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Distinct functions of capsid protein in assembly and movement of tobacco etch potyvirus in plants

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Tobacco etch potyvirus engineered to express the reporter protein β-glucuronidase (TEV-GUS) was used for direct observation and quantitation of virus translocation in plants. Four TEV-GUS mutants were generated containing capsid proteins (CPs) with single amino acid substitutions ($R_{154}D$ and $D_{198}R$), a double substitution (DR), or a deletion of part of the N-terminal domain (ΔN). Each modified virus replicated as well as the parental virus in protoplasts, but was defective in cell-to-cell movement through inoculated leaves. The $R_{154}D$, $D_{198}R$ and DR mutants were restricted essentially to single, initially infected cells. The ΔN variant exhibited slow cell-to-cell movement in inoculated leaves, but was unable to move systemically due to a lack of entry into or replication in vascular-associated cells. Both cell-to-cell and systemic movement defects of each mutant were rescued in transgenic plants expressing wild-type TEV CP. Cell-to-cell movement, but not systemic movement, of the DR mutant was rescued partially in transgenic plants expressing TEV CP lacking the C-terminal domain, and in plants expressing CP from the heterologous potyvirus, potato virus Y. Despite comparable levels of accumulation of parental virus and each mutant in symptomatic tissue of TEV CP-expressing transgenic plants, virions were detected only in parental virus- and ΔN mutant-infected plants, as revealed using three independent assays. These data suggest that the potyvirus CP possesses distinct, separable activities required for virion assembly, cell-to-cell movement and long-distance transport.

Key words: cell-to-cell movement/filamentous virus/positive-strand RNA/systemic infection

Introduction

A rapidly growing body of evidence demonstrates that systemic infection by most plant viruses includes two distinct processes: cell-to-cell movement through plasmodesmata and long-distance movement between organs through the vascular system (Hull, 1991; Maule, 1991). It is well established that plant viruses spread intercellularly by an active process involving the interplay of specific viral and host factors. As a result, some virus–host combinations lead to systemic infection, while others result in restriction of virus to inoculated cells or organs (Huber *et al.*, 1977; Motoyoshi and Oshima, 1977; Sulzinski and Zaitlin, 1982).

Although the nature of the host factors required for virus movement is understood poorly, the roles of two classes of viral proteins in virus transport have been identified. 'Movement proteins' (MPs) facilitate cell-to-cell transfer of virions or genomes, although the functional nature of the MPs from different viruses may be distinct. In some cases, such as the rod-shaped tobacco mosaic tobamovirus (TMV), movement may be facilitated by interaction of MP with the non-assembled viral genome and plasmodesmata (Deom *et al.*, 1992). In some other cases, such as the isometric cowpea mosaic comovirus (CPMV), the MP promotes formation of tubules extending between cells through which virions pass (van Lent *et al.*, 1990; Kasteel *et al.*, 1993). Interestingly, movement defects of many viruses in certain hosts can be complemented by heterologous viral MP functions provided *in trans* by other co-replicating viruses (Atabekov and Taliansky, 1990) and transgenic plants (Deom *et al.*, 1987), or by MP genes expressed in chimeric viruses (de Jong and Ahlquist, 1992). In addition to MP, capsid protein (CP) is also involved in the translocation of many plant viruses. For example, the CP is required for long-distance, but not cell-to-cell, movement of TMV (Takamatsu *et al.*, 1987; Dawson *et al.*, 1988; Saito *et al.*, 1990), whereas CPs (assembled virions) are required for both cell-to-cell and systemic transport of CPMV (Wellink and van Kammen, 1989). The nature of the CP–host interactions potentiating cell-to-cell or long-distance transport in these different situations is not clear.

The potyviruses comprise the largest group of plant viruses, with at least a few hundred members infecting numerous dicot and monocot crops worldwide (Matthews, 1991). Their positive-strand RNA genomes are expressed via the production of a large polyprotein that undergoes co- and post-translational proteolysis catalyzed by three virus-encoded proteinases (Figure 1) (Riechmann *et al.*, 1992). The single CP of potyviruses derives from the C-terminus of the polyprotein and belongs to a large family of plant virus CPs forming filamentous capsids (Allison *et al.*, 1986; Domier *et al.*, 1986; Dolja *et al.*, 1991). The CP is a three-domain protein with variable N- and C-terminal regions exposed on the virion surface and susceptible to mild trypsin treatment, and a central domain forming the core subunit structure (Allison *et al.*, 1985; Shukla *et al.*, 1988). The N-terminal domain, although not involved directly in the maintenance of virion architecture, is required for transmission of potyviruses by aphids (Atreya *et al.*, 1991). Despite the progress in understanding potyvirus molecular biology, the movement functions have not been identified.

The objective of this paper was to probe the role of potyviral CP in virus movement using tobacco etch virus expressing β-glucuronidase (TEV-GUS). This system permits the visualization of virus translocation from

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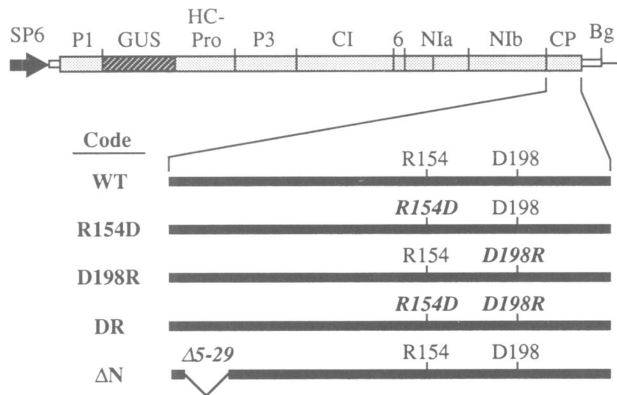

Fig. 1. Diagrammatic representation of pTEV7DA-GUS and mutagenized derivatives. The non-coding (open boxes) and coding sequences of the TEV genome (shaded) and GUS (hatched) are shown at the top. Vertical lines above the TEV map indicate sequences coding for proteolytic processing sites, whereas the line designated Bg represents the *Bgl*II restriction site engineered at the 3′-end of the TEV sequence. The positions of codons for amino acid residues at positions 154 and 198 in the wild-type (WT) or mutagenized (bold, italicized) coat protein (CP) sequences are indicated in the expanded diagram. The sequence for codons 5–29 deleted in the ΔN mutant is indicated. Abbreviations: P1, protein 1 or 35 kDa proteinase; HC-Pro, helper component-proteinase; P3, 50 kDa protein 3; CI, cylindrical inclusion protein; 6, 6 kDa protein; NIa, nuclear inclusion 'a' protein; NIb, nuclear inclusion 'b' protein; SP6, SP6 polymerase promoter.

inoculated cells to neighboring cells and to systemic tissues (Dolja *et al.*, 1992). We found that mutations affecting the CP subunit debilitated virus movement, but that movement defects were independent of virus assembly. The requirements for CP movement function were investigated with a complementation approach using transgenic plants expressing different forms of CP. These and other data presented indicate that, in addition to virion assembly, potyviral CPs have distinct functions in cell-to-cell and long-distance movement.

Results

Construction of TEV-GUS genomes carrying mutations in the CP coding sequence

Two different types of CP mutants of TEV-GUS were generated using the full-length cDNA plasmid, pTEV7DA-GUS, from which infectious RNA transcripts were produced. In the first type, two charged amino acid residues, invariant among CPs of plant filamentous viruses and suggested to play a critical role in the folding and assembly of CP subunits (Dolja *et al.*, 1991), were targeted. The $R_{154}D$ mutant contained a substitution of Asp for Arg_{154}, while the $D_{198}R$ mutant contained a substitution of Arg for Asp_{198}. The DR mutant combined both mutations (Figure 1). The CP mutant of the second type, ΔN, lacked the N-terminal sequence between amino acid residues 5 and 29, comprising most of the trypsin-sensitive domain (Allison *et al.*, 1985).

Normal RNA amplification of the CP mutants in tobacco protoplasts

Transcripts capped with m^7GpppG were produced from the linearized parental pTEV7DA-GUS and mutant plasmids using bacteriophage SP6 polymerase. *Nicotiana tabacum* cv. 'Xanthi nc' protoplasts were transfected and viral RNA

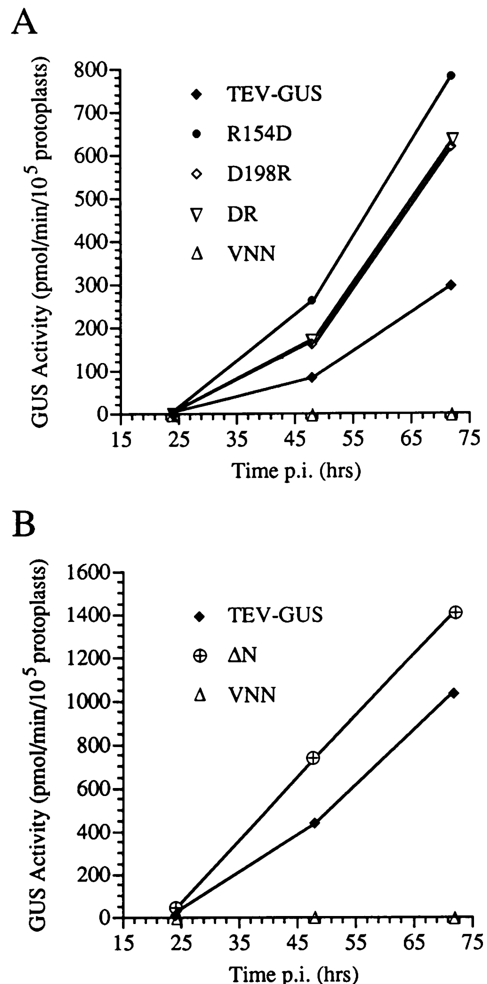

Fig. 2. Time course of GUS activity accumulation in tobacco protoplasts transfected by RNA transcripts of parental TEV-GUS and CP mutants ($R_{154}D$, $D_{198}R$, DR and ΔN). The mean values of GUS activity from two (**panel A**) or four (**panel B**) contemporaneous transfections from a typical experiment (three experiments total) are plotted. Data shown in panels A and B were collected in experiments conducted at different times. The VNN mutant contained a wild-type CP sequence, but harbored a mutation in the NIb sequence that rendered the virus replication inactive.

amplification was evaluated by measuring GUS activity at 24, 48 and 72 h post-inoculation (h.p.i.). Increase of GUS activity over time in protoplasts was shown previously to be dependent on TEV-GUS RNA amplification (Carrington *et al.*, 1993). Also, it was demonstrated that TEV-encoded protein and RNA accumulate in proportion to one another in infected leaves (Dolja *et al.*, 1993), further validating the use of GUS as a surrogate marker for viral RNA amplification in the TEV-GUS system. To distinguish between GUS activity resulting from translation of input transcripts and progeny RNA synthesized during virus multiplication, replication-defective transcripts from pTEV7DA-GUS/VNN were used as a negative control. This mutant (termed VNN) contains Val-Asn-Asn in place of the highly conserved Gly_{347}-Asp_{348}-Asp_{349} motif in the NIb polymerase, inactivating viral RNA replication (Carrington *et al.*, 1993).

GUS activity in protoplasts transfected with the VNN mutant transcripts was negligible at all time points, indicating relatively low levels of translation of input RNA in the absence of replication (Figure 2). In contrast, GUS activity

Table I. Systemic infection of non-transgenic and transgenic tobacco plants with TEV-GUS and capsid protein mutants

Inoculum[a]	Non-transgenic 'Burley 49' plants	Transgenic plants[b]			
		FL3.3	ΔN29	ΔC18	E11
TEV-GUS	11/13[c]	7/8	4/5	6/6	3/3
TEV-GUS/DR	0/11	6/8	0/3	0/4	0/3
TEV-GUS/ΔN	0/9	12/12	0/4	0/4	–[d]
TEV-GUS/R154D	0/8	5/8	–	–	–
TEV-GUS/D198R	0/8	6/8	–	–	–

[a]Plants were inoculated with RNA transcripts of corresponding plasmids as described in Materials and methods.
[b]The FL3.3 plants expressed wild-type TEV CP, while ΔN29 and ΔC18 plants expressed CP missing 29 N-terminal or 18 C-terminal amino acid residues, respectively. The E11 plants were expressors of potato virus Y CP.
[c]Number of systemically infected plants/number of plants inoculated. Infection of plants was tested by symptom appearance and by fluorometric GUS assays using inoculated and systemic leaves. Results of 1–3 separate experiments were combined.
[d]Not determined.

in cells transfected with parental transcripts (TEV-GUS) increased over time to a level three orders of magnitude higher than the activity in VNN mutant-transfected cells at 72 h.p.i. Each of the CP mutants induced GUS activity to a level comparable to or exceeding that of TEV-GUS (Figure 2). This indicated that the CP modifications had no negative effect on virus RNA amplification and expression at the single-cell level, and perhaps even stimulated expression due to potential encapsidation defects (see below).

Impairment of cell-to-cell and long-distance movement of mutant viruses

Despite normal RNA amplification activity of each CP mutant in protoplasts, none was able to establish systemic infection in *N.tabacum* cv. 'Burley 49' (B49) plants (Table I). This result could be ascribed to defects in cell-to-cell or long-distance movement, or both. The ability of the CP mutants to spread from cell to cell was tested by vacuum infiltration of transcript-inoculated leaves with the chromogenic GUS substrate, X-gluc, 3 days post-inoculation (d.p.i.). The parental TEV-GUS spread to form infection foci with average diameters of ~10 epidermal cells (Figure 3A and Table II). In sharp contrast, movement of the DR, $R_{154}D$ and $D_{198}R$ mutants out of primarily infected cells was virtually arrested (Figure 3B–D and Table II). With very few exceptions, the infection foci were restricted to single epidermal cells. The average infection focus size in ΔN mutant-inoculated leaves was limited to 2.4 cells, indicating a low but measurable degree of cell-to-cell movement. Statistical analyses revealed that each mutant was significantly different from the parental TEV-GUS ($P < 0.0001$), and that the $R_{154}D$, $D_{198}R$ and DR mutants were significantly different from the ΔN mutant ($P < 0.02$). The $R_{154}D$, $D_{198}R$ and DR mutants, however, were indistinguishable ($P > 0.20$).

The lack of systemic movement of the ΔN mutant, which was able to move from cell to cell slowly, may have been due to an inability to reach vascular-associated cells. To examine this possibility, B49 plants were inoculated with TEV-GUS or ΔN transcripts, propagated for 12 d.p.i., and analyzed using the *in situ* GUS assay. Infection foci containing TEV-GUS were relatively large and expanded along vascular tissue (Figure 3M). The ΔN mutant formed infection foci that overlapped, but did not spread along, vascular tissue (Figure 3N and O). Non-inoculated, upper leaves from the same plants were symptomless and did not contain GUS activity (Table I), again indicating an inability of the ΔN mutant to move long distance. The same results were obtained when the propagation period was extended to 18 d.p.i. (data not shown).

Complementation of movement defects in transgenic plants expressing wild-type TEV CP

Transgenic B49 tobacco plants (FL3.3) expressing the full-length TEV CP rescued the movement defects of each CP mutant. In contrast to the single-cell limitation of $R_{154}D$, $D_{198}R$ and DR mutants on B49 plants, infection foci containing these three mutants were 5.1–6.1 epidermal cells in diameter on FL3.3 plants at 3 d.p.i. (Figure 3G–I and Table II). The slow cell-to-cell movement defect of the ΔN mutant was also rescued to an extent similar to the three point mutants (Figure 3J and Table II). The differences between infection focus diameters on B49 and transgenic FL3.3 plants were highly significant for each mutant ($P < 0.0001$). Although the cell-to-cell movement defect of each mutant was complemented in the transgenic plants, infection foci were still only 50–60% the size of those containing parental TEV-GUS (Table II).

In addition to complementing the cell-to-cell movement defects of the CP mutants, the FL3.3 transgenic plants rescued the long-distance movement defects of each modified virus (Table I). GUS activity was quantitated using a fluorometric assay with total-leaf extracts from FL3.3 plants systemically infected by parental and mutant viruses at 10 d.p.i. The DR mutant accumulated to a level ~55% that of parental TEV-GUS, while $R_{154}D$ and $D_{198}R$ variants accumulated to a level of ~7% (Figure 5A). Accumulation of the ΔN mutant in systemic leaf tissue was most restricted (~1.5% of TEV-GUS; Figure 5A). Except for the pairwise comparison of $R_{154}D$ and $D_{198}R$ mutants, the differences between activity levels induced by each of the viruses were statistically significant ($P < 0.0005$). The varying levels of activity correlated with the extent of symptom appearance in systemic leaves, with the ΔN mutant-infected FL3.3 plants exhibiting the most mild disease (data not shown).

In contrast to activity in total-leaf samples, GUS activity levels in the extracts of symptomatic tissue excised from systemically infected FL 3.3 leaves at 10 d.p.i. were similar for parental TEV-GUS and all four CP mutants (Figure 5B; $P > 0.08$ for all combinations). In leaves subjected to the *in situ* histochemical assay, the decrease in extent of mutant virus infection was exhibited most obviously by a limitation

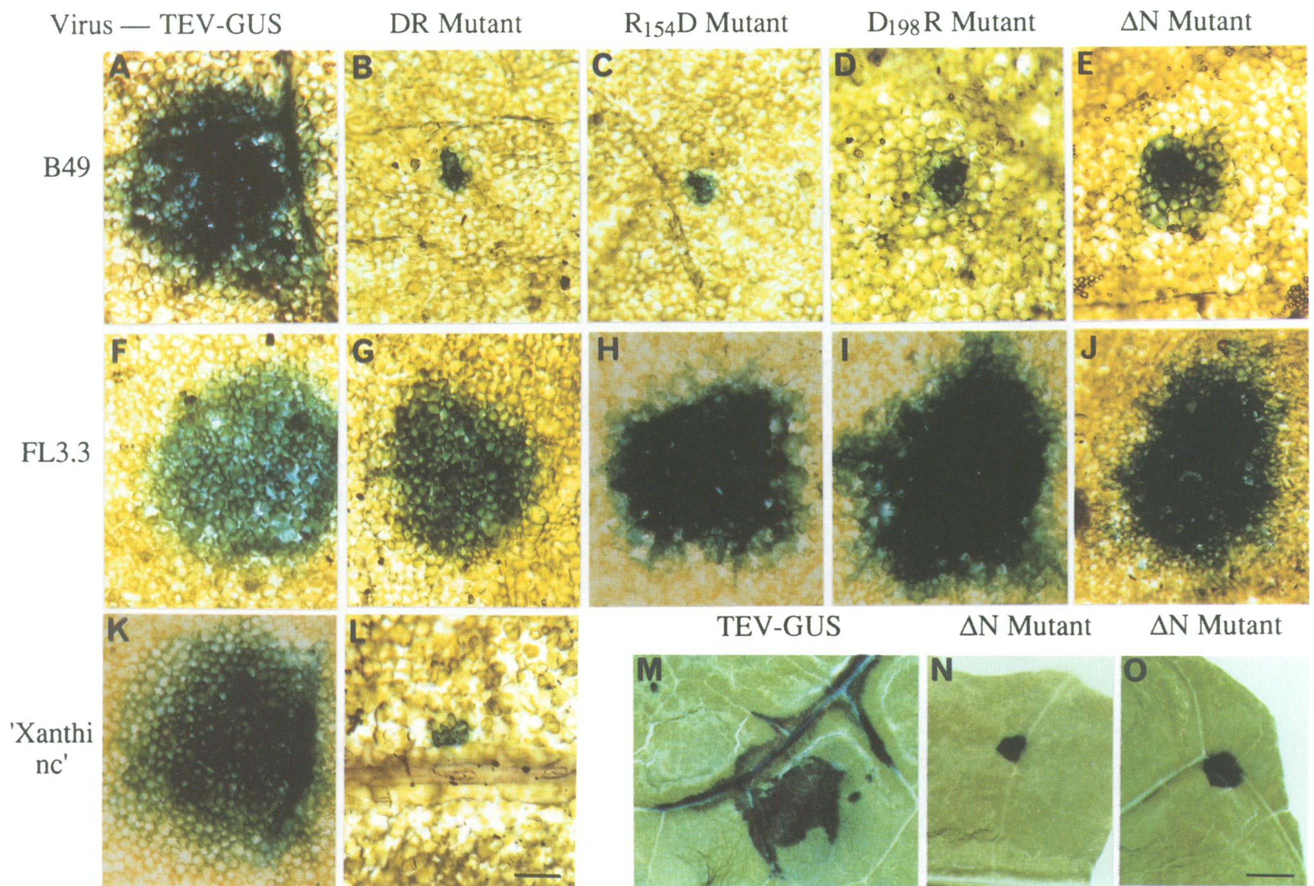

Fig. 3. *In situ* localization of GUS activity in leaves of non-transgenic and transgenic tobacco plants inoculated with parental TEV-GUS and CP mutants. **(Panels A–L)** The transcripts corresponding to the parental TEV-GUS or mutant viruses used to infect plants are shown at the top of each vertical column. The non-transgenic *N.tabacum* cv. 'Burley 49' (B49), *N.tabacum* cv. 'Xanthi nc' and transgenic FL3.3 plants infected by the transcripts are shown at the left of the horizontal rows. Infection foci were photographed 3 d.p.i. The bar in panel L equals 200 μm. **(Panels M–O)** Macroscopic views at 12 d.p.i. of leaves of non-transgenic *N.tabacum* cv. 'Burley 49' inoculated with parental TEV-GUS or ΔN mutant transcripts. The presence of GUS activity is indicated by the blue histochemical reaction.

Table II. Average diameters of foci formed on inoculated leaves of non-transgenic and transgenic tobacco plants infected with TEV-GUS and capsid protein mutants[a]

Inoculum	Non-transgenic 'Burley 49' plants	Transgenic plants			
		FL3.3	ΔN29	ΔC18	E11
TEV-GUS	10.7±4.1[b]	10.3±4.3	10.9±4.5	9.8±4.4	9.2±3.8
TEV-GUS/DR	1.0±0.2	5.1±2.5	1.4±0.6	3.4±1.6	3.1±0.9
TEV-GUS/ΔN	2.4±1.2	5.4±2.2	2.7±1.3	2.6±1.0	–[c]
TEV-GUS/R_{154}D	1.1±0.3	5.9±2.6	–	–	–
TEV-GUS/D_{198}R	1.4±0.7	6.1±2.9	–	–	–

[a]The inocula and plants were the same as in Table I.
[b]The diameters of foci were determined by *in situ* GUS assays using light microscopy of leaves 3 d.p.i. The diameters are expressed in numbers of epidermal cells (±SD). The number of foci measured varied between 30 and 70, with the exception of R_{154}D and D_{198}R mutants on 'Burley 49' plants, where only 8 and 13 foci, respectively, were found.
[c]Not determined.

in the number and size of infected areas per leaf (data not shown). Taken together, this suggests that each virus replicates to a comparable level within infected cells, but that the invasiveness into systemic tissues differs between parental and mutant viruses.

Complementation in plants expressing truncated forms of TEV CP or CP of a heterologous potyvirus

To characterize the roles of the N- and C-terminal domains of transgenic TEV CP in complementation of cell-to-cell and long-distance movement, plants expressing truncated CP derivatives lacking the N-terminal 29 (ΔN29) and C-terminal 18 (ΔC18) residues were infected by parental TEV-GUS and the DR and ΔN mutants. *In situ* GUS assays revealed equivalent spread of TEV-GUS on inoculated leaves of B49 and both types of transgenic plants at 3 d.p.i. (Figure 4A–C and Table II). The DR mutant was virtually limited to single cells after infection of B49 and ΔN29 plants, but was able to form infection foci with diameters of 3.4 epidermal cells on ΔC18 plants (Figure 4E–G and Table II), indicating that

Fig. 4. *In situ* localization of GUS activity in leaves of non-transgenic and transgenic tobacco plants inoculated with parental TEV-GUS and CP mutants. The non-transgenic *N.tabacum* cv. 'Burley 49' (B49) and transgenic plants (ΔN29, ΔC18 and E11) are shown at the top of each vertical column. The transcripts corresponding to the parental TEV-GUS or mutant viruses used to infect plants are shown at the left of each horizontal row. Infection foci were photographed 3 d.p.i. The presence of GUS activity is indicated by the blue histochemical reaction.

only the ΔC18 plants could rescue (partially) the cell-to-cell movement defect. The ΔN mutant, on the other hand, was restricted to infection sites with diameters of 2.4–2.7 epidermal cells on B49 and both truncated CP-expressing plant types (Figure 4I–K and Table II). The ability of TEV-GUS and the two mutants to move systemically in the different transgenic plants was determined by symptom appearance and fluorometric GUS assays. Parental TEV-GUS infected B49, ΔN29 and ΔC18 plants systemically, whereas both the DR and ΔN mutants were restricted to inoculated leaves of all plant types (Table I). The fact that the ΔN mutant on each plant type, as well as the DR mutant on ΔC18 transgenic plants, were able to move slowly in inoculated leaves, but were incapable of long-distance transport, suggests that movement from cell to cell is not sufficient for systemic spread.

To test the specificity of complementation in transgenic plants, TEV-GUS and the DR mutant were used to inoculate plants (E11) expressing a wild-type CP from potato virus Y, a distinct potyvirus. As in the other transgenic plants, parental TEV-GUS cell-to-cell and long-distance transport

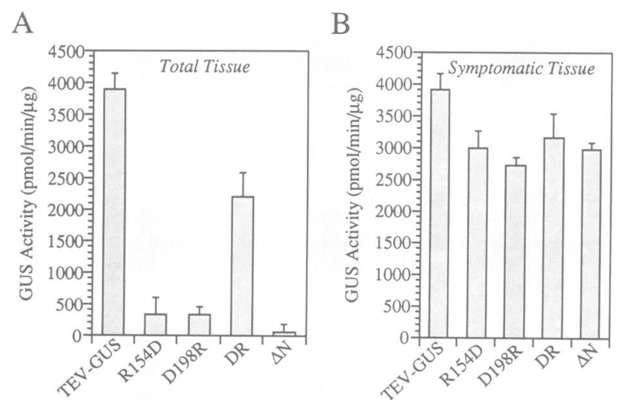

Fig. 5. GUS activity in systemically infected leaves of transgenic FL3.3 tobacco plants inoculated with transcripts corresponding to parental TEV-GUS and CP mutants. The levels of GUS activity in extracts of total leaves (**panel A**) or symptomatic tissue only (**panel B**) were quantified 10 d.p.i. using a fluorometric assay. Symptomatic tissue showing mild chlorosis was identified visually. All samples were collected from developmentally equivalent leaves. Mean GUS activity levels and standard deviations are shown.

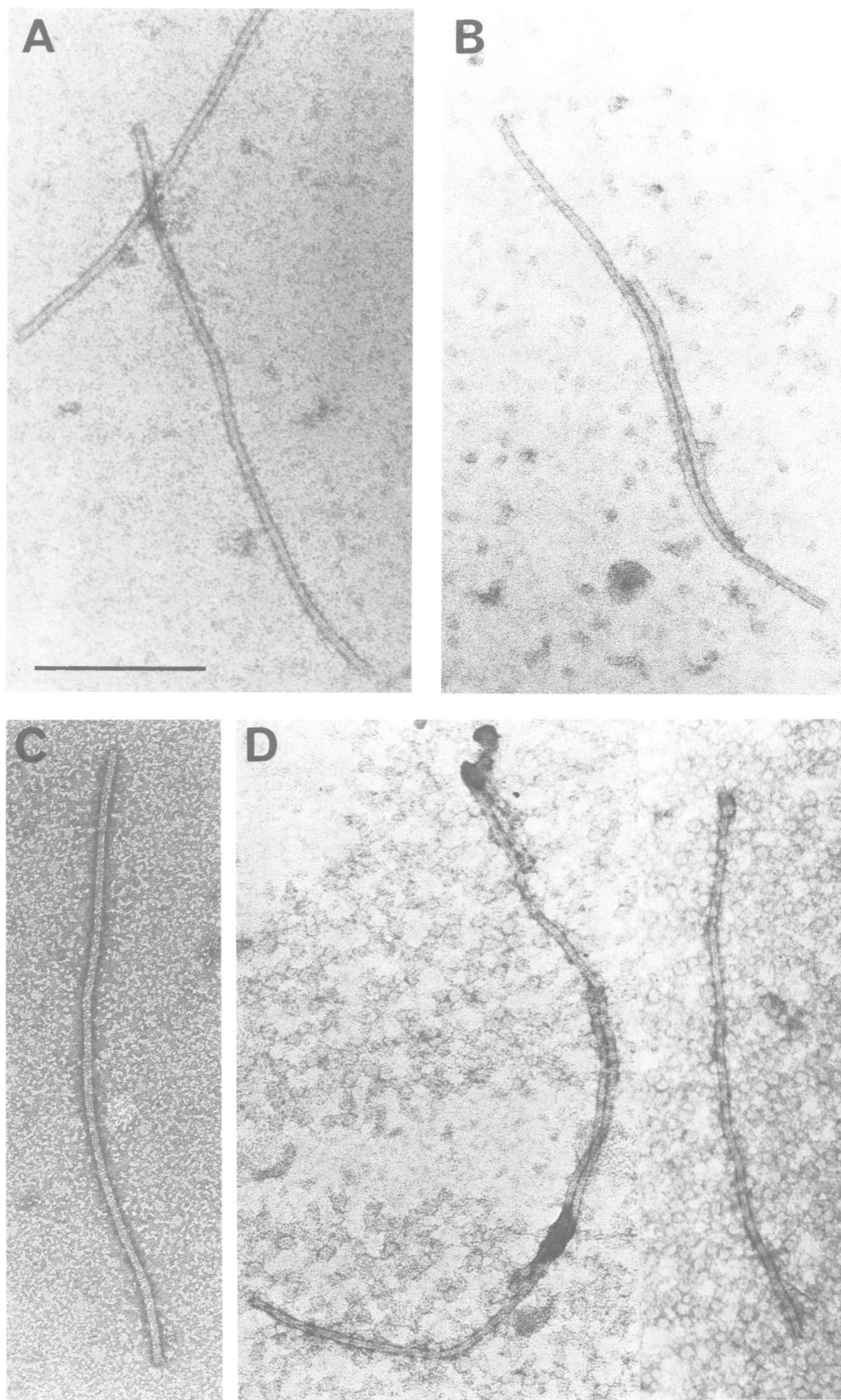

Fig. 6. SSEM of TEV-GUS and ΔN mutant virus from infected plants and protoplasts. Micrographs were taken from partially purified preparations of parental TEV-GUS (**panels A** and **B**) and ΔN mutant (**panel C**) isolated from infected FL3.3 transgenic plants, or from lysates of non-transformed tobacco protoplasts inoculated with ΔN mutant transcripts (**panel D**, a composite photograph). Grids were coated with anti-TEV serum prior to application of samples. Bar equals 200 nm.

was unaffected in E11 plants (Figure 4D; Tables I and II). Cell-to-cell movement of the DR mutant was stimulated in E11 plants, but to a lesser degree than in FL3.3 plants expressing the homologous CP (Figure 4H; Tables I and II). The E11 plants were unable to rescue the systemic movement defect of the DR mutant (Table I), again suggesting the lack of a simple correlation between short- and long-distance transport.

Assembly of virus particles in DR and ΔN mutant-infected protoplasts and plants

The point and deletion mutations analyzed thus far clearly have effects on cell-to-cell and long-distance transport. Do these mutations also affect virion assembly, and is virus movement a simple function of virion formation? To address these questions, mutant virus assembly was assayed by three independent methods. Serologically specific electron microscopy (SSEM) with anti-TEV serum was used to assay for virus particles in non-transgenic protoplasts inoculated by parental TEV-GUS and the DR and ΔN mutants. Virus particles were detected in cells infected by TEV-GUS and the ΔN mutant (Figure 6), but not in cells infected with the DR variant (data not shown). Extracts from symptomatic areas of FL3.3 transgenic plants systemically infected by TEV-GUS and all four CP mutants were also examined directly for virions using SSEM. Virus particles were detected in TEV-GUS- and ΔN mutant-infected plants, but not in DR-, R_{154}D- and D_{198}R-infected plants (Table III). This suggests that the N-terminal domain of CP is dispensable for virus assembly *in vivo*, and that the single and dual mutations in the core region inhibit virion formation even in the presence of wild-type CP supplied *in trans*.

The failure to detect virions in extracts from protoplasts and transgenic plants infected by the core region mutants may have been due to their low concentrations and to the limits of sensitivity of the SSEM method. To analyze more concentrated preparations, virus was partially purified from symptomatic tissue of FL3.3 plants infected by parental TEV-GUS and ΔN and DR mutants, and examined by SSEM. Again, virions were observed only in TEV-GUS and ΔN mutant preparations (Figure 6A–C), but not in DR mutant preparations. These concentrated extracts were also subjected to immunoblot analysis using anti-TEV serum. Capsid protein of normal size was detected in TEV-GUS preparations, but not in preparations from non-infected or DR mutant-infected plants (Figure 7, lanes 7–9), even though CP expressed in non-infected and DR mutant-infected transgenic plants was readily detected in total protein extracts (lanes 2–4). Interestingly, both total protein and concentrated virus preparations from ΔN mutant-infected transgenic plants contained predominantly the truncated CP along with minor amounts of full-length CP expressed from the transgene (lanes 5 and 10), confirming the assembly competence of both the mutant virus-encoded ΔN CP and the transgenic full-length CP.

The results of SSEM and immunoblot analyses suggested that core subunit mutants accumulated in a non-virion form, even though their cell-to-cell and long-distance transport functions were rescued by wild-type CP provided *in trans*. If so, this hypothetical form should exhibit greater sensitivity to degradation, compared to normal virions, by host nucleases after homogenization of tissue samples. To test this hypothesis, symptomatic FL3.3 leaf tissue infected by parental TEV-GUS and DR and ΔN mutants was homogenized in neutral buffer and incubated at 37°C for 0, 15 or 60 min. The residual infectivity in the homogenates was compared by bioassay inoculation of FL3.3 plants. The number of infection foci, as revealed using the *in situ* histochemical GUS assay, was counted at 4 d.p.i. In the first experiment, TEV-GUS infectivity was stable, whereas the DR mutant infectivity declined dramatically over the 60 min incubation period (Figure 8A). The differences between infectivity were significant ($P < 0.016$) at each time point for the DR mutant, but not for TEV-GUS ($P > 0.05$). In the second experiment, infectivity of both TEV-GUS and the ΔN mutant was stable, with no statistically significant changes ($P > 0.05$) for either virus over the course of incubation. These results bolster the conclusion that the ΔN mutant was assembly competent while the DR mutant was not.

Table III. Detection of virions in symptomatic tissue of transgenic FL3.3 plants by SSEM

Inoculum[a]	Number of virions/300 μm^2 [b]
TEV-GUS	36.5 ± 5
TEV-GUS/DR	0
TEV-GUS/R_{154}D	0
TEV-GUS/D_{198}R	0
TEV-GUS	25 ± 3
TEV-GUS/ΔN	137 ± 65

[a]Same as in Tables I and II. The data are from two independent experiments and are grouped accordingly.
[b]Average number of virions/field (± SD) from six photographic fields covering 300 μm^2 each.

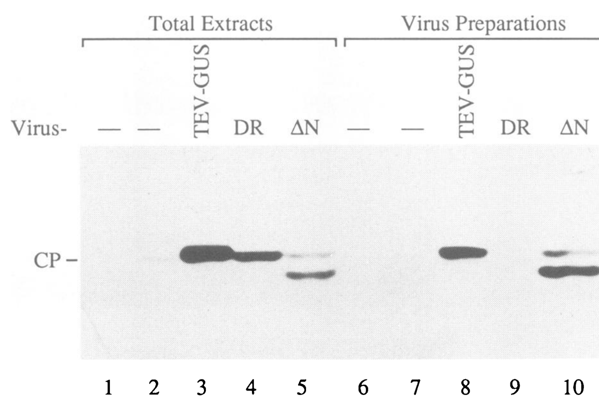

Fig. 7. Immunoblot analysis of capsid protein in plants infected by parental TEV-GUS and CP mutants. Total protein extracts (lanes 1–5) and partially purified virus preparations (lanes 6–10) from the same plants were analyzed. Samples were prepared from non-transgenic, non-infected *N.tabacum* cv. 'Burley 49' plants (lanes 1 and 6), non-infected transgenic FL3.3 plants (lanes 2 and 7), or from transgenic FL3.3 plants infected with parental TEV-GUS (lanes 3 and 8), DR mutant (lanes 4 and 9) and ΔN mutant (lanes 5 and 10). The immunoblot was processed using anti-TEV serum. Note that the ΔN mutant virus encodes a truncated CP.

Discussion

Mutations affecting TEV assembly and movement

Application of the TEV-GUS system permitted the visualization and quantitation of virus translocation in plants. We describe in this paper several CP mutants of TEV exhibiting normal RNA amplification levels in protoplasts, but dramatic defects in cell-to-cell and long-distance movement in tobacco plants, demonstrating unequivocally that the TEV CP performs specific functions in virus translocation.

Two types of TEV mutants were constructed, based on experimental and predicted structural properties of the CP subunit (Allison *et al.*, 1985; Shukla *et al.*, 1988; Dolja *et al.*, 1991). Single or double point mutations were targeted to affect the conserved residues Arg_{154} and Asp_{198}, which

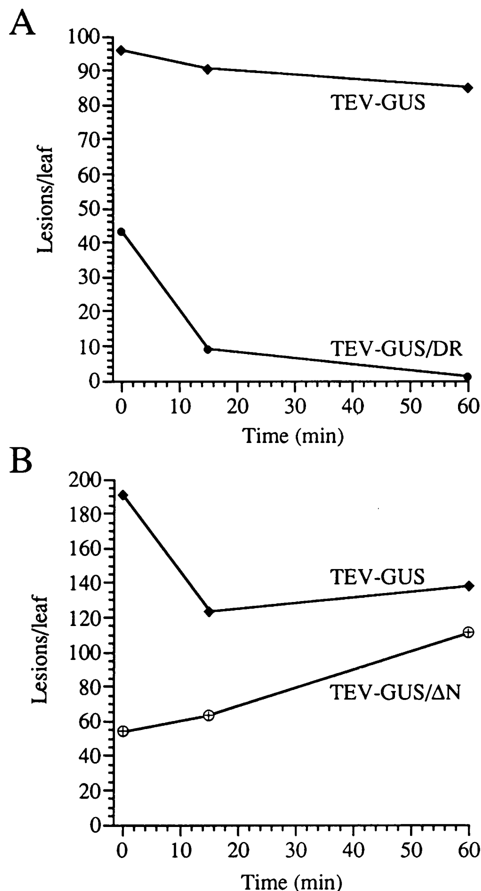

Fig. 8. Analysis of stability of parental TEV-GUS and CP mutants in sap extracts of systemically infected transgenic FL3.3 tobacco plants. The number of infectious units/75 μl sap extract after incubation at 37°C for 0, 15 or 60 min was measured by bioassay using transgenic FL3.3 plants. Infection foci were counted at 4 d.p.i. using the *in situ* GUS assay. The experiments in **panels A** and **B** were conducted at different times. The mean values from four inoculated leaves are shown for each time point.

were suggested to interact via a salt bridge (Dolja *et al.*, 1991), within the predicted α-helical core. The TEV CP point mutants described here replicated well in protoplasts, but failed to form detectable virions, suggesting a functional role for these residues in virus assembly or stability. The dual-substitution DR mutant, containing a CP in which the Arg and Asp residues were switched, was constructed initially to test the salt-bridge model through a compensatory mutation strategy. As the DR mutant failed to assemble in transfected protoplasts, we were unable to support the salt-bridge model. A similar conclusion was reached using another potyvirus (Johnsongrass mosaic virus) in which the assembly of mutant CP subunits containing equivalent single and dual substitutions was assayed in a prokaryotic expression system (Jagadish *et al.*, 1993). Strikingly, each of the assembly-deficient $R_{154}D$, $D_{198}R$ and DR mutants was arrested in movement out of primarily infected epidermal cells of tobacco plants.

The second type of CP mutant contained a deletion of most of the variable, trypsin-sensitive N-terminal sequence, which comprises a domain exposed on the virion surface. Previous biochemical analysis suggested that this part of CP plays no essential role in maintaining proper virion architecture or infectivity (Allison *et al.*, 1985; Shukla *et al.*, 1988). The fact that the ΔN mutant formed virions with normal appearance at the electron microscopic level supports these suggestions. The ΔN virus exhibited movement defects, although these were distinct from those of the core mutants. While the ΔN variant was impaired at the level of cell-to-cell transport, it nonetheless could move at a slow rate. Long-distance movement, on the other hand, was completely inhibited.

Cell-to-cell movement

Several regions of the TEV CP may be involved in cell-to-cell movement. Mutations affecting the core domain ($R_{154}D$, $D_{198}R$ and DR) had the most debilitating effect on cell-to-cell movement. These mutations may have inhibited an interaction with viral and/or host factor(s) necessary for movement, or may have asserted their effects on movement through inhibition of virion assembly. Delivery of wild-type CP by transgenic host plants rescued the cell-to-cell movement defects of these mutants, although the level of movement was less than that of parental TEV-GUS. Given that the ΔN mutant was capable of transport in the absence of transgenic CP, the N-terminal domain appeared to play only a minor role in cell-to-cell movement. However, the slow-movement phenotype of the ΔN mutant and the lack of complementation by the ΔN29 CP-expressing transgenic plants suggests that the N-terminal domain has an accessory role in promoting local movement. The role of the variable C-terminal domain was less clear in view of the partial complementation of the DR mutant, and the lack of complementation of the ΔN virus, in ΔC18 transgenic plants. In preliminary experiments, cell-to-cell movement of a TEV-GUS mutant expressing a C-terminal truncated CP was debilitated, but not inactivated, suggesting a potential accessory role of this domain in virus transport (data not shown). Interestingly, the cell-to-cell movement defect of the DR mutant was complemented partially by PVY CP, indicating the absence of strict specificity of the transport function of CP between potyviruses.

What is the infectious entity moving from cell to cell in potyvirus-infected plants, and what precise role does the CP play in this process? Two possibilities are viable with the data available. Cell-to-cell movement may require the assembly and translocation of virions between host cells. Alternatively, a non-virion ribonucleoprotein may be the transported form, analogous to that proposed for several other viruses that do not require virus assembly prior to movement (Deom *et al.*, 1992). If the latter is true, the potyviruses differ in that CP may participate in the formation of the ribonucleoprotein or may interact with host or other viral factors to potentiate transport in a manner comparable to a 'movement protein'. The fact that the ΔN mutant formed virions, but was partially inhibited in cell-to-cell movement, indicates that virion assembly is not the only role of CP during translocation. Additionally, the lack of detectable virions in DR mutant-infected transgenic plants supplying wild-type CP may also support the idea of a transport-facilitating role of CP distinct from encapsidation. However, we cannot exclude that cell-to-cell movement of the DR mutant in transgenic plants was due to the formation of virions at a low, non-detectable level. Discrimination between these possibilities may be approached by analysis of mutants with a wild-type CP, but with defective RNA sequences involved in assembly. Unfortunately, such RNA sequences have yet to be identified.

Long-distance movement

Several lines of evidence indicate that long-distance transport of TEV involves more stringent requirements than cell-to-cell movement. First, although the ΔN mutant virus could form virions and spread from cell to cell, albeit more slowly than parental virus, into the vicinity of the major vascular tissue in inoculated leaves of non-transgenic plants, no systemic translocation was detected. This long-distance movement defect was rescued by wild-type, transgenic CP, confirming the role of CP in systemic transport and the separability of this function from assembly. Second, despite the similar levels of cell-to-cell movement of all mutants in the presence of wild-type CP in transgenic plants, dramatic differences were measured in the invasiveness of various mutants in systemically infected leaves. Finally, in contrast to complementation of cell-to-cell transport, transgenic complementation of the long-distance movement defects of DR and ΔN mutants occurred only with the homologous wild-type CP and not with truncated or PVY-derived CP. These differential effects suggest that short- and long-distance transport require distinct types of virus−host interactions within vascular and non-vascular tissues.

Unlike parental virus, the ΔN mutant did not spread along veins of inoculated leaves or move systemically in non-transgenic plants. This phenotype may have been due to inhibition of entry into vascular-associated cells comprising the phloem system. It may also have resulted from an inability to move between phloem-associated cells or to enter the sieve elements for long-distance translocation. Finally, this phenotype may be explained by the lack of egress from sieve elements or phloem-associated cells. All of these possibilities may reflect the involvement of a phloem-specific host factor that recognizes the N-terminal domain to facilitate transport, or a quantitative difference between the recognition of the truncated CP by epidermal/mesophyll cells and phloem-associated cells.

As with cell-to-cell transport, the nature of the infectious unit translocating through the vascular system is not clear. The DR mutant accumulated to the highest extent in systemic tissue of transgenic plants, despite the lack of detectable virion formation, provoking the idea that long-distance transport involves a non-virion form. This mutant stands in contrast to the ΔN variant which formed virions, but was the least invasive in systemic transgenic tissue. Several rod-shaped viruses, including tobraviruses (Harrison and Robinson, 1986) and hordeiviruses (Petty et al., 1990), can be transported through the vasculature in a non-virion form, although in these cases the capsid protein is a non-participant in the process. Regardless of whether or not virions are transported, the wide disparities between the invasiveness of the different viruses in transgenic plants may be explained by a *trans*-inhibitory effect of the mutant viral CPs. Accordingly, the DR mutant CP may be the least likely to interfere competitively with the transgenic CP for host- or virus-encoded transport factors, whereas the ΔN variant CP may be the most effective competitor and thus the most poorly transported. These ideas are consistent with the data and conclusions of Lindbo and Dougherty (1992a) concerning the inhibitory effects of transgenic CPs lacking the N- and C-terminal domains on the systemic invasion of wild-type TEV.

Recently, the filamentous potexviruses, which contain a CP with similarity to the potyvirus CP, were also found to require CP for local and systemic spread (Chapman et al., 1992; Forster et al., 1992), although the particular role of virus assembly was not clear. Interestingly, deletion of the variable N-terminal region of potato virus X CP, a mutation similar to that in our ΔN virus, resulted in a virus capable of assembly and long-distance transport (Chapman et al., 1992). This may indicate that the functional role(s) of CP in the movement of potyviruses and potexviruses is different, or that the movement factors interacting with capsid protein are distinct. At present, it is not known if CP is the only viral protein required for movement of potyviruses, or if additional proteins cooperate with CP to perform specific movement functions. In any case, the mechanisms of cell-to-cell and long-distance movement may be distinct for the potyviruses.

Materials and methods

Mutation of the TEV capsid protein gene

Point and deletion mutations were introduced into the CP coding sequence within pTL7SN-SP, a plasmid containing cDNA representing TEV nucleotides (nts) 7166 [according to the numbering of Allison et al. (1986)] to the 3′ poly(A) tail. It was constructed by transfer of the *Sal*I−*Pvu*II fragment from pTEV7D (Dolja et al., 1992) into plasmid pTL7SN described previously (Oh and Carrington, 1989). The mutations were generated by the oligonucleotide-directed method of Kunkel et al. (1987). The Arg_{154} codon (AGG) of CP at nts 8977−8979 was changed to an Asp codon (GAC) to generate the $R_{154}D$ mutant. The Asp_{198} codon (GAC) at nts 9109−9111 was changed to an Arg codon (AGG) to produce the $D_{198}R$ mutant. The DR double mutant contained both point mutations combined in one plasmid. The ΔN mutant was generated by oligonucleotide-directed, 'loop-out' mutagenesis of the sequence coding for amino acid residues 5−29 of CP (nts 8530−8604). Each of these mutagenized CP sequences was introduced into pTEV7DA-GUS by replacement of the homologous *Sal*I−*Bgl*II restriction fragment consisting of nts 7166 to the 3′ end of the poly(A) tail, resulting in pTEV7DA-GUS/$R_{154}D$, pTEV7DA-GUS/$D_{198}R$, pTEV7DA-GUS/DR and pTEV7DA-GUS/ΔN, respectively. The TEV genomes represented by these cDNAs contained a bacteriophage SP6 promoter immediately upstream of the viral sequence, and the coding sequence for GUS fused in frame between the P1 and HC-Pro coding sequences (Figure 1). As demonstrated previously, proteolysis by the P1 and HC-Pro proteinases results in excision of a GUS−HC-Pro fusion protein from the viral polyprotein in cells infected by TEV-GUS.

The plasmid pTEV7DA-GUS/VNN was described previously (Carrington et al., 1993). Transcripts from this plasmid are replication defective due to a mutation resulting in substitution of Val−Asn−Asn for the highly conserved $Gly_{347}-Asp_{348}-Asp_{349}$ motif in NIb, the RNA-dependent RNA polymerase.

In vitro transcription, inoculation of plants and transfection of protoplasts

RNA transcripts capped with m^7GpppG were synthesized using bacteriophage SP6 polymerase and applied manually onto carborundum-dusted leaf surfaces of young tobacco plants (*N.tabacum* cv. 'Xanthi nc' or 'Burley 49') as described previously (Dolja et al., 1993). For some plant inoculation experiments, as well as for all protoplast transfections, concentrated inoculum was prepared by precipitation of RNA from transcription reactions with an equal volume of 4 M LiCl, and resuspension in 1/5 of the original volume. *Nicotiana tabacum* cv. 'Xanthi nc' protoplasts were isolated as described previously (Carrington and Freed, 1990). RNA transcripts (~10 μg in 10 μl of distilled water) were introduced into protoplasts (7.5×10^5) using the polyethylene glycol-mediated transfection procedure (Negrutiu et al., 1987).

Production and analysis of *N.tabacum* cv. 'Burley 49' transgenic plant lines expressing full-length CP of TEV (line FL3.3) and its truncated forms lacking 29 N-terminal residues (line ΔN29 8.1) or 18 C-terminal residues (line ΔC18 7.9) were described previously (Lindbo and Dougherty, 1992a,b). Transgenic line E11 was produced using *N.tabacum* cv. 'K326', and expressed (H.Smith and W.G.Dougherty, unpublished) potato virus Y CP.

GUS assays in vitro and in situ

To measure GUS activity in protoplasts, 2.5×10^5 cells were harvested at 24, 48 and 72 h.p.i., resuspended in 4 vols of GUS assay buffer consisting of 40 mM sodium phosphate, 10 mM EDTA, 0.1% Triton X-100, 0.1% sodium lauryl sarcosine and 0.07% β-mercaptoethanol (pH 7.0) and lysed

by one freeze–thaw cycle. Extracts from tobacco leaves were prepared by grinding tissue in 1.5 ml microcentrifuge tubes with a Contes pestle or in a mortar using 5 or 10 vols of GUS assay buffer. Fluorometric GUS assays using protoplast or leaf extracts, as well as *in situ* GUS assays, were performed as described previously (Dolja *et al.*, 1992). The StatView 4.0 program (Abacus Concepts, Inc.) was used for statistical analysis of the data.

Purification of virions, immunoblot analysis and assays of virus infectivity

Partially purified virus was prepared from 1.5 g samples of symptomatic leaf tissue of FL3.3 tobacco plants as described previously (Dolja *et al.*, 1993). These preparations, as well as total SDS-soluble proteins extracted from symptomatic leaf tissue by grinding in 4 vols of protein dissociation buffer (0.625 M Tris–HCl, 2% SDS, 10% 2-mercaptoethanol, 10% glycerol, pH 6.8), were subjected to immunoblot analysis with anti-TEV sera (dilution 1:1000) after SDS–PAGE as in previous studies (Restrepo *et al.*, 1990).

To compare the infectivity and stability of virus from leaves of FL3.3 transgenic tobacco plants systemically infected with TEV-GUS or the DR and ΔN mutants, symptomatic areas from leaves were excised with a razor blade and ground in 5 vols of 10 mM Tris–HCl, 1 mM EDTA (pH 7.6) on ice. The extracts were used immediately for mechanical inoculations of FL3.3 plants (75 μl of extract/leaf). The remaining extract was incubated at 37°C for 15 or 60 min prior to plant inoculation. The number of infection foci was determined on inoculated leaves at 4 d.p.i. by *in situ* GUS assays. In each experiment, a total of four leaves were inoculated with extracts from each time point.

Serologically specific electron microscopy

The ability of parental TEV-GUS and the CP mutants to assemble into virus particles was tested by SSEM according to Derrick (1973). Symptomatic leaf tissues or protoplast pellets were ground in 5 vols of 10 mM Tris–HCl, 1 mM EDTA (pH 7.6) and the extracts were immediately applied to EM grids precoated with anti-TEV serum (1:500 dilution). After 1 h incubation at room temperature, the grids were rinsed for 30 s in a stream of distilled water, stained with 2% aqueous uranyl acetate and blotted dry. The preparations were viewed with a Zeiss 10C electron microscope at 60 kV.

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