Several symptom-modulating mutations in the coat protein of turnip crinkle carmovirus result in particles with aberrant conformational properties

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Particles of several symptom-modulating TCV coat protein (CP) mutants were pretreated at pH 5.5, 7.5 or 8.5 and their conformations compared by agarose gel electrophoresis to those of wild-type particles. Particles of two mutants were swollen under conditions in which wild-type particles remained contracted; particles of one mutant were contracted under conditions in which wild-type particles were swollen; a portion of the particles of one mutant was contracted and another portion swollen under conditions in which wild-type particles remained contracted; and particles of one mutant, that elicited wild-type symptoms, comigrated with wild-type particles under all conditions tested. The results of in vitro translation experiments with mutant particles were essentially similar to those with wild-type particles, despite conformational differences at pH 5.5 and 8.5. These results suggest that more than the swollen conformation is required for in vitro translation, and that particle conformation may play a role in symptom elicitation.

Turnip crinkle carmovirus (TCV) is a 30 nm icosahedral plant virus that consists of a single-component, positive-sense RNA of 4051 bases (Carrington et al., 1989) and 180 copies of a single, 38 kDa coat protein (CP) subunit for which the three-dimensional structure has been determined to 3.2 Å (Hogle et al., 1986; for review see Morris & Carrington, 1988). TCV, tomato bushy stunt tombusvirus (TBSV) and southern bean mosaic sobemovirus (SBMV), among others, swell when the pH is shifted from slightly acidic to slightly basic after divalent cations are removed by chelation (Adolph, 1978; Golden & Harrison, 1982; Hull, 1977; Incardona & Kaesberg, 1964; Robinson & Harrison, 1982; for review see Heaton & Morris, 1992). SBMV, cowpea chlorotic mottle bromovirus, brome mosaic bromovirus and alfalfa mosaic virus (AIMV) were reported to undergo cotranslational disassembly in vitro after slightly basic pretreatments (Brisco et al., 1985, 1986; Shields et al., 1989).

The CPs of several, morphologically diverse plant viruses are involved in virus-host interactions (Cruz & Baulcombe, 1993; Culver & Dawson, 1989a, b; Heaton et al., 1991; Neelaman et al., 1991; Shintaku et al., 1992). We previously reported that amino acid substitutions in the TCV CP hinge resulted in the attenuation of symptoms in Nicotiana benthamiana Domin. (Heaton et al., 1991). We show here that several symptom-attenuating substitutions in the TCV CP hinge affect gross particle morphology which opens the possibility that conformation-dependent interactions by assembled particles play a role in symptom elicitation. We also show that, even though particles of one mutant were 'permanently' swollen and those of another 'permanently' contracted, the mutant particles behaved like wild-type particles in in vitro translation reactions after various pH pretreatments.

Wild-type TCV and mutants were propagated in N. benthamiana in plant growth chambers at 22 °C with a 16 h light, 8 h dark photoperiod. Particles were purified from fresh leaf tissue as described for carnation mottle carmovirus by Lommel et al. (1982). RNA was extracted from particles as described by Carrington & Morris (1984). TCV CP mutants TCV-R1, -R2, -1S, -2Q and -SV and the symptoms they elicit were described previously (Heaton et al., 1991; TCV-SV was called TCV-AccV in Heaton et al., 1991) and are summarized in Table 1. The mutants were reconstructed by oligonucleotide-directed mutagenesis (Heaton et al., 1991), and N. benthamiana plants were inoculated with in vitro-synthesized RNA as described (Heaton et al., 1991). Various pH treatments, with and without added calcium, and agarose gel electrophoresis were as described (Heaton, 1992).
Table 1. Summary of mutant hinge substitutions, particle conformation, symptoms in Nicotiana benthamiana and in vitro translation products

<table>
<thead>
<tr>
<th>Mutant†</th>
<th>AA change</th>
<th>Particle conformation*</th>
<th>Translation products† (pH 5.5/8.5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>Gly Ser</td>
<td>Contracted</td>
<td>Wild-type</td>
</tr>
<tr>
<td>TCV-R1</td>
<td>Ser Val</td>
<td>Swollen</td>
<td>Swollen</td>
</tr>
<tr>
<td>TCV-R2</td>
<td>Gln Val</td>
<td>Swollen</td>
<td>Swollen</td>
</tr>
<tr>
<td>TCV-1S</td>
<td>Ser Ser</td>
<td>Contracted</td>
<td>Wild-type</td>
</tr>
<tr>
<td>TCV-2Q</td>
<td>Gln Ser</td>
<td>Contracted</td>
<td>Attenuated</td>
</tr>
<tr>
<td>TCV-SV</td>
<td>Gly Val</td>
<td>Contracted/ swollen</td>
<td>None/p38, p27</td>
</tr>
</tbody>
</table>

* Particle conformations (contracted or swollen) were determined by agarose gel electrophoresis as described by Heaton (1992).
† TCV mutants, their hinge sequences, and symptoms elicited in N. benthamiana were reported by Heaton et al. (1991).
‡ In vitro translation reactions were programmed with particles pretreated at pH 5.5 or 8.5 without CaCl₂ or EDTA. NT, Not tested.

Migration patterns of wild-type TCV particles were reported previously (Heaton, 1992). Particles of TCV-R1 and -R2 migrated primarily as the swollen form, or as ribonucleoprotein (RNPs) consisting of genomic RNA and approximately six CP subunits (Wei et al., 1990), even after pH pretreatments in which wild-type particles were contracted (Fig. 1a, b). TCV-R1 and -R2 particles dissociated to RNPs to a greater degree than wild-type particles upon treatment with EDTA and increasing pH. Unlike wild-type particles, increasing the pH to 8.5 in the presence of 5 mM-CaCl₂ also resulted in partial dissociation of TCV-R1 and -R2. A minor amount of the contracted form of TCV-R1 was seen at pH 5.5 in 5 mM-CaCl₂. After pretreatments of pH 7.5 and 8.5 in the presence of CaCl₂, particles of TCV-R1 and -R2 migrated primarily as the swollen form, and dissociation to RNPs increased with increased pH (Fig. 1b).

TCV-1S particles had migration patterns very similar to those of wild-type particles. (Fig. 1a; in other experiments the intact virus band of TCV-1S pretreated at pH 8.5 in the presence of EDTA was more intense than the band in Fig. 1a). There was no detectable dissociation when TCV-1S particles were pretreated at pH 5.5 in the presence of 5 mM-CaCl₂. After pretreatments of pH 7.5 and 8.5 in the presence of CaCl₂, particles of TCV-R1 and -R2 migrated primarily as the swollen form, and dissociation to RNPs increased with increased pH (Fig. 1b).

TCV-1S particles had migration patterns very similar to those of wild-type particles. (Fig. 1a; in other experiments the intact virus band of TCV-1S particles pretreated at pH 8.5 in the presence of EDTA was more intense than the band in Fig. 1a). There was no detectable dissociation when TCV-1S particles were pretreated at pH 5.5 in the absence of EDTA; however, unlike wild-type particles, TCV-1S particles partially dissociated with the addition of EDTA at pH 5.5 (Fig. 1a). In the presence of 5 mM-CaCl₂, TCV-1S particles remained contracted after all three pH pretreatments with very little dissociation (Fig. 1b).

TCV-2Q particles migrated as contracted forms after all three pH pretreatments (Fig. 1a, b), and the dissociation of TCV-2Q particles increased with increasing pH (Fig. 1a). TCV-SV particles migrated as both swollen and contracted forms after pretreatment at pH 5.5 without EDTA (Fig. 1a). With the addition of 5 mM-EDTA to pH 5.5, 7.5 and 8.5 pretreatments, however, TCV-SV particles migrated as the swollen form (Fig. 1a). The dissociation of TCV-SV particles increased with increasing pH (Fig. 1a). With the addition of 5 mM-CaCl₂ to pretreatments at pH 5.5, 7.5 and 8.5, TCV-SV particles migrated in both forms (Fig. 1b), and there was no detectable dissociation.

For in vitro translation of virus particles, particles were preincubated at pH 5.5 or pH 8.5 in 0.1 M-Tris base for 30 min on ice. The volume was increased to 50 µl, and particles were centrifuged through a 100 µl sucrose pad (20%, w/v, in 0.01 M-sodium acetate pH 5.5) in a microcentrifuge to remove ribonucleoproteins (RNPs) consisting of genomic RNA and approximately six CP subunits (Wei et al., 1990). Particle pellets were re-suspended in 12 µl 0.01 M-sodium acetate pH 5.5 and the suspensions were used to programme a rabbit reticulocyte lysate translation system according to the manufacturer's instructions (Promega). To resolve the products in SDS-10% polyacrylamide gels, two volumes of 2× dissociation buffer (0.125 M-Tris–HCl, pH 6.8, 4% SDS, 20% glycerol and 10% 2-mercaptoethanol) were added to each aliquot, and samples were boiled for 3 min before loading.

The identities of the translation products were verified by immunoprecipitation. SDS was added to translation reactions to a final concentration of 2%, and the reaction mixtures were boiled for 2 min. After the addition of 315 µl TNET buffer (0.125 M-Tris–HCl, pH 7.5, 0.15 M-NaCl, 1 µM-EDTA, 2% Triton X-100 and 0.02% NaN₃, and 1 µl phenylmethysulphonyl fluoride (Sigma), at 8 mg/ml, the mixture was centrifuged for 5 min in a microcentrifuge. Purified IgG (1 mg/ml),
Fig. 1. Ethidium bromide-stained agarose gels of wild-type and mutant TCV particles after pretreatment at various pHs. (a) Lanes containing wild-type ('TCV') and mutant particles are labelled across the top. Lanes beneath the lines contain particles of the virus named above the line. The number above each lane indicates the pH at which the particles were pretreated and ' + E' indicates that 5 mM-EDTA was present in the pretreatment. The positions of dissociation products (RNP), swollen particles and contracted particles are indicated on either side of the figure. (b) The labelling scheme is as described for (a). All of the pretreatments in (b) included 5 mM-CaCl2 and lacked EDTA. The positions of dissociation products (RNP), swollen particles and contracted particles are indicated on either side of the figure.

raised against intact TCV according to the method of Kendall et al. (1988), or against p27 (kindly provided by Dr T. J. Morris, University of Nebraska-Lincoln, USA) was added, and the mixture was incubated at 37 °C with vigorous shaking. After 3 h, 40 μl of Protein A-Sepharose CL-4B (Pharmacia), resuspended 1:10 in TNT buffer (5 mM-Tris–HCl, pH 7.5, 0.15 M-NaCl, 1% Triton X-100 and 0.02% NaN3) was added. The mixture was incubated for 1 h at 37 °C with vigorous shaking, and centrifuged in a microcentrifuge through a 1 ml sucrose pad (15%, w/v, in TNT buffer). Sepharose beads were washed three times in TNT buffer +0.1% SDS. Beads were resuspended in 20 μl 2× dissociation buffer and boiled for 2 min before electrophoresis in SDS–10% polyacrylamide gels.

RNA extracted from wild-type particles and translated as a control produced approximately equal amounts of polypeptides with apparent molecular masses of 38 kDa and 27 kDa (Fig. 2). These polypeptides were identified by immunoprecipitation as the coat protein (p38) and the N-terminal domain of the putative replicase (p27; data not shown). No TCV-specific polypeptides were detected in in vitro translations programmed with wild-type TCV, TCV-R1 or TCV-2Q particles pretreated at pH 5.5 (Fig. 2). After pretreatment at pH 8.5, however, wild-type TCV, TCV-R1 and TCV-20 particles programmed the synthesis of approximately equal amounts of p27 and p38. A time course of the accumulation of translation products over a 60 min period showed that both p38 and p27 were synthesized to detectable levels within 15 min (data not shown). The translation of TCV-R1, which nearly always comigrated with swollen wild-type TCV, resulted in more intense bands than those resulting from the translation of either wild-type TCV or TCV-2Q.

Like the RNA of other icosahedral plant virus particles (Brisco et al., 1985, 1986; Shields et al., 1989), TCV RNA was translated after the particles were swollen by slightly alkaline pretreatments. Exactly how swelling makes the RNA of icosahedral viruses available for the initiation of translation is unknown; however, a model
contains the products of reactions programmed with wtTCV. TCV-R1 or TCV-2Q particles pretreated at the pH indicated above each lane. The pretreatments lacked CaCl₂, and EDTA. The positions of p38 (CP) and p27 (putative replicase) are indicated on either side of the figure. The pretreatments lacked CaCl₂, and EDTA. The positions of p38 (CP) and p27 (putative replicase) are indicated on either side of the figure.

has been proposed (Heaton & Morris, 1992). The possibility that mutant particles with aberrant conformational properties would not require, or respond to, pH pretreatments prompted their inclusion in the in vitro translation experiments. Even though TCV-R1 particles were swollen after pretreatment at pH 5.5 in the absence of EDTA, its RNA was not detectably translated in vitro. When TCV-R1 particles were pretreated at pH 8.5, however, the p27 and p38 bands were more intense than those produced by similarly pretreated wild-type or TCV-2Q particles.

A plausible explanation for the lack of translation of TCV-R1 particles after a pH 5.5 pretreatment is that most of the RNA is bound to the CP N-terminal domains in the interior of assembled particles, while only RNA with high affinity for CP remains bound at pH 8.5 (Munowitz et al., 1980; Wei et al., 1990). This pH-induced switch in the CP–RNA complex, therefore, appears necessary for translation, even though mutant particles assume the swollen conformation. Accounting for the seemingly more efficient translation of TCV-R1 after slightly alkaline pretreatments is difficult. While precautions were taken to minimize the addition of translatable RNPps to the in vitro reactions, the possibility that mutant particles dissociated during incubations in the reticulocyte lysate was considered. After 60 min of in vitro translation, virus particles were resolved in agarose gels, blotted to nitrocellulose, and hybridized with a radioactive, TCV-specific nucleic acid probe. These hybridization analyses showed no detectable RNP bands (data not shown).

Fig. 2. SDS-10% polyacrylamide gel of total in vitro translation products. No RNA and extracted TCV RNA were added as negative and positive controls, respectively. Lanes labelled ‘wt’, ‘R1’ and ‘2Q’ contain the products of reactions programmed with wtTCV, TCV-R1 or TCV-2Q particles pretreated at the pH indicated above each lane. The pretreatments lacked CaCl₂ and EDTA. The positions of p38 (CP) and p27 (putative replicase) are indicated on either side of the figure.

wild-type particles and the increased porosity may have allowed translation after pretreatment at pH 8.5. It is unlikely that TCV-2Q particles dissociated in the translation reactions, because hybridization experiments showed no detectable RNP bands (data not shown).

While the CPs of several plant viruses are involved in virus–host interactions (Cruz & Baulcombe, 1993; Culver & Dawson, 1989a, b; Heaton et al., 1991; Neeleman et al., 1991; Shintaku et al., 1992), neither the polymeric states nor the mechanisms through which the CPs interact with their hosts are known. The disorganization of chloroplasts induced in mature tobacco leaves inoculated with specific TMV CP mutants appeared to be associated with cytoplasmic aggregates of nonassembled TMV CP, rather than CP within chloroplasts (Lindbeck et al., 1991). Other point mutations in the ‘elicitor region’ of the TMV CP bring about a hypersensitive response in N. sylvestris plants that carry the N’ gene (Saito et al., 1987; Knorr & Dawson, 1988; Culver & Dawson, 1989b). The ‘elicitor region’ surrounds the interface between adjacent subunits (Culver, 1991), but it is not yet clear if the plant recognizes a structurally altered aggregate or a lower aggregate concentration since the mutations may weaken subunit–subunit interactions (Dawson, 1992).

Shintaku et al. (1992) concluded that chlorosis elicited by certain CMV CP mutants is associated with a local secondary structure of the CP subunit, but the polymeric state in which the CMV CP interacts is unknown. In the case of TCV, free CP was suggested as the most likely elicitor of attenuated symptoms (Shintaku et al., 1992), since the symptom-modulating mutations are not located on the particle’s surface and, therefore, are not available for interactions with cellular components. This possibility cannot be eliminated, but we show here that substitutions in the TCV hinge, which resulted in attenuated symptoms, also altered the gross conformational properties of mutant particles. TCV particles, therefore, may be the polymeric state in which TCV CP interacts with the host.

Although the stabilities of the TCV mutant particles have not been extensively tested in vitro, particles of three of the four symptom-modulating mutants, TCV-SV and especially TCV-R1 and -R2, dissociated more readily than wild-type TCV in the presence of EDTA. Particles of one of the symptom-modulating mutants, TCV-2Q, dissociated to about the same degree as wild-type particles, and to a lesser degree than those of TCV-1S which elicited wild-type symptoms. None of the mutants showed detectable RNP bands when pretreated at pH 5.5 without EDTA or added calcium, and particle yields of all of the mutants were approximately equal to the yields of wild-type particles. Perhaps attenuated symptoms are the result of less stable particles, but how the stabilities of
these mutant particles compare with wild-type particles in vivo is unknown.

The mutants described here have substitutions adjacent to a putative calcium-binding site (Carrington et al., 1987). In a separate study (Lasako & Heaton, 1993), substitutions of residues thought to interact directly with calcium ions resulted in the generation of ribonuclease A-resistant particles. Cell-to-cell movement was greatly reduced and there was no detectable systemic movement suggesting that the TCV CP plays a role in movement. However, the timing of the appearance of symptoms in N. benthamiana infected with the mutants in this study was indistinguishable from that of wild-type virus, suggesting that differences in the rates of movement in vivo probably cannot account for the less severe symptoms elicited by these TCV mutants. In at least two animal virus systems, mutant receptors functioned normally in virus entry and uncoating, but infected cells showed attenuated cytopathic effects (Cao et al., 1994; Morrison et al., 1994), leading to the suggestions that virus–receptor interactions may play a role in the induction of cytopathic effects. Both TCV and TBSV are capable of animal virus-like receptor interactions with red blood cells, and preliminary results indicate that TCV binds some component of crude plant extracts (M. H. Walter & L. A. Heaton, unpublished results). Perhaps intracellular, conformation-dependent interactions between plant virus particles and an unknown host component(s) play a role in symptom elicitation.

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References


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