

## **Structural characterization of *Tobacco etch virus* coat protein mutants**

**A. E. Voloudakis<sup>1,\*</sup>, C. A. Malpica<sup>1,\*</sup>, M.-E. Aleman-Verdaguer<sup>1</sup>,  
D. M. Stark<sup>1</sup>, C. M. Fauquet<sup>1</sup>, and R. N. Beachy<sup>1,2</sup>**

<sup>1</sup>Department of Cell Biology, The Scripps Research Institute,  
La Jolla, California, U.S.A.

<sup>2</sup>Donald Danforth Plant Science Center, St. Louis, Missouri, U.S.A.

Received June 4, 2003; accepted October 2, 2003  
Published online December 8, 2003 © Springer-Verlag 2003

**Summary.** The assembly of Tobacco etch potyvirus (TEV) coat protein (CP) and truncated mutants in *Escherichia coli* was studied. CP from which 28, 63 or 112 amino acids were deleted from the N-terminus polymerized into potyvirus-like particles (PVLPs). These structures were more rigid and progressively smaller in diameter than those produced by full length TEV-CP. CP from which 175 N-terminal amino acids were removed, failed to polymerize. A fragment containing amino acids 131 to 206 of TEV-CP is sufficient for PVLV assembly in *E. coli*.

To determine the function of the highly conserved amino acids Ser152, Arg154, and Asp198 point mutants were generated. The mutant CP $\Delta$ 63(Asp198Glu) exhibited different spectral properties following circular dichroism analysis showing a lower amount of  $\alpha$ -helix compared to the wild type molecule. No differences were observed in spectra obtained from fluorescence spectroscopy. The point mutants bind RNA *in vitro* to the same degree as the wild type protein. However, while the wild type and the Arg154Gln mutant CP were each able to form PVLVs in *E. coli*, the Asp198Glu and the double mutant Ser152Pro/Arg154Gln mutants did not. These results suggest that the Asp198Glu mutation has an altered secondary structure which affects the capacity of the protein to polymerize but did not affect *in vitro* protein-RNA interactions.

### **Introduction**

The potyviruses form the largest family of plant pathogenic viruses and infect a wide variety of host plants. Potyviruses have flexuous, rod-shaped particles, measuring 12 to 15 nm in diameter and 700 to 900 nm in length. Their genomes

\*These authors contributed equally to the present work.

are single stranded, positive sense RNA molecules of approximately 10,000 nucleotides, and are encapsidated by nearly 2,000 molecules of a single type of coat protein (CP) [17]. Potyvirus CPs vary considerably in size due to differences in sequences near the amino-termini [17, 1]. In many cases, the amino-termini are unique [17, 21, 22] and contain virus-specific epitopes [18, 20]. Treatment of purified potyvirus particles with trypsin removes residues from both the amino and carboxy terminal regions of the CP, implying that these domains are exposed on the surface of the virus [16]. Shukla and Ward proposed that filamentous viruses, including the potyviruses, share a common architecture with that of the tobamoviruses [19, 22].

The dissociation and reassembly of potyvirus particles *in vitro* was studied in detail by McDonald and co-workers in the 1970's [7, 13, 12] and demonstrated that Potato virus Y (PVY) CP can assemble to form long flexuous particles in the absence of RNA. The repeat distance of the stacked rings in these studies was 4 nm, which is significantly greater than the 3.1–3.3 nm helical pitch of the native virus [13]. Addition of RNA to the stacked rings resulted in the assembly of short virus-like particles (VLPs) whose helical pitch was the same as that of native virus particles with 7 to 8 CP subunits per ring [7]. When the Johnsongrass mosaic potyvirus (JGMV) CP was expressed in *Escherichia coli* and *Saccharomyces cerevisiae* the protein self-assembled [9]. Potyvirus like particles (PVLPs) also were observed following production of JGMV-CP in insect cells [5] and mammalian cells [10].

There is a high degree of conservation amongst potyvirus CP sequences in the central part of the CP molecules (core region), and indicates the importance of this region for structure and/or assembly of CP molecules. A model for the secondary structure of the PVY-CP [19, 22] was proposed based on computer predictions. The model predicts strong similarities with the known tertiary structure of *Tobacco mosaic virus* (TMV) CP, i.e. a four helix bundle protein [14]. Based on the predicted similarities of the two proteins it is predicted that the highly conserved Arg154 and Asp198 interact with each other to form a salt bridge that keeps the molecule folded correctly [3]. When the two analogous residues of JGMV-CP (i.e. Arg194 and Asp234) were mutated, the assembly process was disrupted [8]. A tertiary computer model structure of TEV-CP based on the structural similarities of CPs of TMV and TEV was developed [24].

In this paper we determine the minimum requirements of TEV-CP for assembly into PVLPs in *E. coli*. In addition, the function of the conserved amino acids, namely Arg154, Asp198 and Ser152 was determined based on studies of their secondary structure using CD spectroscopy and fluorescence spectroscopic analysis and RNA-protein interactions.

## Materials and methods

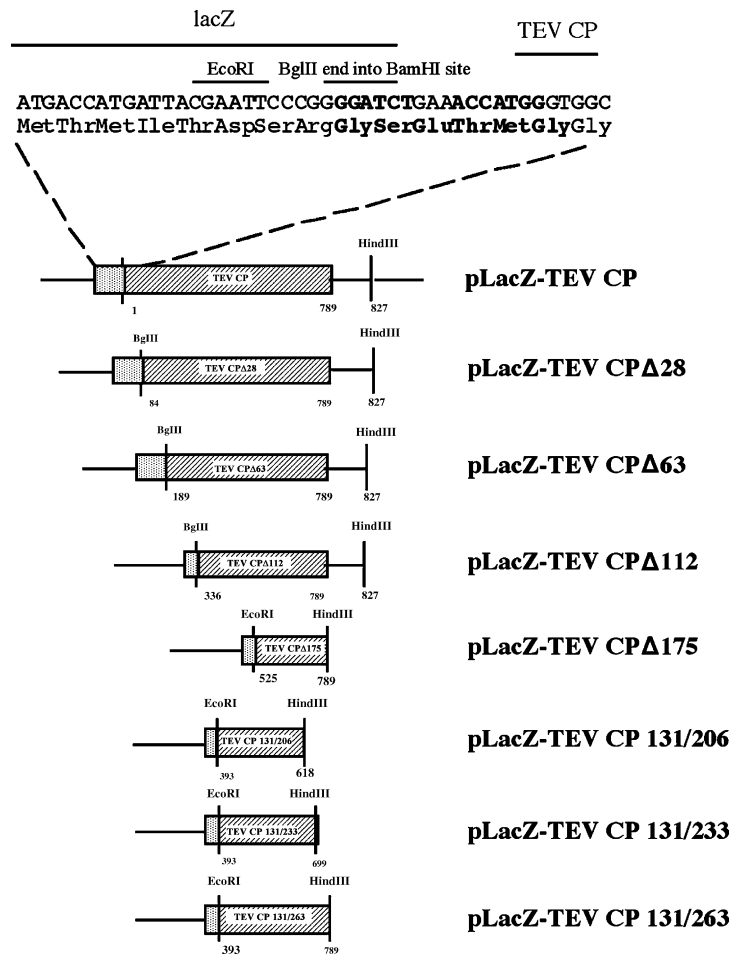
### *Construction of truncated and point mutants of TEV-CP*

Recombinant DNA manipulations were carried out using standard protocols [2]. A cloned cDNA of the TEV-CP, designated pTEV B22, was isolated and characterized using previously

*Tobacco etch virus coat protein mutants*

described methodology [6]. In that work, *in vitro* mutagenesis created a *Bgl*II cloning site and added an AUG start codon upstream of the TEV-CP. Similarly, three truncated mutants of the TEV-CP, e.g. CP $\Delta$ 28 (which removed 28 a.a. from the N-terminus), CP $\Delta$ 63 (which removed 63 a.a.), and CP $\Delta$ 112 (which removed 112 a.a.) were made via oligonucleotide directed mutagenesis to introduce an *Nco*I site at the desired position. Upon digestion with *Nco*I the 5' coding sequences were removed and the remaining DNA was cloned into pEMBL18<sup>+</sup>. PCR mutagenesis was used to construct mutants in which sequences were deleted from both the N- and C-terminus of the TEV-CP to create CP 131-206, CP 131-233, and CP 131-263. PCR products were cloned into pEMBL18<sup>+</sup> with the ligation adding 12 a.a. of  $\beta$ -galactosidase to the amino terminus of the TEV-CP sequences (Fig. 1).

To create amino acid mutations a two step PCR based mutagenesis was performed on CP $\Delta$ 63 [2]. The primers used in the mutagenesis were as follows: P1: 5'-CTATGCGTTCGA ATTCTATGAGC-3', P2: 5'-GTCATAGAATTCGAACGCATAG-3', P3: 5'-TCAACACTGC AGCAAATTATG-3', P4: 5'-CATAATTTGCTGCAGTGTGGA-3', P5: 5'-CCAACACTGC AGCAAATTATG-3' and P6: 5'-CATAATTTGCTGCAGTGTGG-3'. P1 and P2 were used to



**Fig. 1.** Diagrammatic representation of translational gene fusion used to express truncated forms of TEV-CP in *E. coli*. *LacZ* =  $\beta$ -galactosidase, *CP* = coat protein, *TEV* = tobacco etch virus,  $\Delta$  = amino acid deletion. (See Materials and methods)

mutagenize Asp198 to Glu198. P3 and P4 were used to mutagenize Arg154 to Gln154&Ser152 to Pro152. P5 and P6 were used to mutagenize Arg154 to Gln154. In all cases the universal forward and reverse primers were used as the “outside” primers in the PCR reactions. 15 cycles of PCR (94 °C for 1 min, 50 °C for 1 min, 72 °C for 2 min, with a final extension step at 72 °C for 10 min) were performed for the first round whereas for the second round of PCR 25 cycles were used. PCR was performed with a Taq/Pfu ratio of 1:100. All the designed mutations were confirmed by DNA sequencing; no unintended base changes were detected.

The DNA fragments containing point mutations were then introduced into the pET3d vector (Novagen Inc. Madison, WI). *E. coli* strain BL21(DE3) pLysS cells were transformed and tested for overexpression upon induction with IPTG. The truncated forms of TEV-CP were found to be relatively insoluble and accumulated in inclusion bodies, even at low growth temperatures (25 °C). Therefore, the point mutations were introduced into the full length TEV-CP by exchanging the *XhoI-HindIII* domains between the mutants that were constructed in CPΔ63 and the wild type sequence.

#### *Production of wild type, truncated and point mutants of TEV-CP in E. coli*

TEV-CP and truncated mutants were produced in *E. coli* DH5α grown overnight at 37 °C in LB, diluted 1:50 in fresh medium and grown at 37 °C for 2 hrs before induction by IPTG (500 μM) and cultures were incubated an additional 2 h at 37 °C. Total proteins were isolated by boiling 1 ml of cell culture in sample buffer [11]. Fusion proteins were detected by Coomassie blue staining following SDS-PAGE, as well as by western blot analysis using a polyclonal antibody against the core coat protein of JGMV (gift of Dr. D. Shukla). For purification of TEV-CP or PVLPs from *E. coli*, cells from 100 ml of cultures were resuspended in 4 ml of mix I (20% sucrose, 100 mM Tris.Cl pH 8, 10 mM EDTA), treated with lysozyme (84 μl of 5 mg/ml), and the reaction mixture was incubated for 10 min at room temperature, then 15 minutes on ice. The spheroplasts were suspended in 0.8 ml of mix II (100 mM Tris.Cl pH 8, 20% sucrose, 10 mM MgCl<sub>2</sub>, 1 μg/ml each of DNase I and RNase A) and further diluted in water to a final volume of 2 ml to lyse the cells, and for direct immunosorbent electron microscopy (ISEM). Samples were concentrated by ultracentrifugation at 55,000 rpm (275,444 RCF) at 4 °C.

For large scale purification of the TEV-CP point mutants, *E. coli* transconjugants were grown in 500 ml LB at 25 °C until OD<sub>600</sub> = 0.5 to 0.7. Cells were induced by IPTG (1 mM) and following growth for 4 hrs at 25 °C cells were harvested and resuspended in 10 ml lysis buffer (50 mM Tris-Cl, pH 8, 100 mM NaCl, 1 mM EDTA). Lysozyme, PMSF, RNase A and DNase I were added to final concentrations of 1 mg/ml, 2 mM, 10 mg/ml and 10 mg/ml, respectively. A French Press (1,000 PSI) was used to break the cells, and the cell extract was clarified by centrifugation at 15,000 rpm (17,640 RCF) for 10 min. To concentrate the samples, PEG 8000 and NaCl were added to the supernatant to final concentrations of 8% and 0.1 M, respectively. Suspensions were stirred for 1 hr at 4 °C, followed by centrifugation at 10,000 rpm (7,840 RCF) for 10 min. Pellets were resuspended in 3 ml of 50 mM Tris-Cl, pH8 and kept in 20% (V/V) glycerol at -20 °C.

#### *Electron microscopy of E. coli and plant extracts*

ISEM of purified virus and extracts prepared from *E. coli* was carried out on carbon coated grids. The JGMV core coat protein antibody was used at an IgG concentration of 1 μg/ml. The grids were negatively stained with 1% (w/v) uranyl acetate, pH 4, and examined in a Hitachi HU-12A electron microscope at 75 kV. Particle measurements were performed on

## *Tobacco etch virus coat protein mutants*

a minimum of 100 particles per construct and sizes were measured using TMV particles as standard (300 nm × 18 nm).

For the point mutants, 10 µl of the overexpressed TEV-CP sample were placed on a carbon coated grid and stained with 1.5% uranyl acetate. Excess liquid was blotted, grids were air dried and examined at 35,000X magnification with a Phillips CM120 microscope.

TEV was obtained from R. E. Ford (University of Illinois) and increased in *Nicotiana tabacum* cv Xanthi. TEV was purified as described before [4]. Purified virus particles remained infectious when stored in buffered 20% glycerol at -80 °C.

### *Purification of the His-tagged TEV-CP for secondary structure analysis*

The *NcoI-HindIII* fragments of plasmids containing the full length CP, the Δ63 truncation and its point mutants were cloned into the vector pTrcHisB (Invitrogen, San Diego, CA) to result in N-terminal translational fusions of 6 histidine residues (6xHis) with the TEV-CP constructs. *E. coli* XL1-Blue cells harboring the target plasmids were grown at 37 °C and expression of the fusion proteins was induced by IPTG (1 mM). The isolation of the fusion proteins was performed under denaturing conditions (8 M urea) using the protocol by Qiagen Inc. (Valencia, CA) for use of Ni<sup>+</sup> superflow resin, with the exception that extracts were passed twice through a French Press after the addition of the guanidine solution. Protein purification was performed on an FPLC (Pharmacia, Inc.) with the fusion proteins eluted at approximately pH 5.9. Urea was removed from the samples by a stepwise decrease (4 M, 2 M, 1 M) of urea, followed by dialysis against 10 mM Tris, pH8, overnight at 4 °C with two additional buffer changes. Samples were centrifuged at 14,000 rpm (15,366 RCF) for 5 min to remove precipitated material. The supernatant was subjected to circular dichroism (CD) analysis using an Aviv circular dichroism spectropolarimeter, MODEL 60DS (AVIV Associates Inc.). Spectra were collected between 200 to 260 nm in 0.5 nm wavelength steps and an average time 3.0 sec at 25 °C. Three scans per sample were obtained, averaged and the baseline, corresponding to 10 mM Tris, was subtracted to obtain the final values. Protein concentrations were estimated with the Bradford method (BIORAD, Hercules, CA).

The same samples were subjected to fluorometric analysis using a Hitachi F-2000 fluorescence spectrophotometer (Hitachi Instruments, Inc.). The excitation wavelength was 295 nm and the emission spectra were collected between 300 to 500 nm in a 1-cm quartz cuvette at room temperature. There were three scans per sample from which the average values were calculated.

### *In vitro protein-RNA interactions of wild type and point mutants of TEV-CP*

Protein-RNA interactions of the TEV-CP molecules were studied *in vitro* by a blotting assay. His-tagged proteins used in this study were purified as above. *Tobacco mosaic virus* (TMV) CP, rice yellow mottle sobemovirus (RYMV) CP and TEV-CP (isolated from tobacco) were used as controls in this experiment. Equal amounts of proteins were run on SDS-PAGE minigels, electroblotted to nitrocellulose and renatured according to Reichel et al. [15]. A full length transcript of the TEV genome was made radiolabelled using α <sup>32</sup>P-CTP (Amersham, Inc.) and SP6 RNA polymerase using pTEV7DLC (gift of Dr. J.C. Carrington). 42 µl of probe were added in 10 ml of binding buffer [15] and incubated for 2 hrs at room temperature. Membranes were then washed twice with 25 ml of binding buffer for 5 min with rotation [15], air dried and exposed to X-OMAT X-ray films. X-ray films were developed after an exposure time ranging between 30 min to 2 hrs.

## Results

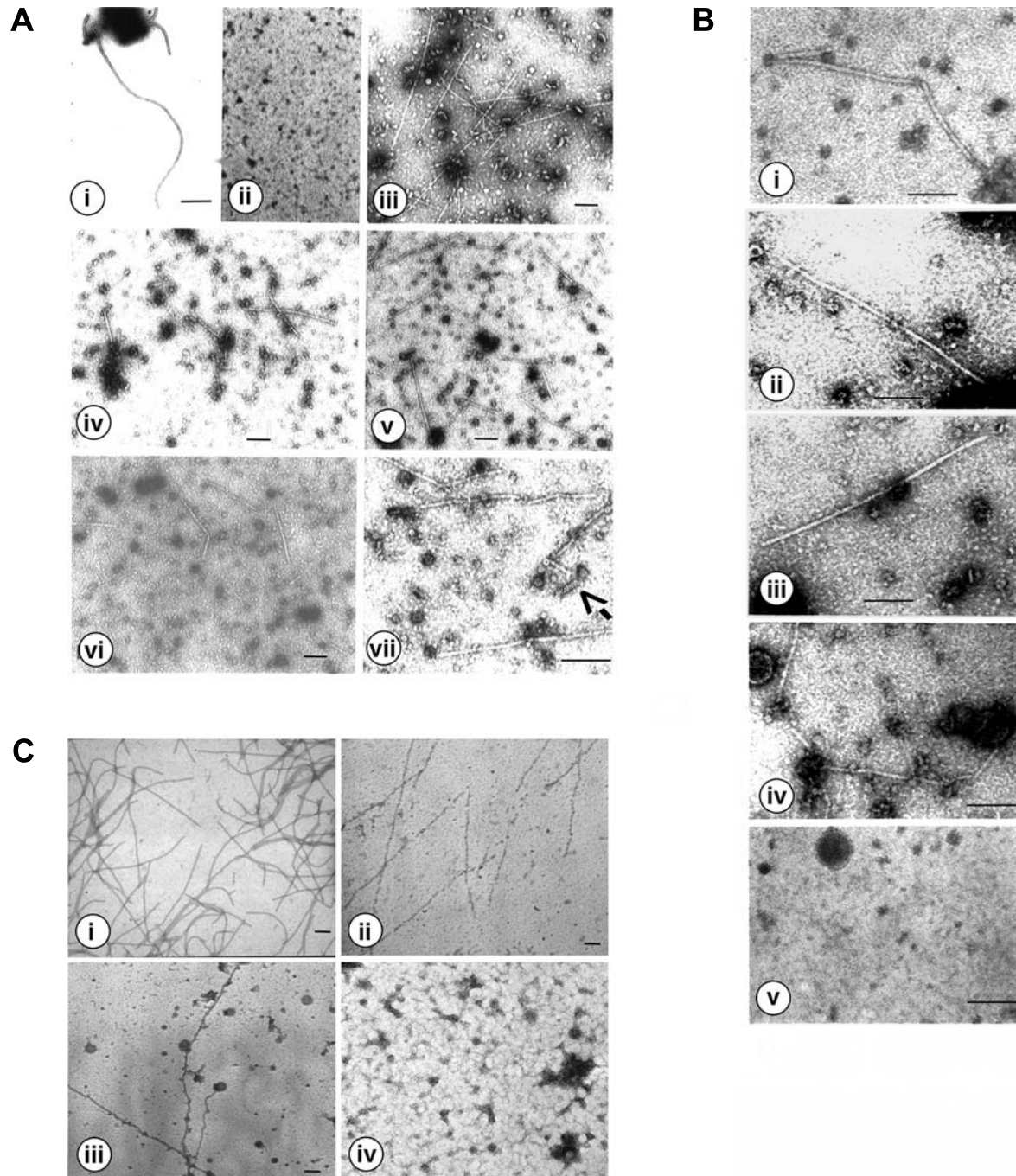
### *Expression of full length, truncated and point mutants of TEV-CP in E. coli*

N- and C-terminal deletions of the TEV-CP (Fig. 1) were constructed in order to determine the minimum requirements for protein self-assembly. All gene constructs were expressed in *E. coli* and encoded a fusion protein (with 12 a.a. of  $\beta$ -galactosidase) of the expected size (data not shown). Soluble proteins recovered from extracts of *E. coli* expressing each of the truncated proteins were subjected to ISEM to trap and visualize the proteins. *E. coli* that encodes the antisense of the TEV-CP cistron was used as negative control. All samples were diluted to contain an equal amount of total *E. coli* protein (100  $\mu$ g/ml).

Full length TEV-CP assembled to form PVLPS that were readily detected by ISEM (Fig. 2A-iii). TEV-CP N-terminal truncations, CP $\Delta$ 28, CP $\Delta$ 63 and CP $\Delta$ 112 yielded PVLPS (Fig. 2A-iv, 2A-v, 2A-vi, respectively), although the numbers of particles per  $\mu$ g of extracted protein were lower than those produced by TEV-CP. PVLPS also were observed when extracts were prepared from transgenic plants expressing similar constructs and similarly examined (data not shown). At higher magnification (Fig. 2A-vii, arrow), the particles showed a stacked-ring structure similar to that of PVY-CP when assembled in the absence of RNA [13]. The PVLPS were highly heterogeneous in length. CP $\Delta$ 28 formed fewer (Fig. 2A-iv) and thinner virus-like particles with diameters of  $9.6 \pm 0.6$  nm compared with  $12 \pm 0.6$  nm for full length TEV-CP (Table 1). Particles formed by CP $\Delta$ 63 (Fig. 2A-v) had diameters of  $8.4 \pm 0.5$  nm, while CP $\Delta$ 112 formed very few particles (Fig. 2A-vi) and had diameters of  $7.2 \pm 0.5$  nm (Table 1). CP $\Delta$ 175 failed to assemble in *E. coli* (Fig. 2B-v). Some of the very short rods were oriented perpendicularly to the grid surface in the EM preparation and made it possible to measure the diameter of the cavity in the center of the structure. The core was  $2.4 \pm 0.2$  nm in all PVLPS observed.

Similar studies were carried out with TEV-CP from which sequences were deleted from the C-terminus (full length CP contains 263 a.a.). The cDNA encoding CP from which 131 a.a. were deleted from the N-terminus (to produce CP 131–263) were further mutagenized to produce CP that ended at amino acids 233 or 206 i.e., CP 131–233 or CP 131–206, respectively (Fig. 1). All fusion proteins assembled into short, rigid PVLPS as shown in Fig. 2B with diameters from 6.4 to 7 nm (Table 1).

It was suggested that Arg154 and Asp198, which are highly conserved amongst potyvirus CPs, are important for virus assembly [3]. Proline is known to disrupt  $\alpha$ -helices, and to alter a possible  $\alpha$ -helix in the vicinity of Arg154, Ser152 was mutagenized to Pro152. We constructed mutants of the TEV-CP and CP $\Delta$ 63, in which Arg154 and/or Asp198, and Ser152 were altered. To characterize the ability of the point mutants to self-assemble, typical micrographs of negatively stained specimens from samples of purified TEV, and CP and point mutants of the CP produced in *E. coli* are shown in Fig. 2C. The wild type TEV-CP and the mutant TEV-CP(Arg154Gln) produced filamentous particles (Fig. 2C, ii and iii) that were long (highly heterogeneous in length) and less flexuous than virions (Fig. 2C, i).



**Fig. 2.** Electron micrographs of PVLPS isolated from *E. coli* via immunosorbent electron microscopy. **A** N-terminal truncations of TEV-CP. (i) purified TEV, (ii) truncated antisense construct, (iii) PVLPS produced by TEV-CP, (iv) PVLPS produced by CP $\Delta$ 28, (v) PVLPS produced by CP $\Delta$ 63, (vi) PVLPS produced by CP $\Delta$ 112, (vii) different forms of PVLPS produced by CP $\Delta$ 63 that were observed. **B** N- and C-terminal truncations of TEV-CP. (i) PVLPS observed from CP a.a. 112–263, (ii) PVLPS observed from CP a.a. 131–263, (iii) PVLPS observed from CP a.a. 131–233, (iv) PVLPS observed from CP a.a. 131–206, (v) extracts from CP a.a. 176–263. **C** Point mutants of TEV-CP. (i) TEV isolated from *Nicotiana benthamiana*; (ii) PVLPS isolated from *E. coli* that produced TEV-CP; (iii) PVLPS produced by TEV-CP(Arg154Gln); and (iv) extracts of *E. coli* expressing TEV-CP(Asp198Glu). Bars = 0.1 mm

**Table 1.** Assembly of potyvirus-like particles in *E. coli*

CP construct	Length (nm)	Diameter (nm)
Purified TEV	730 ± 10	12 ± 0.6
LacZ-TEV CP	90–750	12 ± 0.6
LacZ-TEV CPΔ28	50–750	9.6 ± 0.6
LacZ-TEV CPΔ63	40–560	8.4 ± 0.5
LacZ-TEV CPΔ112	35–400	7.2 ± 0.5
LacZ-TEV CPΔ175	N.A.	N.A.
LacZ-TEV CP 131–206	N.D.	6.4 ± 0.5
LacZ-TEV CP 131–233	N.D.	6.8 ± 0.5
LacZ-TEV CP 131–263	N.D.	7.0 ± 0.5

<sup>a</sup>N.A. = not applicable; particles were not produced by this construct

<sup>b</sup>N.D. = not determined

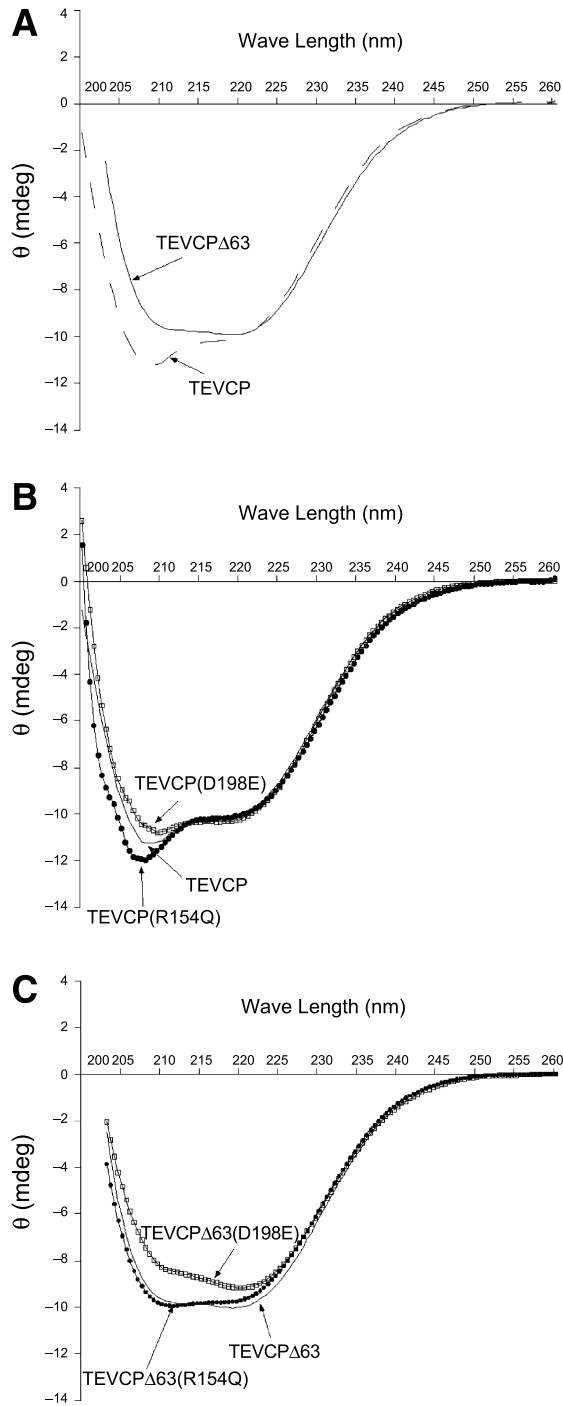
There were no particles observed for the mutant TEV-CP(Asp198Glu) (Fig. 2C, iv) and TEV-CP(Arg154Gln & Ser152Pro) (data not shown). These data suggest that Asp198 and Ser152 are crucial amino acids for the ability of the TEV-CP molecule to assemble and polymerize into PVLPs.

#### *Secondary structure analysis of wild type and point mutants of TEV-CP*

The His-tagged TEV-CP and CPΔ63 and their point mutants were expressed in *E. coli* and purified as described above. The purified proteins were renatured following dialysis to remove urea and subjected to CD analysis; the results are shown in Fig. 3. Based on the shape of the CD spectra, all the proteins exhibit a highly helical conformation [23]. There was a significant difference between the spectra of the TEV-CP and CPΔ63 (Fig. 3A). The data suggest that the full length molecule has more random coil and  $\alpha$ -helical structure compared to the truncated mutant. These results presumably reflect the fact that the N-terminal 63 a.a., which is not involved in the assembly of the CP (Table 1), is missing from the truncated mutant.

The affect of mutant Asp198Glu and Arg154Gln was determined in wild type CP and in CPΔ63. As shown in Fig. 3B and Fig. 3C the mutation Arg154Gln did not affect the overall  $\alpha$ -helicity of either CP molecule, while the mutation Asp198Glu reduced the degree of  $\alpha$ -helicity in both molecules, with stronger effects in CPΔ63. Each of the proteins was subjected to fluorescence spectroscopy to study the possibility that mutations of single amino acids altered the structure of the renatured proteins to expose a phenylalanine or a tryptophan residue. The fluorescence emission spectra of the mutants were not different from the non-mutated CP molecules (data not shown). These data indicate that Asp198Glu and Arg154Gln mutations did not cause a change in structure that resulted in the change in UV excitation under these conditions.

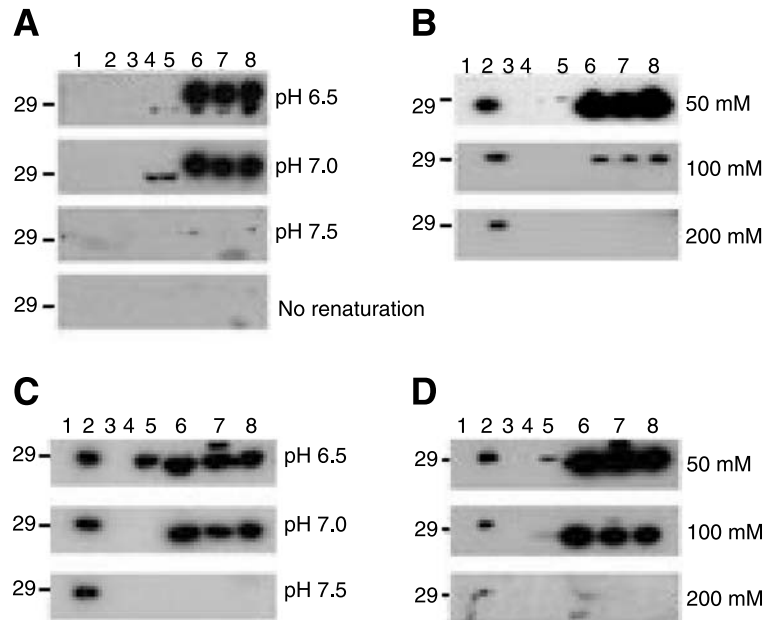
*Tobacco etch virus coat protein mutants*



**Fig. 3.** CD spectra of the His-tagged purified proteins. **A** CD spectra comparison of the TEV-CP and CP $\Delta$ 63, **B** CD spectrum analysis of the full length TEV-CP and its mutants, **C** CD spectrum analysis of the CP $\Delta$ 63 and its mutants

*In vitro protein-RNA interactions of wild type  
and point mutants of TEV-CP*

To determine whether the interactions of TEV-CP with RNA were affected by the point mutations, protein-RNA binding assays were performed on the His-



**Fig. 4.** Northwestern analysis of point mutants of TEV-CP. **A** The full length TEV-CP with different pH: 1, TMV; 2, HMW markers; 3, Vector protein extracts; 4, TEV; 5, TEV in urea; 6, TEV-CP; 7, TEV-CP(Asp198Glu); 8, TEV-CP(Arg154Gln). **B** the full length TEV-CP with different salt (NaCl) concentrations: 1, TMV; 2, RYMV; 3, HMW markers; 4, Vector protein extracts; 5, TEV; 6, TEV in urea; 7, TEV-CP; 8, TEV-CP(Asp198Glu); 9, TEV-CP(Arg154Gln). **C** the CP $\Delta$ 63 with different pH: 1, TMV; 2, RYMV; 3, HMW markers; 4, Vector protein extracts; 5, TEV; 6, CP $\Delta$ 63; 7, CP $\Delta$ 63(Asp198Glu); 8, CP $\Delta$ 63(Arg154Gln). **D** the CP $\Delta$ 63 with different salt (NaCl) concentrations: 1, TMV; 2, RYMV; 3, HMW markers; 4, Vector protein extracts; 5, TEV; 6, CP $\Delta$ 63; 7, CP $\Delta$ 63(Asp198Glu); 8, CP $\Delta$ 63(Arg154Gln)

tagged TEV-CPs. As shown in Fig. 4 both TEV-CP and CP $\Delta$ 63 bound TEV RNA *in vitro*. CP mutants Asp198Glu and Arg154Gln bound TEV RNA to a similar degree as the wild type molecules (compare lanes 7 and 8 with lane 6 in all panels of Fig. 4). To determine whether there is a difference in RNA-binding behavior between the mutants, the effect of pH (6.5, 7.0, and 7.5) and salt concentration (50, 100 and 200 mM NaCl) on the binding capacity of the proteins to RNA was studied. Binding solutions varied in the pH. Increasing the pH reduced binding to RNA, but there was no difference between the mutants and the wild type CP (panels A and C, Fig. 4). The salt concentration of the binding buffer was varied to estimate the difference in strength of the protein-RNA interactions. Increasing NaCl concentration from 50 to 200 mM NaCl abolished the binding of proteins to the RNA, but there were no observed differences between the mutants and the wild type CP molecules (panels B and C, Fig. 4). TMV-CP did not bind TEV RNA but RYMV-CP bound strongly to TEV RNA; interaction was apparently stronger than that of TEV-CP (lane 2 in panel B and C, Fig. 4). It also was observed that the signal obtained in the RNA-protein binding assay by the His-tagged proteins was more consistent and significantly higher than that obtained

from CP isolated from virions. The presence of urea in the solution of His-tagged proteins was not responsible *per se* for the difference in the signal, nor was the fact that TEV-CP was isolated from plants, since non His-tagged proteins, recovered from inclusion bodies, showed similar levels of interaction (data not shown). Therefore, we suggest that the presence of the His-tag in the N-terminus of the proteins rendered them more amenable to renaturation under the conditions used in this experiment.

## Discussion

The prediction that amino acid sequences conserved amongst potyviruses provide a structural skeleton for the assembly of potyvirus CPs, as postulated by Shukla and Ward [22] was confirmed by using TEV-CP, N- and C-terminal truncated mutants. Our studies demonstrate that a domain of TEV-CP essential for capsid assembly was affected by deleting amino acids 112 to 175. As presented in Fig. 2 amino acids 131 to 206 of TEV-CP are sufficient for the assembly of virus-like particles in *E. coli*. The sequences that comprise this region contain those that are most highly conserved amongst the potyvirus CPs. These results were not unexpected since it was demonstrated that the amino and carboxyl-terminal amino acids of the CPs of other potyvirus were removed by treating virus particles with trypsin, although such treatment did not alter particle morphology [19]. However, the trypsin-resistant core of TEV as reported by Shukla et al. [19] comprises residues 30–245; the data presented here indicate that additional amino acids (i.e, residues 206–245) are dispensable for protein folding and subunit interactions, making the TEV-CP truncations in this study of considerable interest. The length of the PVLPS produced by the truncated CP mutants varied greatly, most probably due to the absence of viral RNA. The diameter of PVLPS formed by truncated proteins were smaller in diameter than were particles produced by wild type TEV-CP (Table 1); such differences are presumably due to the removal of the “external features” attributed to the C- and N-terminal amino acid sequences rather than to other structural rearrangements.

In the absence of X-ray diffraction data for any potyvirus CP, it is difficult to speculate about the position of the amino acids mutated in this study. However, based on the high degree of conservation of Arg154 and Asp198, it was proposed that these a.a. have a significant role in the assembly of the TEV-CP, possibly through formation of a salt bridge. The effect of the mutations of these a.a. on secondary structure was therefore determined. The ability of the mutant Arg154Gln to self assemble demonstrated that the change in charge at this position is not sufficient to disrupt the assembly of PVLPS; this indicates that a salt bridge between these a.a. may not be existent. In contrast, mutations Asp198Glu and Ser152Pro&Arg154Gln restricted PVLPS formation, and suggests that changes in the volume and/or charge of the amino acid at positions 198 and 152 were not tolerated structurally. As a consequence TEV-CP(Asp198Glu) and TEV-CP(Ser152Pro&Arg154Gln) are assembly deficient mutants. A similar conclusion was drawn when the CP of JGMV was mutated to produce Arg194Lys

in JGMV, Arg194 is homologous to Arg154 in TEV, and was found to produce PVLPS in *E. coli* [8]. In contrast, mutations of Arg154Asp and Asp234Glu resulted in molecules that do not self-assemble *in vitro*, suggesting that these amino acids are crucial for the three dimensional structure of the CP [8]. Attempts to restore the potential salt bridge in JGMV-CP in the double mutant Arg154Asp/Asp234Glu were not successful and challenges the hypothesis that a salt bridge is formed between Arg154 and Asp234 [8].

Both fluorescence spectroscopy and circular dichroism were applied to elucidate potential differences in secondary structures of the wild type and mutant TEV-CP. No significant differences were detected in the fluorescence emission spectra between the mutant and the wild type proteins analyzed. However, since the spectra were similar for all the mutants tested, we concluded that if conformational changes occurred, they did not expose an aromatic amino acid that caused a difference in the fluorescence emission spectra.

Several conclusions were drawn from the CD spectra obtained for TEV-CP, CP $\Delta$ 63 and the point mutants. The spectral minima of the full length TEV-CP (at 208–210 nm) were different from the spectra obtained for CP $\Delta$ 63 (Fig. 3A). In addition, the CD spectra of TEV-CP was shifted towards lower wavelengths. These observations suggest that wild type CP contains higher amounts of  $\alpha$ -helix (based on values at 208 to 210 nm) and higher amounts of random coil (shift of the spectra to wavelength between 200 and 208 nm) than CP $\Delta$ 63. This is presumably due to the presence of the 63 N- terminal amino acids on wild type CP. This interpretation is in agreement with the prediction that the termini of the potyvirus CPs have a rather unstructured motif [24] and are exposed in the external part of the molecule and virus particle (i.e. both termini are removed by trypsin digestion) [19].

CD spectra for the full length constructs were similar for wild type and point mutants, although the Asp198Glu mutant had less  $\alpha$ -helix than wild type CP or Arg154Gln mutant CP. Similarly, for the truncated mutant CP $\Delta$ 63, the assembly deficient mutant Asp198Glu also exhibited different CD spectra compared to wild type CP $\Delta$ 63. Based on the values at 222 nm and 208 to 210 nm it can be concluded that the assembly deficient mutant is altered in secondary structure and has a reduced content of  $\alpha$ -helix. A similar change in the spectra is less obvious in the context of the full length molecules. It is also possible that such a change in the CD spectra in full length CP is “masked” by the increased random coil in the molecule.

Interactions between TEV-CP and viral RNA were studied by an *in vitro* binding assay. In these assays the CP $\Delta$ 63 bound TEV RNA as efficiently as the full length TEV-CP. This suggests that the N-terminal portion of the CP is not necessary for protein-RNA interactions. Similarly, CP mutants bound RNA to the same extent as the wild type CP; pH and salt dependence had similar effects on protein-RNA interactions for the wild type protein and point mutants (Fig. 4). These results are not in contradiction with the predicted RNA binding site [18] as being located within the inner part of the core CP, an area opposite to the position of the point mutants made in this study [24]. Therefore, mutations do not seem to affect the structure of the presumed RNA binding sequence. Alternatively, the

effect of the point mutants on the structure of the molecule is not significant to alter its RNA binding capability.

We described here the minimum region of the TEV-CP that is required for self assembly *in vivo*. In addition, two conserved amino acids of potyvirus CPs were mutagenized to determine the possible role that they play in the assembly of the TEV-CP. The mutant Asp198Glu is an assembly deficient mutant while the mutant Arg154Gln is not. We also described the *in vitro* binding of a potyvirus CP with RNA. Based on the assay used, we observed no differences in RNA binding of the assembly competent and assembly deficient mutants. To identify the role of individual amino acids in TEV structure and in interactions with RNA a 3D structure of a potyviral CP at high or low resolution is needed.

### Acknowledgments

We thank Drs. Cheng-Ming Chang (University of California, San Diego) and Malcom Wood (TSRI) for skillful electron microscopy, Christoph Reichel for his significant input in the northwestern analysis and Linda Tennant for helpful assistance in the circular dichroism analysis. The authors wish to thank Drs. Jonathan Levin, INRA, Hal Padgett and Christoph Reichel for reviewing this manuscript. C. A. Malpica was sponsored by a graduate fellowship from the French Ministry for Research and Technology (MRT) and the French Research Institute for the Development in Cooperation (ORSTOM). M.-E. Aleman-Verdaguer was a recipient of a student fellowship from the Ministère de la Recherche of France and from ORSTOM. Major support was provided by NIH grant AI27161 to RNB.

### References

1. Aleman-Verdaguer M-E, Goudou-Urbino C, Dubern J, Beachy RN, Fauquet C (1997) Analysis of the sequence diversity of the P1, HC, P3, N1b and CP genomic regions of several yam mosaic potyvirus isolates: implications for the intraspecies molecular diversity of potyviruses. *J Gen Virol* 78: 1253–1264
2. Ausubel FM, Brent R, Kingston RE, Moore DD, Siedman JG, Smith JA, Struhl K (1987) *Current protocols in molecular biology*. Wiley, New York
3. Dolja VV, Boyko VP, Agranovsky AA, Koonin EV (1991) Phylogeny of capsid proteins of rod-shaped and filamentous RNA plant viruses: Two families with distinct patterns of sequence and probably structure conservation. *Virology* 184: 79–86
4. Dougherty WG, Hiebert E (1980) Translation of potyvirus RNA in a rabbit reticulocyte lysate: Identification of nuclear inclusion proteins as products of tobacco etch virus RNA translation and cylindrical on prion as a product of the potyvirus genome. *Virology* 104: 174–182
5. Edwards SJ, Hayden MB, Hamilton RC, Haynes JA, Nisbet IT, Jagdish MN (1994) High level production of potyvirus-like particles in insect cells infected with recombinant baculovirus. *Arch Virol* 136: 375–380
6. Eggenberger AL, Stark DM, Beachy RN (1989) The nucleotide sequence of a soybean mosaic virus coat protein-coding region and its expression in *Escherichia coli*, *Agrobacterium tumefaciens* and tobacco callus. *J Gen Virol* 70: 1853–1860
7. Goodman RM, McDonald JG, Horne RW, Bancroft JB (1976) Assembly of flexuous plant viruses and their proteins. *Philos Trans R Soc London B Biol Sci* 276: 173–179
8. Jagdish MN, Huang D, Ward CW (1993) Site-directed mutagenesis of a potyvirus coat protein and its assembly in *Escherichia coli*. *J Gen Virol* 74: 893–896

9. Jagadish MN, Ward CW, Gough KH, Tulloch PA, Whittaker LA, Shukla DD (1991) Expression of potyvirus coat protein in *Escherichia coli* and yeast and its assembly into virus-like particles. *J Gen Virol* 72: 1543–1550
10. Hammond JM, Sproat KW, Wise TG, Hyatt AD, Jagadish MN, Coupar BE (1998) Expression of the potyvirus coat protein mediated by recombinant vaccinia virus and assembly of potyvirus-like particles in mammalian cells. *Arch Virol* 143: 1433–1439
11. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680–685
12. McDonald JG, Banroft JB (1977) Assembly studies on potato virus Y and its coat protein. *J Gen Virol* 35: 251–263
13. McDonald JG, Beveridge TJ, Bancroft JB (1976) Self-assembly of protein from a flexuous virus. *Virology* 69: 327–331
14. Namba K, Stubbs G (1986) Structure of Tobacco mosaic virus at 3.6Å resolution: implications for assembly. *Science* 231: 1401–1406
15. Reichel C, Maas C, Schulze S, Schell J, Steinbiss HH (1996) Cooperative binding to nucleic acids by barley yellow mosaic bymovirus coat protein and characterization of a nucleic acid-binding domain. *J Gen Virol* 77: 587–592
16. Shukla DD, Ford RE, Tosic M, Jilka J, Ward CW (1989) Possible members of the potyvirus group transmitted by mites or whiteflies share epitopes with aphid-transmitted definitive members of the group. *Arch Virol* 105: 143–151
17. Shukla DD, Frenkel MJ, Ward CW (1991) Structure and function of the potyvirus genome with special reference to the coat protein coding region. *Can J Plant Pathol* 13: 178–191
18. Shukla DD, Jilka J, Tosic M, Ford RE (1989) A novel approach to the serology of potyviruses involving affinity-purified polyclonal antibodies directed towards virus-specific N termini of coat proteins. *J Gen Virol* 70: 13–23
19. Shukla DD, Strike PM, Tracy SL, Gough KH, Ward CW (1988) The N and C termini of the coat proteins of potyviruses are surface-located and the N terminus contains the major virus-specific epitopes. *J Gen Virol* 69: 1497–1508
20. Shukla DD, Tosic M, Jilka E, Ford RE, Toler RW, Langham MAC (1989) Taxonomy of potyviruses infecting maize sorghum and sugarcane in Australia and the United States as determined by reactivities of polyclonal antibodies directed towards virus-specific N-termini of coat proteins. *Phytopathology* 79: 223–229
21. Shukla DD, Ward CW (1989) Identification and classification of potyviruses on the basis of coat protein sequence data and serology. *Arch Virol* 106: 171–200
22. Shukla DD, Ward CW (1989) Structure of potyvirus coat proteins and its application in the taxonomy of the potyvirus group. *Adv Virus Res* 36: 273–314
23. Venyaminov SY, Yang JT (1986) Determination of protein secondary structure. In: Fasman GD (ed), *Circular dichroism and the conformational analysis of biomolecules*. Plenum Press, New York
24. Voloudakis AE, Eliopoulos E, Aleman-Verdaguer M-E, Beachy RN (2000) A three dimensional model of tobacco etch virus coat protein based on the secondary structure and homology studies with Tobacco mosaic virus coat protein. *EMBO Workshop, Kolymbari, Crete, Greece, May 28–June 1, 2000*

Author's address: R. N. Beachy, Donald Danforth Plant Science Center, 975 North Warson Road, St. Louis, MO 63132, U.S.A.; e-mail: RnBeachy@danforthcenter.org