro*-transcribed viral RNA (lane Tr), or from plant inoculated with TMV-G5.24 virus particles (lane Vi). (c) Immunogold labeling of TMV-G5.24 and TMV-U1 using the monoclonal MAb5 antibody raised against the G5-24 peptide. Electron microscopy was performed on Phillips CM100 electron microscope at magnification of 39,000 ×. The scale bar represents 200 nm.

anti-G5-24 (MAb5) was successfully bound to purified TMV-G5.24 in immunogold labeling experiments, as shown in Figure 2(c). These results demonstrate that the G5-24 peptide is exposed on or near the surface of the virion.

In contrast, when the 31D epitope, comprising amino acid residues 404 to 418 from the RV nucleoprotein was similarly inserted into the CP, the resulting virus TMV-31D failed to produce local or systemic infections on *N. tabacum* Xanthi-nn plants. We were unable to detect CP-31D or virus particles in inoculated or non-inoculated leaves. However, Western blot analysis of total protein from BY2 protoplasts inoculated with TMV-31D revealed that the CP-31D accumulated in these cells (Figure 3(b)). Virus particles could not be detected in extracts of the protoplasts. Northern blot analysis confirmed TMV-31D replication with concomitant accumulation of viral genomic and CP subgenomic RNAs in infected BY2 cells (data not shown).

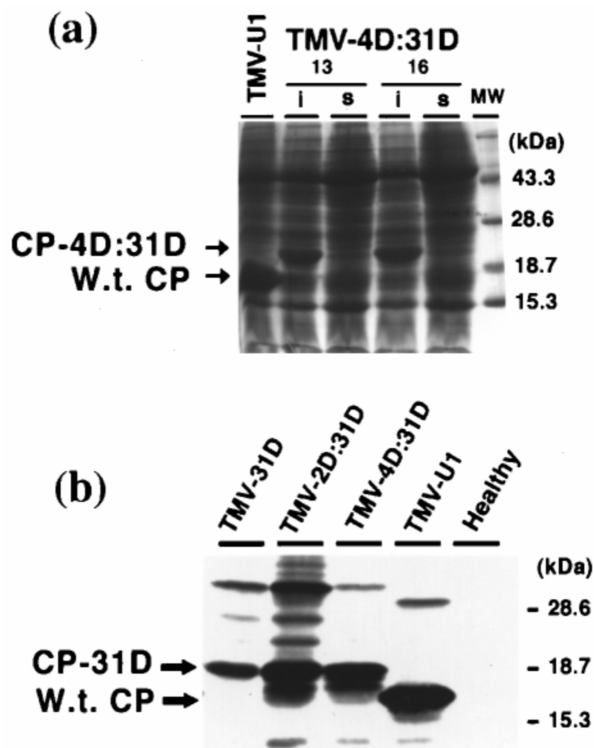

Figure 3. (a) Coomassie blue-stained gel of extracts of tobacco plants inoculated with two independent infectious clones of TMV-4D:31D, clones 13 and 16. The position of the CP-4D:31D is indicated. i, inoculated leaf; s, upper non-inoculated leaf; w.t. CP, wild-type TMV coat protein; Healthy, non infected tobacco plant; Mw, protein molecular mass standards, in kDa. (b) Western blot analysis of the accumulation of the CP-31D, CP-2D:31D and CP-4D:31D in infected BY2 protoplasts using anti-TMV antibody. The positions of molecular mass protein markers are shown in kDa.

We addressed the basis for the lack of accumulation of the CP-31D in inoculated leaf tissues by looking at the composition of the peptide 31D. Peptide 31D contains a high content of basic amino acids (three Arg residues and one Lys residue; Figure 1(b)), resulting in a peptide with a predicted pI of 12 (Table 1). This resulted in a pI:charge value of the hybrid CP-31D molecule of 9.14: + 2; the pI:charge value of w.t. CP is 5.31:-2. Using site-directed mutagenesis we introduced two Asp residues in the CP-31D between Ser154 of the CP and the 31D peptide. The resulting virus clone, referred to as TMV-2D:31D (Figure 1(b)), encodes CP-2D:31D with a pI:charge value of 7.09:0. CP-2D:31D failed to accumulate in inoculated *N. tabacum* Xanthi-nn plants, and in infected BY2 protoplasts CP-2D:31D accumulated to slightly higher levels compared with CP-31D (Figure 3(b)). Two additional Asp residues were inserted in CP-2D:31D to give rise to TMV-4D:31D (Figure 1(b)), bringing the pI:charge value of the chimeric CP to 5.43: − 2 (Table 1). TMV-4D:31D infected *N. tabacum* Xanthi-nn plants and the CP-4D:31D accumulated to high levels in inoculated leaves (Figure 3(a)) and assembled to form virus-like particles. However, the infection did not spread to upper leaves.

Analysis of TMV-MHV hybrids

The peptide 5B19 (amino acid residues 900 to 909; Buchmeier *et al*., 1984; Luytjes *et al*., 1989) from murine hepatitis virus (MHV) spike glycoprotein was inserted between Ser154 and Gly155 of the TMV CP (Koo *et al*., 1999). Virus that carries the hybrid CP-5B19 produced a systemic infection on *N. tabacum* Xanthi-nn, and virus particles were purified from infected plants (Koo *et al*., 1999). However, most of virus particles formed by TMV-5B19 were insoluble and co-purified with cell debris. The hybrid TMV-RB19, which contains an Arg residue between Ser154 of the CP and the 5B19 peptide (Figure 1(a); this virus was created to facilitate release of the MHV peptide by trypsin digestion) produced necrotic local lesions on *N. tabacum* Xanthi-NN similar to those produced by TMV-U1. On the systemic host *N. tabacum* Xanthi-nn, TMV-RB19 also produced a hypersensitive response, and the inoculated plants developed necrotic local lesions that limited the virus to the inoculated leaves (Figure 4(a)).

To determine if the necrotic reaction was caused by the position at which the epitope was inserted or due to this peptide *per se*, the RB19 epitope was inserted in-frame after Thr158 (last amino acid residue of CP) to generate TMV-RB19-2. When TMV-RB19-2 was applied to Xanthi-nn plants, necrotic local lesions similar to those observed with TMV-RB19 developed on the inoculated leaves (data not shown). These results suggest that the inserted peptide is responsible for the observed phenotype and not its site of insertion in the CP.

Table 1. Effect of the insertion of the different peptides on the TMV CP isoelectric point:total charge value

Hybrids	Peptide	Peptide pI[a]	pI/total charge of hybrid CP	Mw of hybrid CP[b] (kDa)	Local movement on Xanthi-nn[c]	Systemic movement on Xanthi-nn[d]
CP-5B19L	PLLGCIGSTCAEDGN	3.84	5.01/ − 4	18.9	Yes	Yes
CP-5B19	LLGCIGSTCA	5.94	5.31/ − 2	18.4	Yes	Yes
CP-RB19	RLLGCIGSTCA	8.59	5.64/ − 1	18.5	HR	No
CP-RB19E	RLLGCIGSTCAE	6.42	5.34/ − 2	18.7	Yes	Yes
CP-31D	AVYTRIMMNGGRLKR	12	9.14/ + 2	19.2	No	No
CP-2D:31D	DDAVYTRIMMNGGRLKR	10.81	7.09/0	19.4	No	No
CP-4D:31D	DDGDDAVYTRIMMNGGRLKR	7.32	5.43/ − 2	19.7	Yes	No
CP-G5.24	PPDQLVNLHDFRSDEIEHLVVEE	4.40	4.92/ − 8	20.2	Yes	Yes
CP wt	–	–	5.31/ − 2	17.5	Yes	Yes

[a] Predicted isoelectric point.
[b] Molecular mass deduced from the gene sequence.
[c] Local and [d]systemic infectivity of viruses carrying the different hybrid CPs on *N. tabacum* Xanthi-nn plants: HR, hypersensitive response; No, little or no CP and virus accumulation in inoculated leaves.

The size of the necrotic lesions that developed on Xanthi-nn leaves inoculated with TMV-RB19 was compared to those induced by w.t. TMV or by TMV-RB19 on Xanthi-NN plants (Figure 4(b)). The local lesions on Xanthi-nn were about 1.5 to two times larger than those that developed on Xanthi-NN plants.

Cell death in BY2 protoplasts that were infected with TMV-RB19 was significantly greater than in protoplasts infected by w.t. TMV or TMV-5B19 (Figure 4(c)). As shown in Figure 4(d), infection with TMV-RB19 caused cell death starting between 24 and 36 hours post-inoculation (h.p.i.) regardless of whether the protoplasts were held at 24 °C or 31 °C. At 48 h.p.i. about 65 % to 80 % of cells were not viable. In comparison, protoplasts that were infected with w.t. TMV exhibited viability of about 60 % through 48 h.p.i.

Analysis by electron microscopy of extracts from BY2 cells infected with TMV-RB19, revealed the presence of virus particles similar in appearance to w.t. TMV; in contrast to w.t. TMV, TMV-RB19 was always attached to cell debris (data not shown).

The peptide RB19 has a predicted pI of 8.59, and when inserted in the CP it results in pI:charge value of 5.64: − 1 for CP-RB19. When a Glu residue was inserted between the RB19 peptide and Gly155 of the CP (Figure 1), the calculated pI:charge value of the hybrid CP-RB19E was 5.34:-2 (equivalent to the w.t. CP; Table 1). The resulting hybrid, referred to as TMV-RB19E, did not induce a hypersensitive response on the inoculated leaves of Xanthi-nn plants (Figure 4(a)) and led to systemic infection. Likewise, the virus did not increase the rate of cell death in infected BY2 protoplasts (Figure 4(c) and (d)). Typical mosaic symptoms were produced by TMV-RB19E infection on Xanthi-nn plants, and virus particles were purified from systemically infected leaves.

Protein extracts prepared from infected plants and CP from purified virus were separated on SDS-PAGE and analyzed by Coomassie blue staining and Western immunoblot assays, using anti-TMV antibody or the monoclonal anti-5B19 antibody (MAb5B19) (Figure 5(a)). CP-RB19E hybrid protein of the predicted molecular mass (18.7 kDa) was present at high levels in infected plants and in assembled virus (Figure 5(a)). Electron microscopy following immunogold labeling using MAb5B19 showed specific labeling of TMV-RB19E, whereas no labeling was obtained with w.t. TMV (Figure 5(b)). In addition, treating purified TMV-RB19E particles with trypsin released the RB19E peptide from the core of CP (data not shown). These results confirm that the peptide is exposed on the surface of virus particles.

Analysis of induced cell death in protoplasts and hypersensitive response in Xanthi-nn

Brederode *et al.* (1991) reported that infection by TMV of the necrotic local lesion host *N. tabacum* Xanthi-NN genotype activates high levels of expression of the acidic PR2 gene both locally and systemically, and low levels of expression of the basic PR2 gene locally, but not systemically. We investigated whether or not the PR2 genes are activated in BY2 protoplasts and in *N. tabacum* Xanthi-nn and Xanthi-NN plants following infection by w.t. TMV or TMV mutants. RNA dot blot analyses were performed using total RNA purified from protoplasts (at 24 and 36 h.p.i.), Xanthi-nn (4 d.p.i.), and Xanthi-NN (4 d.p.i.) plants inoculated with TMV-RB19, TMV-RB19E or w.t. TMV-U1. ^{32}P-labeled DNA molecules specific to acidic or to basic PR2 gene were used as probes. There was little or no induction of acidic or basic PR2 mRNA in BY2 protoplasts infected with w.t. TMV or with hybrid viruses (data not shown). In contrast, TMV-RB19 induced accumulation of acidic and basic PR2 gene expression in inoculated leaves of *N. tabacum* Xanthi-nn and Xanthi-NN (Figure 6). In Xanthi-NN plants inoculated with TMV-RB19, basic PR2 gene expression was about two times greater than that on TMV inoculated Xanthi-NN plants, whereas the accumulation of acidic PR2

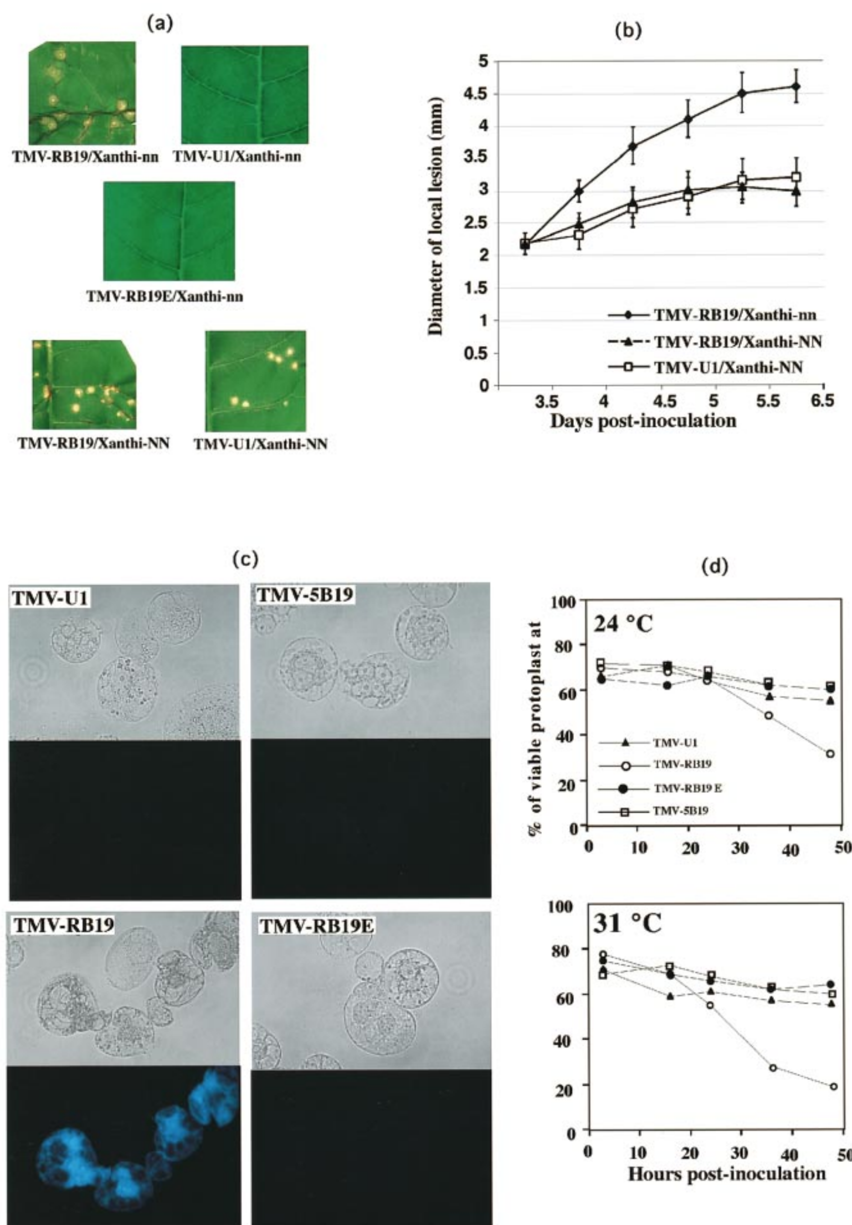

Figure 4. (a) Symptoms of TMV-U1, TMV-RB19 and TMV-RB19E on inoculated leaves of *N. tabacum* Xanthi-nn and Xanthi-NN plants at 5 d.p.i. Plants were inoculated with *in vitro* transcribed viral RNAs and held at 26 °C. (b) Change in diameters of necrotic local lesions produced by TMV-RB19 on inoculated *N. tabacum* Xanthi-nn and by TMV-U1 or TMV-RB19 on *N. tabacum* Xanthi-NN. (c) and (d) Viability of BY2 protoplasts following infection with TMV-U1, TMV-5B19, TMV-RB19 or TMV-RB19E. Protoplasts were inoculated with viral RNA and then incubated at 24 or 31 °C. (c) Light microscopy (top) and fluorescence microscopy (bottom) showing the auto-fluorescence of dead cells at 36 h.p.i. (d) Percentage of viable cells determined microscopically after staining for live cells with FDA.

mRNA was about the same in both plants (Figure 6). On inoculated leaves (IL) of *N. tabacum* Xanthi-nn plants, high levels of mRNAs of acidic and basic PR2 gene expression was observed after inoculation with TMV-RB19, but not by w.t. TMV or TMV-RB19E (Figure 6 and data not shown). Basic PR2 gene expression was induced in inoculated leaves, but not in non-inoculated upper leaves (SL; Figure 6(a)). However, basic PR2 mRNA levels in Xanthi-nn inoculated with TMV-RB19 was about 20 times higher than in Xanthi-nn leaves inoculated with w.t. TMV, and about three to five times higher than in Xanthi-NN leaves inoculated with TMV or TMV-RB19 (Figure 6(a)). In contrast, the accumulation of acidic PR2 mRNA in Xanthi-nn inoculated with TMV-RB19 was similar to that observed on Xanthi-NN inoculated with TMV or TMV-RB19 (Figure 6(b)). These studies demonstrate that the hypersensitive response on Xanthi-nn plants resulting from TMV-RB19 infection triggered the expression of the acidic and basic PR2 genes.

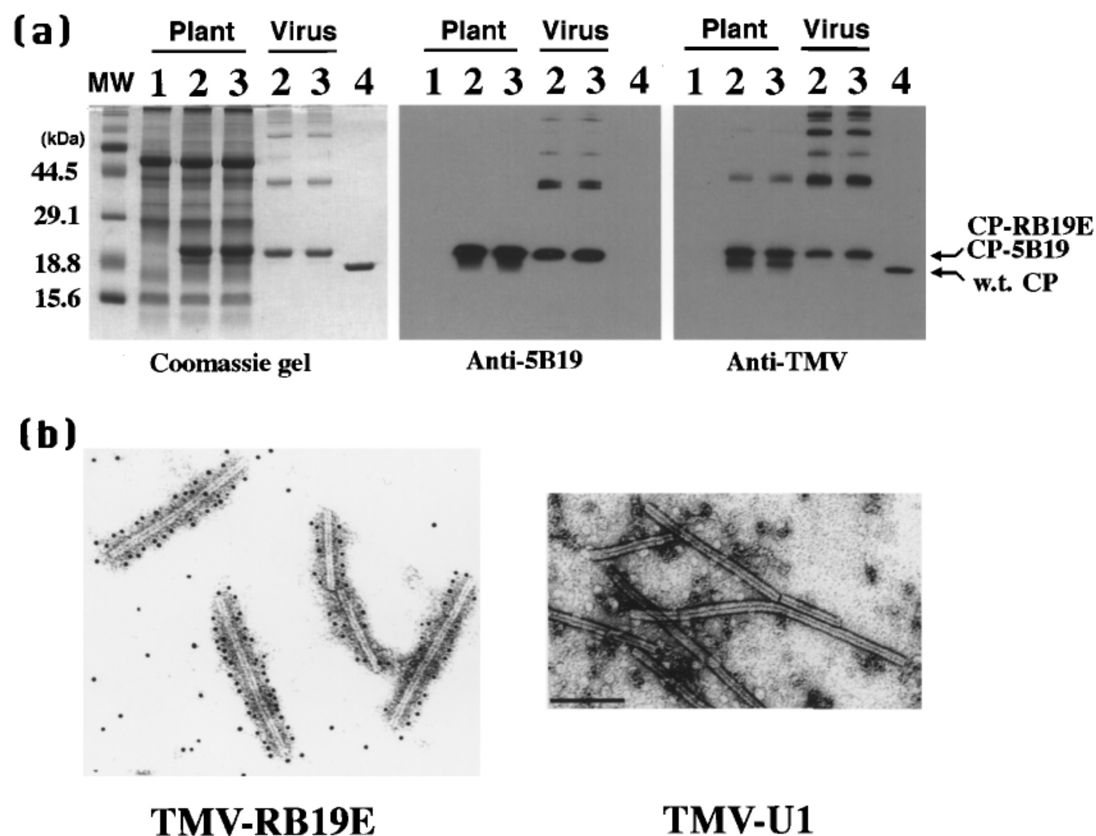

Figure 5. (a) Characterization of CP-5B19 and CP-RB19E. Proteins were extracted from infected leaves or from purified virus, separated by SDS-PAGE, and stained with Coomassie blue or electroblotted to nitrocellulose membrane and reacted with anti-5B19 or anti-TMV antibodies. Samples are from plants inoculated with TMV-5B19 (sample 2), TMV-RB19E (sample 3), TMV-U1 (sample 4) or from healthy plant (sample 1). Mw, protein molecular mass in kDa. (b) Immunogold labeling of TMV-RB19E and TMV-U1 (negative control) using monoclonal antibody raised against the 5B19 peptide. Electron microscopy was performed on Phillips CM100 electron microscope at magnification of 39,000 $\times$. The scale bar represents 200 nm.

Discussion

Expression of immunogenic peptides as fusion proteins with plant virus capsid proteins can lead to production of large amounts of the peptide. TMV CP is a good carrier because it self-assembles in an ordered manner, and accumulates to large amounts in infected tissues. Furthermore, as the atomic structure of TMV is known, one can design proteins that display foreign peptides on the surface of the virion. CP molecules have presumably evolved a variety of functions (e.g. efficient assembly), and in some cases, play an important role in virus movement and/or replication (Matthews, 1991). Mutations in viral CP can cause loss of certain CP functions (Bendahmane *et al.*, 1997). Similarly, display of peptides on the surface of TMV as fusion to the CP can reduce local and systemic virion accumulation in infected plants, and can induce the expression of host defense genes (Beachy *et al.*, 1996; this study).

In this study peptides from two different viruses, rabies virus (a lyssavirus) and murine hepatitis virus (a coronavirus) were produced as fusion proteins with TMV CP. The 23 amino acid residues of peptide G5-24 from the RV glycoprotein, and the ten amino acid residue peptide containing the 5B19 epitope from MHV spike protein, were successfully exposed on the surface of TMV. The respective viruses, TMV-G5.24 and TMV-5B19, systemically infected *N. tabacum* Xanthi-nn. However, insertion of the peptide 31D from the RV nucleoprotein in TMV CP (TMV-31D) reduced the infectivity of the hybrid virus TMV-31D in plants. In BY2 protoplasts TMV-31D replicated and CP-31D accumulated to high levels (Figure 3(b)). The results indicate that the lack of virus accumulation in infected plant leaves may result from interference with local movement. This important finding remains unexplained, since it has been shown that local cell to cell spread of TMV does not require CP. A possible explanation for these results is that leaf cells that are infected are killed before local spread can occur.

TMV-RB19 contained an Arg plus the peptide RB19 (from the MHV S-glycoprotein) inserted in CP. Infection by this virus produced necrotic lesions on *N. tabacum* Xanthi-NN similar to those produced by w.t. TMV. However, in contrast to w.t. TMV, TMV-RB19 induced a hypersensitive response (HR) on the systemic host *N. tabacum* Xanthi-nn. In this case the addition of a single

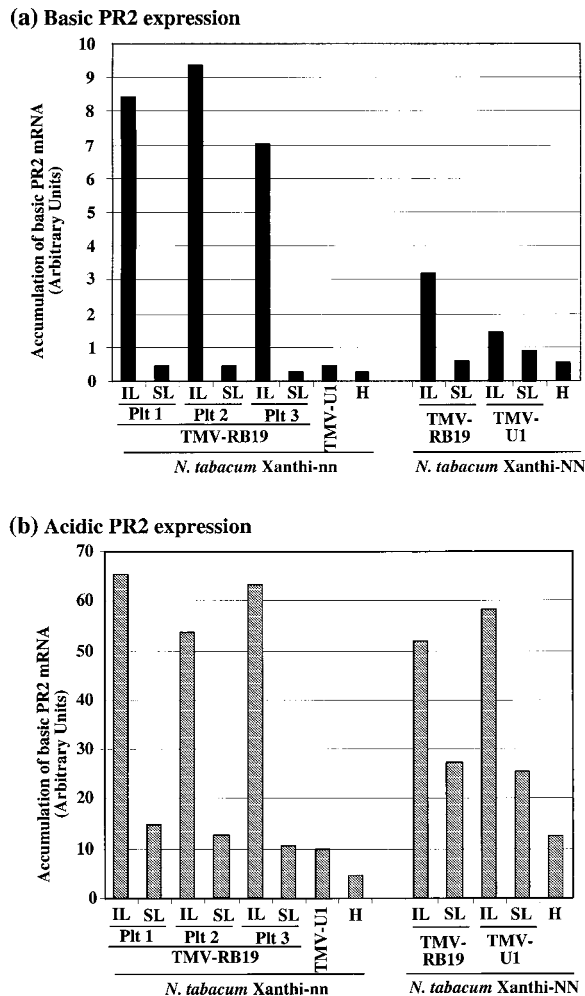

Figure 6. Accumulation of (a) basic and (b) acidic PR2 mRNA in *N. tabacum* Xanthi-nn and Xanthi-NN plants following inoculation with TMV-U1 or TMV-RB19 at 4 d.p.i. RNA dot blots were performed using similar amounts of total RNA and then hybridized with sequences to detect (a) basic or (b) acidic PR2 gene expression. The signal intensity of the isotope label was measured using phosphoimager followed by Multi-Analyst computer program analyses (Bio-Rad, Hercules, CA), and the relative values are displayed as graphs.

positively charged residue to the peptide inserted in the CP resulted in induction of HR in this host.

Comparison of the hybrid CPs that did not cause necrosis on Xanthi-nn plants with those that did, led us to propose that the overall charge of the CP is important in inducing necrosis. The peptides that, when inserted in the CP, resulted in a pI equal to or lower than the w.t. CP (e.g. G5-24, 5B19, and 5B19L; Figure 1(a); Table 1), were successfully displayed on TMV particles, and the chimeric viruses caused systemic infections. In contrast, peptides that resulted in a pI of the CP that is higher than that of w.t. CP resulted in the induction of HR (Table 1). Altering the pI:charge value in hybrid CP to bring it near to that of w.t. CP, 5.31: − 2 (by inserting Asp to Glu residues before or after the peptide) eliminated the necrotic reaction and resulted in local and systemic infection (Table 1). These results confirmed the importance of pI:charge value of the hybrid protein, and show that highly positively charged peptides can influence the interaction of TMV with the host. In contrast, negatively charged epitopes are more tolerated than are positively charged epitopes (e.g. G5-24 and 5B19L peptides; Table 1). These principles were recently applied to successfully generate new hybrid TMV constructs that express the MG3 epitope from the lymphocyte choriomeningitis virus (LCMV) GP1 protein (M.B., J. Paterson, M.B.A. Oldstone & R.N.B., unpublished results), and the 9-14 epitope from the outer membrane F1 protein from *Pseudomonas aeruginosa* (M.B., J. Staczek & R.N.B., unpublished results).

TMV induces local necrotic lesions on *N. tabacum* Xanthi-NN as a result of the *N* resistance gene (Whitham *et al.*, 1994) and an uncharacterized interaction with TMV replicase (Padgett & Beachy, 1993; Padgett *et al.*, 1997; Abbink *et al.*, 1998). Although *N. tabacum* Xanthi-nn lacks the *N* gene, the hybrid virus TMV-RB19 induced HR on these plants. The mechanism of induction of HR by TMV-RB19 on Xanthi-nn plants is not known. However, the signal transduction pathway that results in HR on Xanthi-NN and Xanthi-nn may, at least in a part, be different. This is supported by the observation that TMV-RB19 induces cell death in infected BY2 protoplasts whereas w.t. TMV does not. Furthermore, protoplasts derived from Xanthi-NN plants do not die when infected with TMV (Otsuki *et al.*, 1972). It is known that infection by TMV induces the expression of the basic PR2 gene in the inoculated leaves and the expression of the acidic PR2 gene both locally and systemically in Xanthi-NN (Brederode *et al.*, 1991; Heitz *et al.*, 1994), but not in Xanthi-nn plants that lack the *N* gene (Linthorst, 1991). In contrast to Xanthi-nn plants inoculated with w.t. TMV, Xanthi-nn plants that produce necrotic lesions following inoculation with TMV-RB19 resulted in the expression of the basic and the acidic PR2 genes. These results indicate that the HR observed in the Xanthi-nn plants inoculated with TMV-RB19 may share a portion of the HR signal transduction pathway with the HR in Xanthi-NN.

It is known that the *N′* resistance gene in *Nicotiana sylvestris* does not respond to infection by w.t. TMV. However, certain CP mutants of TMV that are structurally altered to affect its ordered quaternary structure elicit the expression of the *N′* gene (Knorr & Dawson, 1988; Culver *et al.*, 1994). It was suggested that structures created by the mutations interact with an uncharacterized host receptor to elicit the HR (Culver *et al.*, 1994). The HR that results from infection by TMV-RB19 on *N. tabacum* Xanthi-nn may be similar to, or different from, the *N′* elicited HR; additional studies are needed to address this question.

The question remains as to why BY2 protoplasts die as a result of TMV-RB19 infection. An answer

may reside in the fact that TMV-RB19 and TMV-5B19 assemble to form virus particles which are very insoluble in plant cells. Indeed, electron microscopy experiments showed that virus particles were recovered from membrane-rich fractions rather than from a soluble supernatant fraction. This may indicate that insertion of the RB19 or 5B19 peptides at the C terminus of TMV CP creates a domain on the surface of assembled viruses that interacts with and destabilizes cellular membranes, thus causing BY2 cell death. Porta *et al*. (1994) studied the expression of an epitope from foot-and-mouth disease virus (FMDV) on the surface of CPMV and found an unusual affinity of the hybrid CPMV-FMDV with cellular membranes. Porta *et al*. (1994) proposed a direct involvement of three amino acid residues (Arg-Gly-Asp) of the FMDV epitope in affinity with the membrane. The predicted hydrophobicity of the CP-5B19 and CP-RB19 compared to w.t. CP suggests that both proteins may create hydrophobic domains at the C terminus of the hybrid CP that is not present in w.t. CP. This domain may interact with and anchor the CP and/or the virus with cell membranes.

Our data support the conclusion that the pI of the peptide-CP fusion protein influences the interaction of the virus with the host. The TMV CP tolerates negatively charged peptides, but is affected by positively charged epitopes. The data presented here also demonstrate that these effects are reversible, and that bringing the pI:charge value closer to that of w.t. CP reduces or eliminates the ability of the fusion protein to cause cell death in infected plants and protoplasts.

Materials and Methods

Plasmid construction

Construction of TMV-5B19, TMV-5B19L and TMV-RB19

TMV-5B19 and TMV-5B19L containing the nucleotide sequences coding for the peptides containing the 5B19 epitope ($_{900}$LLGCIGSTCA$_{909}$) or the epitope 5B19 plus five neighboring amino acid residues (5B19L: $_{899}$PLLGCIGSTCAEDGN$_{913}$) from murine hepatitis virus (MHV, coronavirus) S-glycoprotein are described by Koo *et al*., (1999). TMV-RB19 harbors the peptide containing an arginine residue plus 5B19 fused between amino acid residues Ser154 and Gly155 of the TMV coat protein (Figure 1; Table 1), and was constructed using the following primers: 5′tAGA TTA TTA GGA TGT ATA GGA TCT ACT TGT GC3′ and its complement 3′gaTCT AAT AAT CCT ATA TAT CCT AGA TGA ACA C5′. The two primers containing the nucleotide-coding sequence of the RB19 peptide were mixed, heated and allowed to anneal. The DNA fragment formed by the annealed primers contains *Ahd*I compatible ends that was used for cloning into the TMV cDNA clone linearized with *Ahd*I. The resulting clone TMV-RB19 contains an arginine residue followed by the nine amino acid residues of the 5B19 epitope between Ser154 and Gly155 of the TMV CP.

Construction of TMV-RB19E

The primers 5′CT TGT GCT GAA GGT CCT GCA ACT TGA GG3′ and its complement 5′CC TTC AGC ACA AGT AGA TCC TAT AC3′ were used in PCR reactions to insert a glutamic acid residue between Ala165 and Gly167 of the hybrid CP:RB19 in the TMV-RB19 cDNA clone. The resulting construct is referred to as TMV-RB19E (Figure 1; Table 1).

Construction of the TMV-31D and TMV-G5.24 clones

The nucleotide sequence coding for the peptide 31D (Dietzschold *et al*., 1990) of the rabies virus nucleocapsid protein ($_{404}$AVYTRIMMNGGRLKR$_{418}$) or for the peptide G5-24 (Dietzschold *et al*., 1990) from the RV glycoprotein ($_{253}$PPDQLVNLHDFRSDEIEHLVVEE$_{275}$) were fused between Ser154 and Gly155 of the TMV CP in the clone pKN2 (Bendahmane *et al*., 1997) *via* a PCR-based reaction using the following primers: 5′CT CGA ATC ATG ATG AAT GGA GGT CGA CTA AAG AGA ggt cct gca act tga ggt agt c3′ and 5′CG ACC TCC ATT CAT CAT GAT TCG AGT ATA AAC AGC aga ggt cca aac caa acc aga ag3′, or 5′CTG CAC GAC TTT CGC TCA GAC GAA ATT GAG CAC CTT GTT GTA GAG GAG ggt cct gca act tga ggt agt c3′ and 5′AAT TTC GTC TGA GCG AAA GTC GTG CAG GTT CAC CAA CTG ATC GGG AGG aga ggt cca aac caa acc aga ag3′, respectively. Sequences in upper case represent the epitope sequence, while those in lower case represent the sequence of the TMV CP. The resulting constructs pCP:31D and pCP:G5.24 contain the 31D or G5-24 epitopes, respectively, inserted between Ser154 and Gly155 in the TMV CP (Figure 1; Table 1).

The hybrids pCP-2D:31D and pCP-4D:31D were constructed by inserting the dipeptide AspAsp or the pentapeptide AspAspGlyAspAsp between Ser154 and Ala155 of the 31D peptide in the pCP:31D construct *via* PCR-based reactions using the following primers: 5′ggt ttg gtt tgg act agt GAC GAC GCT GTT TAT ACT CG 3′ or 5′ ct agt GAC GAC GGT GAC GAC GCT GTT TAT ACT CGA ATC3′ and 5′GTC GTC ACC GTC GTC act agt cca aac caa acc aga aga g3′, respectively. Sequences in upper case represent the epitope sequence, those underlined represent the AspAsp or AspAspGlyAspAsp, and sequences in lower case represent the sequence of the TMV CP.

The pCP-2D:31D construct was engineered to create a new vector with the unique restriction sites *Spe*I and *Ppu*MI to facilitate the cloning of any nucleotide sequence between amino acid residues Ser154 and Gly155 of the TMV CP (Figure 1(c)). The vector is termed pCPs/p.

The fragment *Nco*I-*Kpn*I from the plasmids pCP:31D, pCP-2D:31D, pCP-4D:31D and pCP:G5.24 carrying hybrid CP sequences were used to replace the homologue in the TMV cDNA clone U3/12-4 (Holt & Beachy, 1991) linearized with *Nco*I plus *Kpn*I to generate infectious full-length cDNA clones referred to as pTMV-31D, pTMV-2D:31D, pTMV-4D:31D and pTMV-G5.24, respectively.

Synthesis of *in vitro* transcripts and inoculation of plants

In vitro transcription reactions were used to produce full-length viral RNA from 1 μg of plasmids pTMV-31D, pTMV-2D:31D, TMV-4D:31D, pTMV-G5.24, pTMV-5B19,

pTMV-RB19, pTMV-RB19E or U3/12-4 (wild-type TMV clone) previously linearized with *Kpn*I as described (Holt & Beachy, 1991). Leaves of four-week-old *N. tabacum* Xanthi-NN and Xanthi-nn plants were first dusted with carborundum (320 Grit, Fisher Scientific) and then inoculated with the *in vitro* transcription reaction products.

Purification and analysis of virus particles from infected plants

Wild-type TMV and the hybrid TMV-G5.24 were purified from systemically infected leaves of *N. tabacum* Xanthi-nn plants as described (Asselin & Zaitlin, 1978). To purify hybrid TMV-RB19E, infected plant leaves were ground in liquid nitrogen and homogenized in 20 mM $NaHPO_4$ buffer (pH 7). Cell debris was removed by centrifugation at 3000 *g*. 5 % (w/w) diatomaceous earth (Sigma) was mixed with the supernatant and centrifuged at 10,000 *g* for 30 minutes. The supernatant was then applied to a cushion of 20 % sucrose in 20 mM Tris-HCl (pH 7). After centrifugation at 140,000 *g* for one hour 30 minutes, the pellet was dissolved in 20 mM $NaHPO_4$ buffer (pH 7), and sonicated for one minute. Triton X-100 (5 %, v/v) was then added to the virus solution and the suspension was applied again on a cushion of 20 % sucrose in 20 mM Tris-HCl (pH 7), as above, and subjected to centrifugation. The pellet containing the virus was dissolved in 20 mM $NaHPO_4$ (pH 7).

Immunogold labeling and electron microscopy (EM)

Purified virus at concentrations of 10 to 100 µg/ml was applied to carbon-coated copper EM grids. The grids were then blocked with 0.1 % bovine serum albumin (BSA) solution in 1 × PBS for 15 minutes. The monoclonal antibodies anti-5B19 (MAb5B19; Buchmeier *et al*., 1984) for TMV-RB19E or anti-G5.24 (MAb5; Dietzschold *et al*., 1990) for TMV-G5.24 (diluted 1/50 in 1 × PBS containing 0.1 % BSA) were added for one hour, followed by five washes with 50 µl of 1 × PBS. Goat anti-mouse antibody conjugated with 5 nm gold particles (Ted Pella, Redding, CA) were added to the grid at room temperature for one hour. To remove non-specific binding, six washes with 50 µl of 1 × PBS followed by four washes with water were applied. The grids were then negatively stained with 1 % phosphotungstic acid solution (pH 5.6), for two minutes. Grids were dried and examined on a Phillips CM100 electron microscope at 39,000× magnification.

Preparation and inoculation of tobacco protoplasts

Tobacco protoplasts were prepared from BY2 suspension cell cultures; 2×10^6 protoplasts were inoculated with RNA transcribed *in vitro* from above cDNA clones of w.t. or hybrid viruses *via* electroporation as described by Watanabe *et al*. (1987). Protoplasts were harvested at different times post-inoculation and used for further analyses.

Measurement of cell death in infected protoplasts

Suspensions of protoplasts were stained for live cells using fluorescence diacetate (FDA) staining solution (0.1 ml of 0.05 % FDA stock solution plus 5 ml of protoplast culture medium). One volume FDA staining solution was mixed with one volume of suspended protoplasts in a hemocytometer. After five minutes, live cells were visualized and counted with a Nikon Optiphot2-UD microscope equipped with a Nikon B-2A filter set consisting of a 450-490 excitation filter as described by Kahn *et al*. (1998).

Coomassie blue staining and Western blots

Total protein extracts from leaves or from protoplasts were homogenized in 1 × Laemmli loading buffer (Laemmli, 1970), boiled for four minutes and applied to 12.5 % polyacrylamide gels containing SDS (SDS-PAGE). Proteins were either directly stained with Coomassie blue or analyzed by Western immunoblot using as first antibody the polyclonal rabbit anti-TMV, the monoclonal MAb5B19 mouse anti-5B19 peptide or the monoclonal MAb5 anti-G5-24 peptide using standard methods (Sambrook *et al*., 1989). Goat anti-rabbit or goat anti-mouse antibodies conjugated with horse radish peroxidase or alkaline phosphatase were used as secondary antibodies and the signal was revealed using the chemiluminescence ECL-kit (Amersham) according to the manufacturer's recommendation and as described by Sambrook *et al*. (1989).

Analysis of pathogenesis related (PR2) mRNA expression

Total RNAs were purified from BY2 protoplasts at 24 and 36 h.p.i. with TMV, TMV-RB19 or TMV-RB19E using the standard guanidine thiocyanate extraction method (Sambrook *et al*., 1989). Total RNA was purified from *N. tabacum* Xanthi-nn and Xanthi-NN plants four days post-inoculation with TMV-RB19 or TMV-U1. Leaf discs (1 cm in diameter) containing necrotic lesions were collected and total RNA was purified as follows. Leaf discs were homogenized in the extraction buffer (0.2 M Tris-HCl (pH 7.5), 0.1 M LiCl, 5 mM EDTA, 1 % (w/v) SDS). The homogenate was extracted with phenol/chloroform/isoamyl alcohol and RNA were then precipitated in the presence of 3 M LiCl. The pelletted RNA was resuspended in H_2O and then precipitated in the presence of 2.5 volumes of ethanol. RNA dot blots were performed by applying similar amounts of total RNA onto a nylon membrane; blotted RNAs were hybridized with sequences to detect acidic or basic PR2 genes expression using specific PR2 DNA sequences as described by Karrer *et al*. (1998). After stringent washes the intensity of the radioactive label was measured using a phosphoimager followed by Multi-Analyst computer program analyses according to the manufacturers recommendations (Bio-Rad, Hercules, CA).

Acknowledgments

We thank Drs Judit Szecsi, John Christodoulou, Dean Rochester and Alexandre De Kochko for critical reading of the manuscript. We thank Dr Michael J. Buchmeier (The Scripps Research Institute) for the MAb5B19 antibody. This work was supported by funds from The Scripps Family Chair, Jean & Keith Kellogg, The Seaver Foundation, the Plant Molecular Biology and Biotechnology Research Center (South Korea), and Aline & Sam Skaggs. Other support was provided by grant AI27161 from the National Institutes of Health.

References

Abbink, T. E. M., Tjernberg, P. A., Bol, J. F. & Linthorst, H. J. M. (1998). Tobacco mosaic virus helicase domain induces necrosis in N gene-carrying tobacco in the absence of virus replication. *Mol. Plant Microbe Interact.* **11**, 1242-1246.

Asselin, A. & Zaitlin, M. (1978). Characterization of a second protein associated with virions of tobacco mosaic virus. *Virology,* **91**, 173-181.

Beachy, R. N., Fitchen, J. H. & Hein, M. B. (1996). Use of plant viruses for delivery of vaccine epitopes. *Ann. NY Acad. Sci.* **792**, 43-49.

Bendahmane, M., Fitchen, J. H., Zhang, G. & Beachy, R. N. (1997). Studies of coat protein mediated resistance to TMV: correlation between assembly of mutant coat proteins and resistance. *J. Virol.* **71**, 7942-7950.

Brederode, F. T., Linthorst, H. J. M. & Bol, J. F. (1991). Differential induction of acquired resistance and PR gene expression in tobacco by infection, ethephon treatment, UV light and wounding. *Plant Mol. Biol.* **17**, 1117-1125.

Buchmeier, M. J., Lewicki, H. A., Talbot, P. J. & Knobler, R. L. (1984). Murine hepatitis virus-4 (starin JMH)-induced neurologic disease is modulated *in vivo* by monoclonal antibody. *Virology,* **132**, 261-270.

Burke, K. L., Dunn, G., Ferguson, M., Minor, P. D. & Almond, J. W. (1988). Antigen chimeras of poliovirus as a potential new vaccines. *Nature,* **322**, 81-82.

Culver, J. N., Stubbs, G. & Dawson, W. O. (1994). Structure-function relationship between tobacco mosaic virus coat protein and hypersensitivity in *Nicotiana sylvestris*. *J. Mol. Biol.* **242**, 130-138.

Culver, J. N., Dawson, W. O., Plonk, K. & Stubbs, G. (1995). Site-directed mutagenesis confirms the involvement of carboxylate groups in the disassembly of tobacco mosaic virus. *Virology,* **206**, 724-730.

Dawson, W. O., Bubrick, P. & Grantham, G. L. (1988). Modifications of the tobacco mosaic virus coat protein gene affecting replication, movement, and symptomatology. *Phytopathology,* **78**, 783-789.

Dedieu, J-F., Ronco, J., Van der Werf, S., Hogle, J. M., Henin, Y. & Girard, M. (1992). Poliovirus chimeras expressing sequences from the principal neutralization domain of human immunodeficiency virus type 1. *J. Virol.* **66**, 3161-3167.

Dietzschold, B., Gore, M., Marchadier, D., Nui, H. S., Bunchoten, H. M., Otvos, L., Wunner, W. H., Ertl, H. C. J., Osterhaus, A. D. M. E. & Koprowski, H. (1990). Structural and immunological characterization of a linear virus-neutralizing epitope of rabies virus glycoprotein and its possible use in a synthetic vaccine. *J. Virol.* **64**, 3804-3809.

Durham, A. C. H., Finch, J. T. & Klug, A. (1971). States of aggregation of tobacco mosaic virus protein. *Nature,* **229**, 37-42.

Fitchen, J. H., Beachy, R. N. & Hein, M. B. (1995). Plant virus expressing hybrid coat protein with added murine epitope elicit autoantibody response. *Vaccine,* **13**, 1051-1057.

Goelet, P., Lomonossoff, G. P., Butler, P. J. G., Akam, M. E., Gait, M. J. & Karn, J. (1982). Nucleotide sequence of tobacco mosaic virus RNA. *Proc. Natl Acad. Sci. USA,* **79**, 5818-5822.

Hamamoto, H., Sugiyama, Y., Nakagawa, N., Hashida, E., Matsunaga, Y., Takemoto, S., Watanabe, Y. & Okada, Y. (1993). A new tobacco mosaic virus vector and its use for the systemic production of natigensin-I-converting enzyme inhibitor in transgenic tobacco and tomato. *Bio/Technology,* **11**, 930-932.

Haynes, J. B., Cunningham, J., von Seefried, A., Lennick, M., Garvin, R. T. & Shen, S. (1986). Development of a genetically-engineered, candidate polio vaccine employing the self-assembling properties of the tobacco mosaic virus coat protein. *Bio/Technology,* **4**, 637-641.

Heitz, T., Fritig, B. & Legrand, M. (1994). Local and systemic accumulation of pathogenesis-related proteins in tobacco plants infected with tobacco mosaic virus. *Mol. Plant Microbe Interact.* **6**, 776-779.

Holt, C. A. & Beachy, R. N. (1991). *In vivo* complementation of infectious transcripts from mutant tobacco mosaic virus cDNAs in transgenic plants. *Virology,* **181**, 109-117.

Jagadish, M. N., Hamilton, R. C., Fernandez, C. S., Schoofs, P., Davern, K. M., Kalnins, H., Ward, C. W. & Nisbet, I. T. (1993). High level of production of hybrid potyvirus-like particles carrying repetitive copies of foreign antigens in *Escherichia coli*. *Bio/Technology,* **11**, 1166-1170.

Kahn, T. W., Lapidot, M., Heinlein, M., Reichel, C., Cooper, B., Gafny, R. & Beach, R. N. (1998). Domains of the TMV movement protein involved in subcellular localization. *Plant J.* **15**, 15-25.

Karrer, E., Beachy, R. N. & Holt, C. A. (1998). Cloning of tobacco genes that elicit the hypersensitive response. *Plant Mol. Biol.,* **36**, 681-690.

Koo, M., Bendahmane, M., Lettieri, G. A., Paoletti, A. D., Lane, D. E., Fitchen, J. H., Buchmeier, M. J. & Beachy, R. N. (1999). Protective immunity against murine hepatitis virus induced by intranasal or subcutaneous administration of hybrids of tobacco mosaic virus that carries an MHV epitope. *Proc. Natl Acad. Sci. USA,* **96**, in the press.

Knorr, D. A. & Dawson, W. O. (1988). A point mutation in the tobacco mosaic virus capsid protein gene induces hypersensitivity in *Nicotiana sylvestris*. *Proc. Natl Acad. Sci. USA,* **85**, 170-174.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature,* **227**, 680-685.

Linthorst, H. J. M. (1991). Pathogenesis-related proteins of plants. *Crit. Rev. Plant Sci.* **10**, 123-150.

Lomonossoff, G. P. & Johnson, J. E. (1996). Use of macromolecular assemblies as expression systems for peptides and synthetic vaccines. *Curr. Opin. Struct. Biol.* **6**, 176-182.

Luytjes, W., Geerts, D., Posthumus, R., Meloen, R. & Spaan, W. (1989). Amino acid sequence of a conserved neutralizing epitope of murine coronaviruses. *J. Virol.* **63**, 1408-1412.

Matthews, R. E. F. (1991). *Plant Virology*, 3rd edit., Academic Press, London.

Namba, K., Pattanayek, R. & Stubbs, G. (1989). Visualization of protein-nucleic acid interactions in a virus. Refined structure of intact tobacco mosaic virus at 2.9 Å resolution X-ray fiber diffraction. *J. Mol. Biol.* **208**, 307-325.

Otsuki, Y., Shimomura, T. & Takebe, I. (1972). Tobacco mosaic virus multiplication and expression of the *N* gene in necrotic responding tobacco varieties. *Virology,* **50**, 45-50.

Padgett, H. S. & Beachy, R. N. (1993). Analysis of a tobacco mosaic virus strain capable of overcoming *N* gene-mediated resistance. *Plant Cell*, **5**, 577-586.

Padgett, H. S., Watanabe, Y. & Beachy, R. N. (1997). Identification of the TMV replicase sequence that activates the *N* gene-mediated hypersensitive response. *Mol. Plant Microbe Interact.* **10**, 709-715.

Porta, C., Spall, V. E., Loveland, J., Johnson, J. E., Barker, P. J. & Lomonossoff, G. P. (1994). Development of cowpea mosaic virus as a height-yielding system for the presentation of foreign peptides. *Virology*, **202**, 949-955.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning - A Laboratory Manual*, 2nd edit., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sugiyama, Y., Hamamoto, H., Takemoto, S., Watanabe, Y. & Okada, Y. (1995). Systemic production of foreign peptides on the particle of tobacco mosaic virus. *FEBS Letters*, **359**, 247-250.

Takamatsu, N., Watanabe, Y., Yanagi, H., Meshi, T., Shiba, T. & Okada, Y. (1990). Production of enkephalin in tobacco protoplasts using tobacco mosaic virus RNA vector. *FEBS Letters*, **269**, 73-76.

Turpen, T. H., Reinl, S. J., Charoenvit, Y., Hoffman, S. L., Fallarme, V. & Grill, L. K. (1995). Malarial epitopes expressed on the surface of recombinant tobacco mosaic virus. *Bio/Technology*, **13**, 53-57.

Usha, R., Rohll, J. B., Spall, V. E., Shanks, M., Maule, A. J., Johnson, J. E. & Lomonossoff, G. P. (1993). Expression of an animal virus antigenic site on the surface of a plant virus particle. *Virology*, **197**, 366-374.

Watanabe, Y., Meshi, T. & Okada, Y. (1987). Infection of tobacco protoplasts with *in vitro* transcribed tobacco mosaic virus RNA using an improved electroporation method. *FEBS Letters*, **219**, 65-69.

Whitham, S., Dinesh-Kumar, S. P., Choi, D., Hehl, R., Corr, C. & Baker, B. (1994). The product of the tobacco mosaic virus resistance gene *N*: similarity to Toll and the interleukin-1 receptor. *Cell*, **78**, 1101-1115.

Edited by N-H Chua

(Received 17 December 1998; received in revised form 6 May 1999; accepted 6 May 1999)