The Capsid Protein Gene of Tomato Bushy Stunt Virus Is Dispensable for Systemic Movement and Can Be Replaced for Localized Expression of Foreign Genes

Herman B. Scholthof, T. Jack Morris, and Andrew O. Jackson

Department of Plant Pathology, University of California, Berkeley 94720 U.S.A.
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The requirement of the capsid protein for viability of tomato bushy stunt virus (TBSV), a small, spherical plant virus, was analyzed by inactivating the corresponding gene in full-length cDNA clones followed by bioassays of *in vitro*-generated transcripts. The results demonstrated that the coat protein of TBSV is not involved in replication or gene expression in protoplasts. In addition, cell-to-cell spread of viral RNA as well as long-distance movement in plants occurred in the absence of coat protein expression. Symptoms on systemic tobacco hosts in the absence of an intact coat protein gene were quite typical of a TBSV infection, except that most infected plants survived, whereas infection with the wild-type virus resulted in a lethal necrosis. Viral RNA in which the coat protein gene was replaced with a reporter gene expressed this foreign gene at high levels in protoplasts as well as in the inoculated leaves of plants. Expression of the reporter gene was greatly reduced in upper leaves of systemically infected hosts because the nonviral sequences were rapidly deleted from the genome during the process of systemic infection. The capsid protein of TBSV was not responsible for the elicitation of necrotic lesions on *Chenopodium amaranticolor*. Analyses of β-glucuronidase expression by TBSV on inoculated leaves of this local lesion host suggested that replication and cell-to-cell spread were not affected prior to 1 day postinoculation. The hypersensitive response is probably initiated at some time after establishment of a localized infection and limited cell-to-cell movement.

Additional keywords: GUS and CAT expression, tombusvirus.

Tomato bushy stunt virus (TBSV), the type member of the tombusvirus group, is a small plant virus with a single, positive-sense RNA genome of about 4,800 nucleotides (nt) (Hearne *et al.* 1990). Five major open reading frames (ORFs) have been identified on the genome of tombusviruses that have been subjected to nucleotide sequence analysis (Grieco *et al.* 1989; Hearne *et al.* 1990; Rochon and Tremaine 1989). The genomic RNA (Fig. 1) presumably functions as an mRNA for translation of the 5′ proximal ORFs, the first of which encodes a 33-kDa protein (p33). It is thought that translational readthrough of the p33 stop codon results in translation of the second ORF to yield reduced amounts of a 92-kDa (p92) protein. The second ORF is involved in replication (Scholthof *et al.* 1991a), but the significance of p33 in this process remains unclear. Comparison of the amino acid sequence of the virion capsid protein (Hopper *et al.* 1984) and nucleotide sequence analysis of viral RNA (Hillman *et al.* 1989) revealed that the third gene on the genome encodes the coat protein, which is translated from a subgenomic mRNA (sgRNA) (Fig. 1). Two 3′ distal ORFs code for products of 22 and 19 kDa (p22 and p19), respectively. The p19 gene is nested within p22 and both are presumably translated from a second smaller sgRNA as shown for cucumber necrosis virus (CNV) (Rochon and Johnston 1991), a member of the tombusvirus group that is closely related to TBSV. The functions of p19 and p22 have not been determined, but the CNV analogue of p22 appears to be involved in cell-to-cell movement (Rochon and Johnston 1991).

The viral RNA is encapsidated in a spherical virion assembled from 180 copies of a 41-kDa coat protein subunit (Martelli *et al.* 1988). The capsid structure of TBSV is similar to that of turnip crinkle virus (TCV), a member of the carmovirus group, and the coat protein subunits of both viruses can be subdivided into three analogous domains (reviewed by Harrison 1983). The amino terminus comprises the internally projected basic random domain (R) which is thought to interact with viral RNA. A connecting arm links R to the internal surface of the shell (S) domain, which is connected through a small, flexible hinge, to the carboxyl protruding (P) domain projected outward from the tightly bonded shell.

In addition to forming the capsid shell, the coat protein gene of many RNA plant viruses appears to act pleiotropically in vector specificity (Atreya *et al.* 1991; reviewed by Harrison 1987), host range, and symptom determination (reviewed by Dawson 1992). TBSV is serologically unrelated to CNV, but, except for the distinct coat protein sequences, the rest of the genome has a high degree of sequence similarity (Hearne *et al.* 1990). Fungal transmission is reported for CNV, and, although some evidence suggests that TBSV is a soilborne root pathogen (Gerik *et al.* 1990), this mode of transmission or the involvement

Present address of T. Jack Morris: School of Biological Sciences, University of Nebraska, Lincoln, NE 68588 U.S.A.

Correspondence should be addressed to: A. O. Jackson, Dept. of Plant Pathology, 147 Hilgard Hall, University of California, Berkeley, CA 94720 U.S.A.

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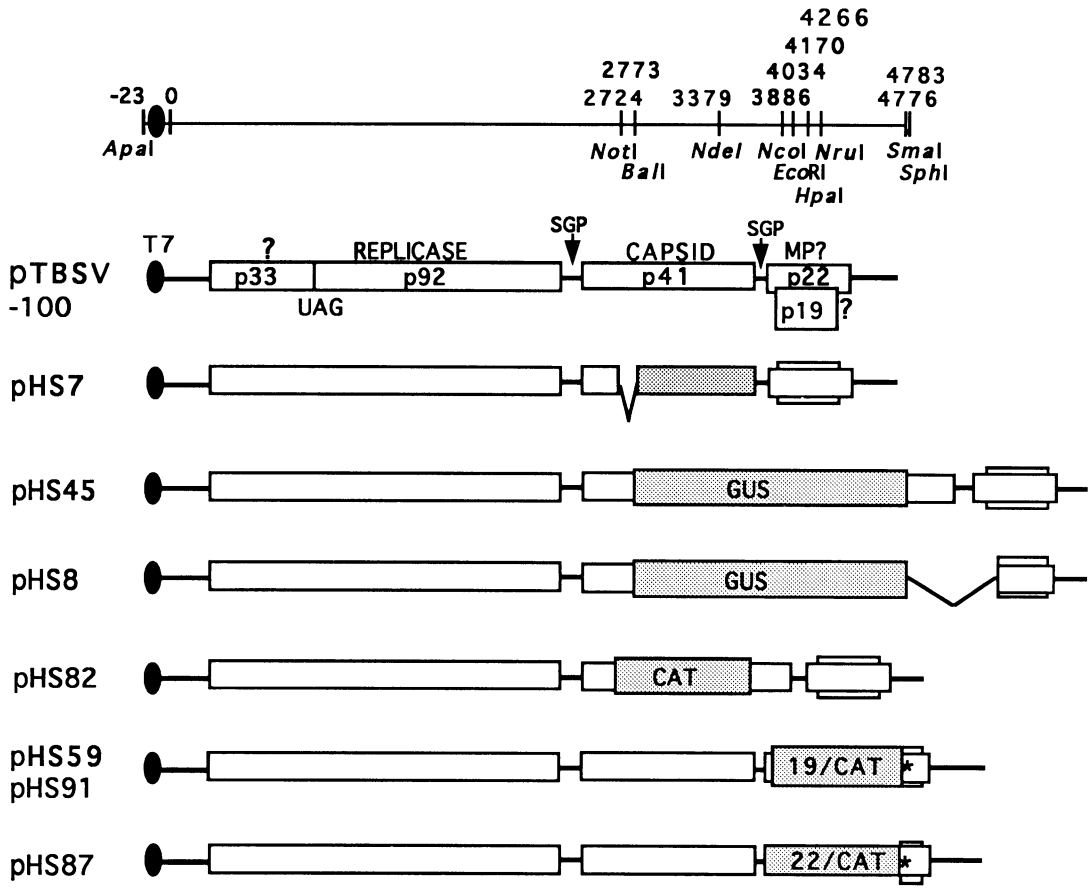
of the coat protein has not been demonstrated.

Some rod-shaped RNA plant viruses like tobacco rattle virus (TRV), tobacco mosaic virus (TMV), and barley stripe mosaic virus (BSMV) are able to incite a systemic infection when the coat protein gene is inactivated (Hamilton and Baulcombe 1989; Dawson *et al.* 1988; Takamatsu *et al.* 1987; Petty and Jackson 1990), although in some cases long-distance movement may be impaired.



Predicted Size of RNAs

	pTBSV -100	pHS7	pHS45	pHS8	pHS82	pHS87 pHS91	pHS59
gRNA:	4780	4730	5980	5325	4905	5275	5175
sgRNAs:	2160	2110	3360	2705	2285	2655	2555
	935	935	935	-	935	1430	1330

Fig. 1. The genomic organization of tomato bushy stunt virus (TBSV) and mutagenized derivatives. The figures represent the cDNA inserts in the various constructs named on the left; the plasmids are omitted. Restriction enzyme sites and positions are designated according to Hearne *et al.* (1990). The position of the T7 promoter is indicated, and boxes denote the viral and reporter genes. The molecular weight of the resulting proteins (p) is given in kDa and their predicted functions are indicated. The question marks (?) indicate that the functions of p33, p19, and p22 are not resolved, although the latter might be the movement protein (MP). The position of the two subgenomic promoters (SGP) for production of mRNAs for p41 and the nested genes (p22/p19) are shown by the arrows. The 50-nt deletion in pHS7 resulted in a frameshift (shaded box) and consequently a stop codon was introduced nine codons downstream of the deletion, in-frame with the 5′ terminus of the coat protein gene. The reporter genes were all fused in-frame with the viral gene in which they were inserted. All reporter gene inserts have a translational stop codon at their 3′ end. The region that is deleted in pHS8, with respect to pHS45, is indicated. A derivative of pHS45 denoted pHS15 (not shown) which lacked an essential component of the replicase (nt 1665–1754) (to be published) was used in some experiments as a replication minus control. The asterisk (*) indicates that a 100-bp fragment from the *Hpa*I site to the *Nru*I site is present downstream of the CAT gene in pHS87 and pHS91, whereas it is absent in pHS59. The organization of the gene fusions was confirmed by sequence analysis. Plasmids were cleaved with *Sma*I prior to *in vitro* transcription unless otherwise indicated in the text. Plasmids pHS8, pHS15, and pHS45 were linearized with *Sph*I because of the presence of an extra *Sma*I site at the 5′ end of the GUS gene insert. The size of the different constructs is drawn approximately to scale, and the predicted sizes of the resulting viral RNAs are tabulated beneath the diagrams.

Other rod-shaped viruses like potato virus X (PVX) (Chapman *et al.* 1992a) and beet necrotic yellow vein virus (BNYVV) (Quillet *et al.* 1989) or the bacilliform, alfalfa mosaic virus (van der Kuyl *et al.* 1991) need a functional capsid protein for movement through the plant. The requirement of the coat protein for infection is especially apparent for spherical viruses, as shown for one or more members in the following groups: bromoviruses (Allison *et al.* 1990; Sacher and Ahlquist 1989), carmoviruses (Heaton *et al.* 1991; Hacker *et al.* 1992), comoviruses (Wellink and van Kammen 1989), cucumoviruses (Suzuki *et al.* 1991), and perhaps luteoviruses (Young *et al.* 1991). However, systemic movement of some spherical plant viruses also occurs in the absence of coat protein as reported for a member of the dianthoviruses (Xiong *et al.* 1993) and for two tombusviruses (Rochon *et al.* 1991; Dalmay *et al.* 1992). The mechanisms or factors determining the requirement or dispensability of the coat protein for different viruses are poorly understood.

In the present investigation, we determined the importance of the capsid protein in the life cycle of TBSV. The results demonstrate that the coat protein has no detectable effect on the level of replication in protoplasts and it is not required for systemic spread of the viral RNA through plants or for the induction of systemic disease symptoms. To enable more detailed studies on replication, gene expression, and virus movement, either the coat protein gene or one of the two nested downstream genes have been replaced with a reporter gene coding for either β-glucuronidase (GUS) or chloramphenicol acetyltransferase (CAT). The results reveal that all recombinant viral RNAs replicate and express the reporter genes in protoplasts. Transcripts containing reporter gene substitutions in the coat protein gene are also infectious in plants, and express the GUS or CAT genes efficiently in the inoculated leaves but poorly in upper systemically infected leaves. The coat protein is not required for elicitation of a hypersensitive response on a local lesion host, which permits the visualization of GUS expression by TBSV before local lesions become visible.

RESULTS

TBSV coat protein independent replication and reporter gene expression in protoplasts.

The replication of wild-type TBSV (pTBSV-100) was compared to that of a coat protein mutant (pHS7) with a 50-nt deletion that yields a downstream frameshift and premature termination nine codons downstream from the deletion (Fig. 1). Replication of *in vitro*-generated transcripts was assayed in protoplasts from cucumber and *Nicotiana benthamiana* as illustrated in Figure 2. The intensity of the genomic RNA bands in the agarose gel in Figure 2 was only slightly less than that of the host ribosomal RNAs, indicating that the wild-type TBSV and the coat protein deletion mutant pHS7 both replicated to very high levels. The analyses illustrated in Figure 2 also revealed that the synthesis of subgenomic (sg) RNAs was not substantially affected by the absence of an intact coat protein gene. Similar high levels of replication and sgRNA synthesis were observed in tomato protoplasts (data not shown). Close inspection of the position of the genomic RNA and the coat protein sgRNA (top and middle bands, respectively, in Fig. 2) revealed that pHS7 RNA migrated slightly faster through the gel than the corresponding wild-type RNAs due to the 50-nt deletion, as expected from the predicted size in Figure 1. The size of the sgRNA for the downstream nested ORFs is not affected because transcription of this RNA initiates downstream of the deletion (Fig. 1).

Since the coat protein of TBSV was not required for replication or synthesis of sgRNAs, we next carried out

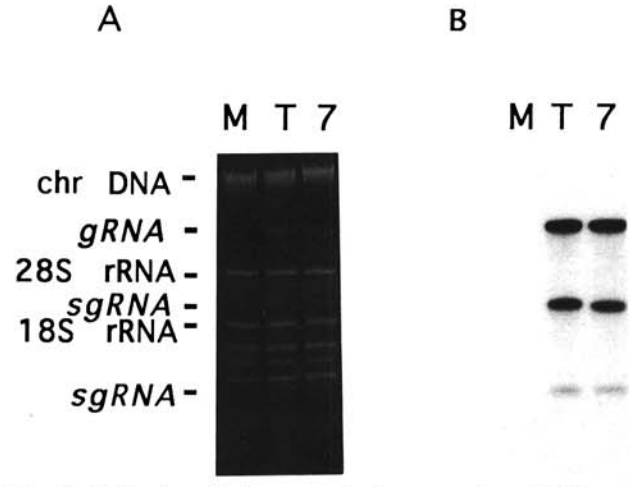


Fig. 2. Replication of the tomato bushy stunt virus (TBSV) coat protein mutant pHS7 in protoplasts. **A,** Ethidium bromide-stained agarose gel with total protoplast nucleic acids. The position of the genomic RNA (gRNA) and sgRNAs are shown as well as those of chromosomal (chr) DNA, 28S and 18S ribosomal RNAs (Jones *et al.* 1990). *Nicotiana benthamiana* protoplasts were mock transfected (M) or transfected with RNA transcribed from pTBSV-100 (T) or pHS7 (7) RNA. **B,** Northern hybridization analyses of a similar experiment as in A, but with cucumber protoplasts. Nick-translated pHS40 was used as a hybridization probe. This plasmid contains TBSV sequences from nt 2500 to 4800.

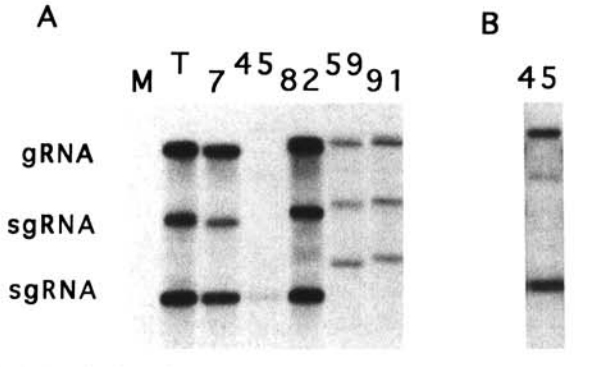


Fig. 3. Replication of viral RNAs containing reporter genes at different positions. **A,** Northern blot analysis of RNA isolated from *Nicotiana benthamiana* protoplasts that were transfected with transcripts from the plasmids (Fig. 1) whose numerical designations are provided above each lane. M and T indicate RNA from mock transfected and pTBSV-100 transfected protoplasts, respectively. The positions of the gRNA and sgRNAs are indicated. Nick-translated pHS49 which contains tomato bushy stunt virus (TBSV) sequences from nt 3800 to 4800 (Scholthof *et al.* 1993), was used as a probe and the autoradiograph was exposed for 2 hr. **B,** Longer exposure (16 hr) of the lane in A containing pHS45 RNA.

experiments to determine whether coat protein substitutions affected replication or translation of viral RNA. For this purpose pHS45 was generated (Fig. 1). This plasmid contains the GUS gene fused in-frame with the 42 amino terminal amino acids of the coat protein. It was presumed that this fusion, which preserved the first 124 nt of the coat protein gene, would minimize the potential interference of the GUS gene with transcriptional or translational regulatory elements at the 5′ end of the coat protein gene. For the same reasons, the CAT gene was inserted in-frame with the first 72 nt of the coat protein gene in plasmid pHS82 (Fig. 1). For comparison, other plasmids which contained the CAT gene in-frame with p19 (pHS59 and pHS91) or with p22 (pHS87) (Fig. 1), were evaluated for replication or reporter gene expression.

The Northern blot analysis of protoplast RNA illustrated in Figure 3 shows that transcripts from pHS45 and pHS82 replicated and that they produced sgRNAs. The reduced migration of pHS45 RNAs (Fig. 3B) compared to those of TBSV, which is especially noticeable for the largest sgRNA, is due to the 1,200-nt increase in size that results from insertion of the GUS gene into the coat protein gene (Fig. 1). In contrast to results with pHS7, which has a simple deletion and frameshift in the coat protein gene, replacement with the GUS gene in pHS45 affected the synthesis of the genomic RNA and in particular that of the larger sgRNA, as indicated by the lower intensities of the bands representing these RNAs (Fig. 3). On the other hand, replacement of the coat protein with the CAT gene in pHS82 did not significantly interfere with replication or production of sgRNAs (Fig. 3). These contrasting results are probably due to differences in the sizes of the respective inserts. The transcript from pHS82 is only slightly larger than TBSV RNA, as the 780-bp CAT gene replaced a 655-bp fragment of the coat protein cDNA (Fig. 1). The signal intensity of bands in Figure 3 representing RNAs from pHS59 and pHS91 appeared lower than for pHS82. However, this effect was not consistent in other trials with different hybridization probes, and most likely was an artifact resulting from the pHS49 probe used in Figure 3. This probe spanned the nested ORFs that were partially deleted in these plasmids, hence, the resulting RNAs from these plasmids were expected to yield a weaker hybridization signal. The sizes of the pHS59 and pHS91 genomic RNAs and coat protein sgRNAs were larger than that of TBSV RNA because the CAT gene insert was about 400 and 500 nt longer, respectively, than the deleted fragment (Figs. 1 and 3). The second sgRNA (bottom band in lanes of Fig. 3) of pHS59 and pHS91 was also longer than the equivalent sgRNA of pHS82 because the CAT gene has been inserted downstream of the initiation site for this second sgRNA in pHS59 and pHS91, but upstream of this site in pHS82 (Fig. 1). The 100 extra nucleotides downstream of the CAT gene in pHS91 compared to pHS59 did not affect replication.

Protoplast extracts were also analyzed for reporter gene expression by assaying GUS and CAT activity. Figure 4 shows that the foreign genes were expressed efficiently, irrespective of which viral ORF was replaced by the reporter gene. We have not yet determined whether expression from the different viral ORFs is temporally regulated. Nor can we make conclusive statements about the comparative levels of CAT gene expression because quantitative differences are probably obscured due to the high CAT activity levels in each case. In separate experiments it was established that the levels of CAT expression from pHS59 (Fig. 1) were similar to those of pHS91 (data not shown). Even though considerable variation existed between the GUS expression assays in Figure 4, the GUS and CAT expression data together demonstrate that all viral genes were expressed at high levels during the transient assay even in constructs lacking a functional coat protein gene.

TBSV coat protein is dispensable for systemic movement and development of disease symptoms.

The preceding experiments demonstrated that none of the essential functions needed to establish a successful infection in single plant cells required the coat protein gene. In the next set of experiments, we investigated the necessity

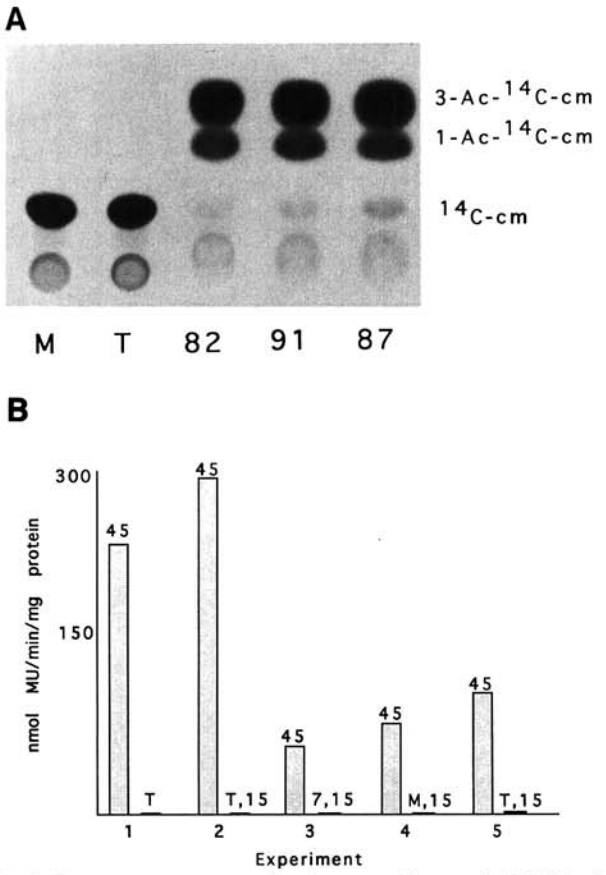


Fig. 4. Reporter gene expression by recombinant viral RNAs in protoplasts. **A,** CAT assay with extracts from cucumber protoplasts transfected with transcripts from plasmids indicated below each lane. Extracts from mock (M) or tomato bushy stunt virus (TBSV) RNA (T) transfected protoplasts were used as a negative control. The locations of ^{14}C-chloramphenicol (^{14}C-cm) and its products that are acetylated by CAT at either the 1 position (1-Ac) or 3 position (3-Ac), are indicated. The 1,3 diacetylated products are not shown. **B,** GUS activity induced by pHS45 RNA in five different experiments. GUS activity is expressed as the rate at which the product (methylumbelliferone, MU) of the GUS reaction (Jefferson 1987) is synthesized in extracts from cucumber protoplasts that were transfected approximately 17 hr previously with RNA from pHS45 (45); pTBSV-100 (T); pHS7 (7); pHS15 (15) or no RNA (mock, M).

of the coat protein for localized movement from cell-to-cell and for long-distance movement in *N. clevelandii* plants. In these experiments transcripts from either pTBSV-100 or pHS7 were inoculated onto plants and 3 wk later, noninoculated leaves were harvested, even though by that time most of the leaf tissue infected with wild-type pTBSV-100 had become necrotic. The leaf tissue was tested for the presence of either virions or virus-specific double-stranded (ds) RNA. As shown in Figure 5, virions could be isolated from a plant inoculated with RNA from pTBSV-100 but, as expected, virions were not present in the two plants inoculated with transcripts from pHS7. However, virus-specific dsRNAs were readily detectable in equivalent amounts (Fig. 5). The electrophoretic patterns again revealed minor differences in sizes of the dsRNAs due to the 50-nt deletion in pHS7, as was previously noted with the corresponding ssRNAs from protoplasts (Fig. 3). Thus, in addition to dispensability of the coat protein gene for replication, subgenomic RNA synthesis and gene expression in protoplasts, the synthesis of coat protein and formation of virions was not required for either localized or systemic movement of TBSV through plants.

Symptoms on *N. benthamiana* and *N. clevelandii* leaves inoculated with wild-type TBSV appeared (on average) at 3 and 4 days postinoculation (dpi), respectively. These symptoms were typified by a few lesions on *N. clevelandii*, and a mild mosaic interspersed with faint yellow spots on *N. benthamiana*. Transcripts derived from mutants of pTBSV-100, which contained a deletion or a frameshift that affected both the p19 and p22 ORFs, were not able to establish a localized or systemic infection (data not shown). Similarly, RNA from pHS59, which has a CAT substitution for p19 and p22, was unable to infect *N. benthamiana* plants. In contrast, *N. benthamiana* plants that were inoculated with pHS7 RNA developed symptoms on the inoculated leaves approximately 1 day after symptoms appeared on plants that were inoculated with wild-type TBSV. The symptoms induced by pHS7 often appeared to be more severe because necrotic lesions and bleached areas developed, which spread as the infection progressed. Bleaching of inoculated leaves was not commonly observed during infection with wild-type TBSV. Symptoms on *N. clevelandii* leaves inoculated with pHS7 RNA normally appeared 2 days after symptoms became visible on plants inoculated with wild-type TBSV RNA. The symptoms elicited by pHS7 on inoculated leaves included the development of necrotic lesions and a generalized necrosis, whereas these symptoms were less prominent on leaves inoculated with wild-type pTBSV-100.

One week after inoculation with wild-type TBSV, young noninoculated leaves of both tobacco hosts started to wilt. This was followed by a top necrosis and, generally, by 14 dpi these plants had died (Fig. 6), although *N. clevelandii* plants could survive longer, especially under warmer greenhouse conditions. Systemic symptoms on plants inoculated with pHS7 RNA generally appeared 3 days later on *N. benthamiana* and 5 days later on *N. clevelandii* than when wild-type RNA from pTBSV-100 was used for inoculum. These systemic symptoms were usually less severe than those elicited by the wild-type virus, but they included a mild mosaic and leaf distortion, localized necrosis, and stunting on *N. benthamiana*. The most severe symptoms can be seen in Figure 6A; the milder response is shown in Figure 6B. Young leaves of *N. clevelandii* plants systemically infected with pHS7 developed yellow areas, leaf curling, and distortion, along with frequent formation of small dark necrotic lesions which eventually culminated in veinal necrosis (Fig. 6C, D). In general, plants inoculated with pHS7 RNA rarely developed the lethal necrosis typical of a wild-type viral infection.

The timing and phenotype of the systemic symptoms induced by pHS82 RNA on *N. benthamiana* and *N. clevelandii* usually resembled those elicited by pHS7 RNA (Fig. 6B–D), but milder symptoms were also observed (Fig. 6A). Symptoms on *N. benthamiana* leaves inoculated with pHS45 RNA appeared approximately 5 days later than

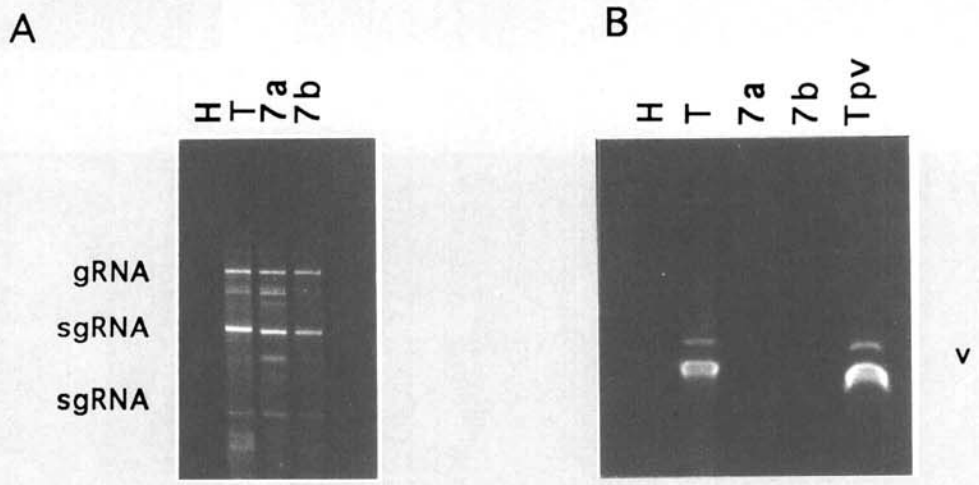


Fig. 5. Analyses of double-stranded (ds) viral RNA or whole virions in sytemically infected *Nicotiana clevelandii* leaves. **A,** Ethidium bromide-stained agarose gel showing the positions of ds gRNA and ds sgRNAs isolated from either healthy (H) plants, plants infected with wild-type TBSV (T) or two plants (a and b) infected with RNA of the defective coat protein mutant pHS7. It is not clear what the additional dsRNA bands represent, but their appearance might be caused by seasonal elevation of greenhouse temperatures (Hillman *et al.* 1985). **B,** Ethidium bromide-stained agarose gel after electrophoresis of whole virus particles. On the right, 9 μg of pure TBSV virus (Tpv) was used as a control. The RNA inside the virions is stained in the bright lower band (v); the upper band may represent virus particle dimers.

symptoms on leaves inoculated with wild-type RNA, and the onset of systemic symptoms was delayed approximately 8 days. The symptoms on inoculated leaves and to a lesser extent those on systemic leaves included yellowing, mosaic patterns, leaf curling and bleaching, or necrosis (Fig. 6).

During several trials, we were not successful in infecting *N. clevelandii* with pHS45 transcripts (Fig. 6) or with RNA from pHS82 linearized with *Sph*I. The treatment with *Sph*I rather than *Sma*I prior to transcription resulted in the addition of at least three extra nucleotides at the 3′ end of the RNA (Fig. 1). However, pTBSV-100 cleaved with *Sph*I yielded transcripts that were infectious on *N. clevelandii* and pHS82 RNA from either *Sma*I or *Sph*I linearized templates replicated to high levels in protoplasts. When the *Sph*I termini of the pHS82 and pHS45 templates were made blunt-ended by DNA polymerase (Klenow fragment) prior to *in vitro* transcription, the RNA became infectious on *N. clevelandii*. Although we do not fully comprehend the rationale, the observations suggest that *N. clevelandii* is a less permissive host for TBSV mutants than *N. benthamiana*. The observation that *N. benthamiana* is a less restrictive host has also been reported for other viruses, as reviewed by Dawson and Hilf (1992).

Reporter gene expression in plants is primarily restricted to inoculated leaves due to the *in vivo* generation of deletions.

Infection of *N. benthamiana* with pHS45 RNA resulted in readily detectable levels of GUS activity (about 50 nmol methylumbelliferone/min/mg of protein) in extracts from inoculated leaves, but this activity was barely detectable in the upper leaves. Leaves that were inoculated with RNA from pHS8 did not have any detectable GUS activity. The viral sequences in pHS8 are similar to those of pHS45, except for a deletion in p19/p22 (Fig. 1). RNA from pHS8 replicated and expressed high levels of GUS in protoplasts as shown in a preliminary report (Scholthof *et al.* 1991a). These data confirm that at least one of the overlapping genes (p19/p22) is necessary for systemic movement.

Leaves were also vacuum-infiltrated with the GUS sub-

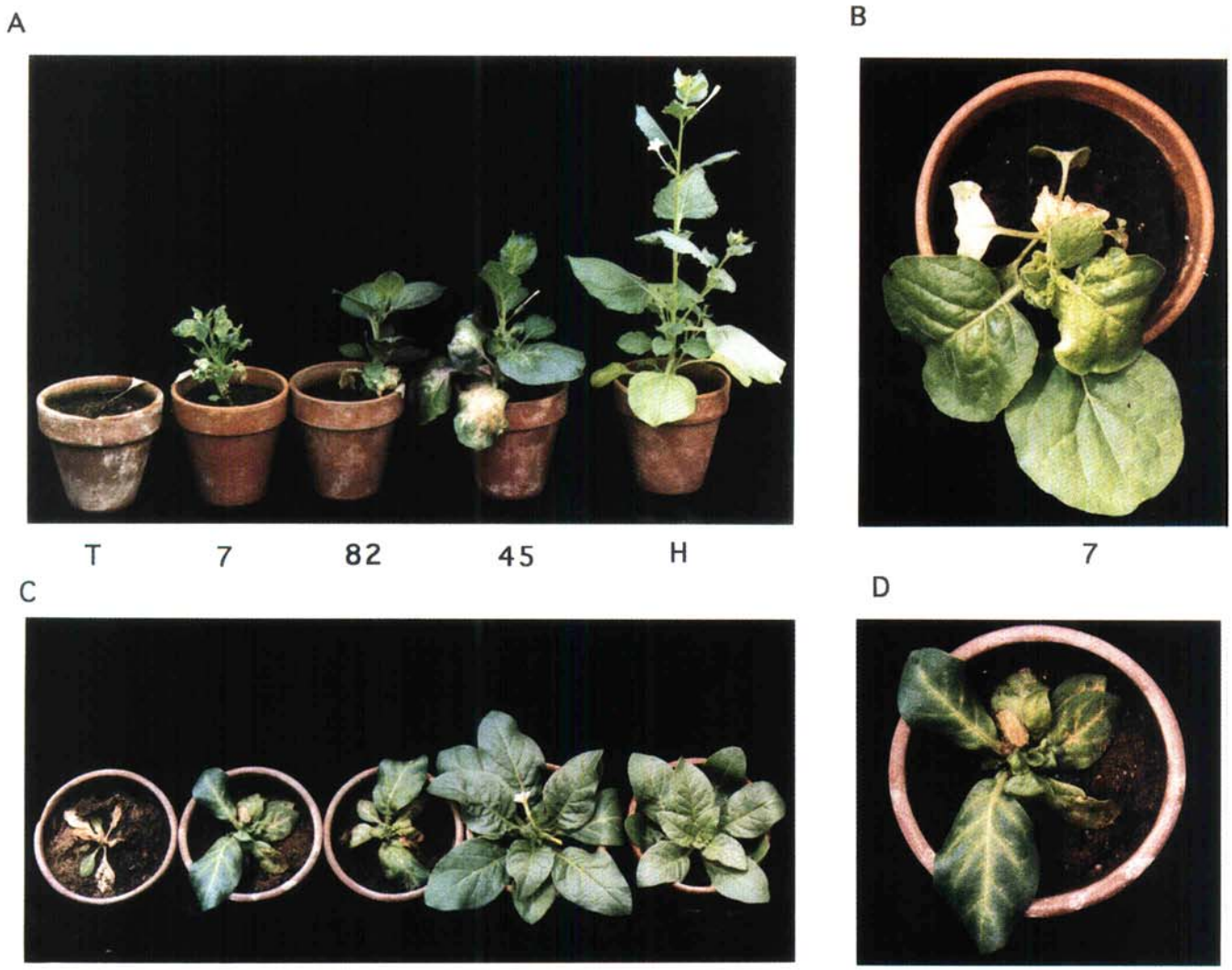


Fig. 6. Infected *Nicotiana benthamina* and *N. clevelandii* plants. **A,** *N. benthamiana* plants 4 wk postinoculation with RNA from (left to right) pTBSV-100, pHS7, pHS82, or pHS45; a healthy plant is shown on the far right. **B,** *N. benthamiana* 3 wk after inoculation with pHS7 RNA. This is a different inoculation experiment than that shown in A. **C,** *N. clevelandii* plants 3 wk after inoculation (same inoculation sequence as in A). **D,** Close-up of plant in C infected with pHS7 RNA.

strate at different times after inoculation to visualize GUS expression *in vivo*. Blue spots indicating replication and expression of pHS45 RNA were detected in inoculated tissue of the fifth and sixth leaves of *N. benthamiana* at 1 dpi (Fig. 7) despite the fact that no signs of virus infection were observed prior to the GUS assay. By 2 dpi, the diameter of the blue spots had increased, and radial expansion of these spots continued (Fig. 7) until about 7 dpi. The staining of the seventh and eighth inoculated leaves of *N. benthamiana* also increased over time, but the virus spread was quite erratic and diffuse in these leaves when compared to patterns developing in the lower leaves (Fig. 7). The blue staining intensity was very low in systemically infected noninoculated leaves, but occasionally a light blue stain became visible upon bleaching of this tissue with ethanol (data not shown).

Since the size of the GUS gene seemed to have an effect on replication (Fig. 3), we speculated that a smaller foreign insert, such as the CAT gene, would be expressed more efficiently in upper leaves. To test this, *N. benthamiana* and *N. clevelandii* plants were inoculated with pHS82 RNA and inoculated leaves and noninoculated upper leaves were harvested every second day for 18 days. Leaf extracts were subsequently assayed for CAT activity and the results at two different times postinoculation are presented in Figure 8. CAT activity was easily detected in extracts from inoculated leaves, but again low levels of foreign gene expression were detected in the upper leaves and enzymatic activity

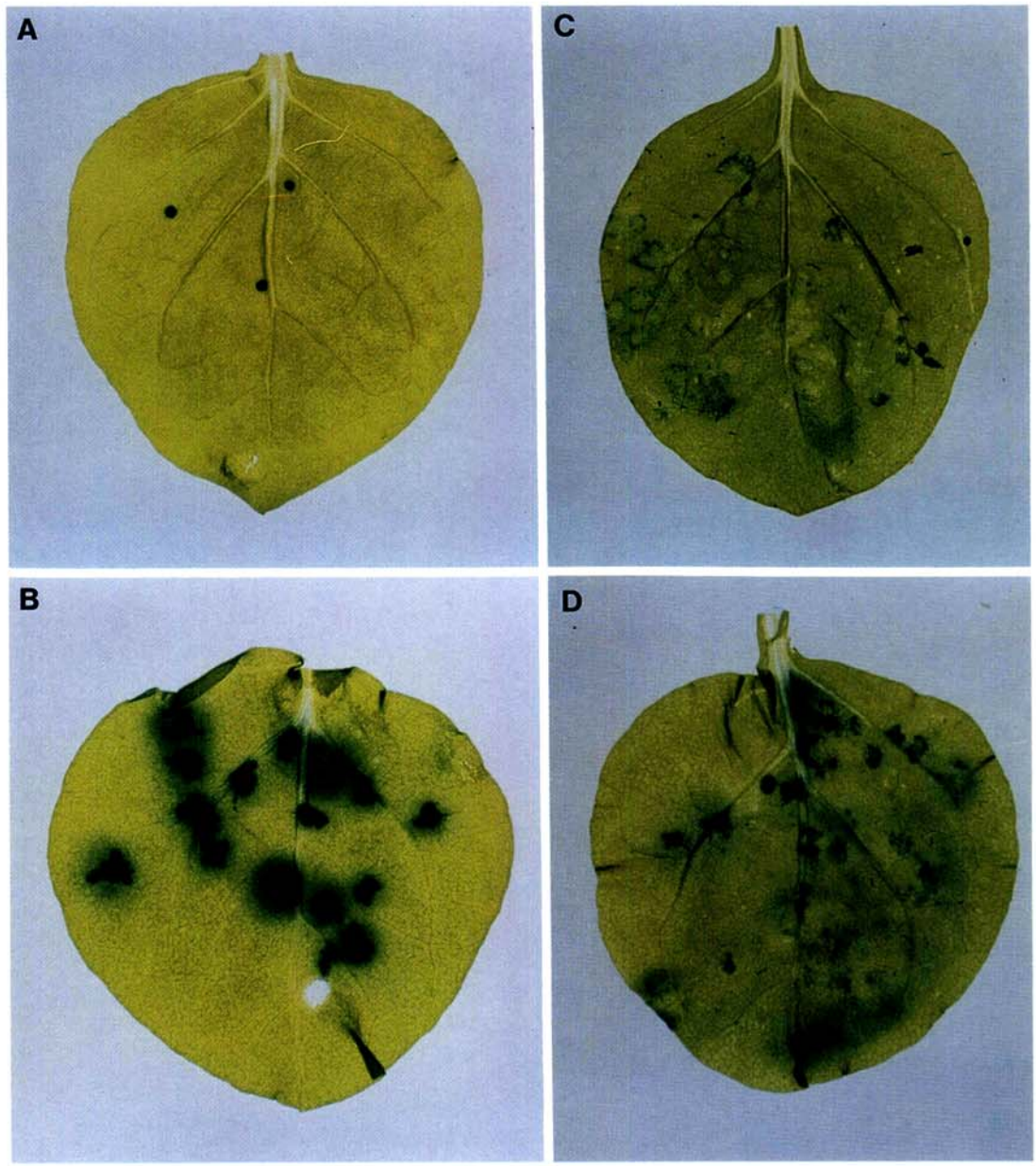


Fig. 7. Localization of GUS expression on *Nicotiana benthamiana* leaves upon inoculation with pHS45 transcripts. The left panel shows inoculated leaves positioned fifth or sixth from the base on the plants, **A,** 1 dpi or, **B,** 5 dpi. The right panel shows inoculated leaves positioned seventh or eighth, **C,** 2 dpi or, **D,** 5 dpi.

failed to increase with time. Instead, CAT expression in inoculated leaves decreased at time points beyond 14 dpi (data not shown).

The intact genomic RNA of pHS82 is similar in size to that of pHS7 RNA (Fig. 4). However, the genomic pHS82 dsRNA that was isolated 21 dpi from systemically infected *N. benthamiana* (Fig. 8) and *N. clevelandii* (not shown) was considerably smaller than that of pHS7. The deletion covered the complete CAT gene because the sgRNA for that gene was nearly the same size as the sgRNA for the downstream ORFs (Fig. 8). Thus, it is very likely that the rapid generation and subsequent selection of deletion mutants was responsible for the low levels of CAT activity in the upper systemically infected leaves. Similarly, deleted forms of the viral RNA were present in root samples which also lacked CAT activity.

Coat protein mutants elicit necrotic local lesions in which GUS expression can be visualized.

Inoculation of *C. amaranticolor* with pTBSV-100 transcripts resulted in the formation of localized necrotic lesions under normal greenhouse conditions (Fig. 9). Local lesions induced by the wild-type pTBSV-100 transcripts and the coat protein deletion mutant (pHS7) were essentially identical in their timing (2 dpi) and shape at 5 dpi (Fig. 9). Transcripts of pHS82 and pHS45, in which the coat protein gene was replaced with either the CAT or GUS gene (Fig. 1), respectively, were also able to elicit local lesions on *C. amaranticolor,* as shown in Figure 9. These lesions were (on average) delayed by 1 day up to a maximum of 2 days for lesions induced by pHS45 RNA. The diameter of the lesions and, in particular, the diameter of the necrotic center were reduced on leaves inoculated

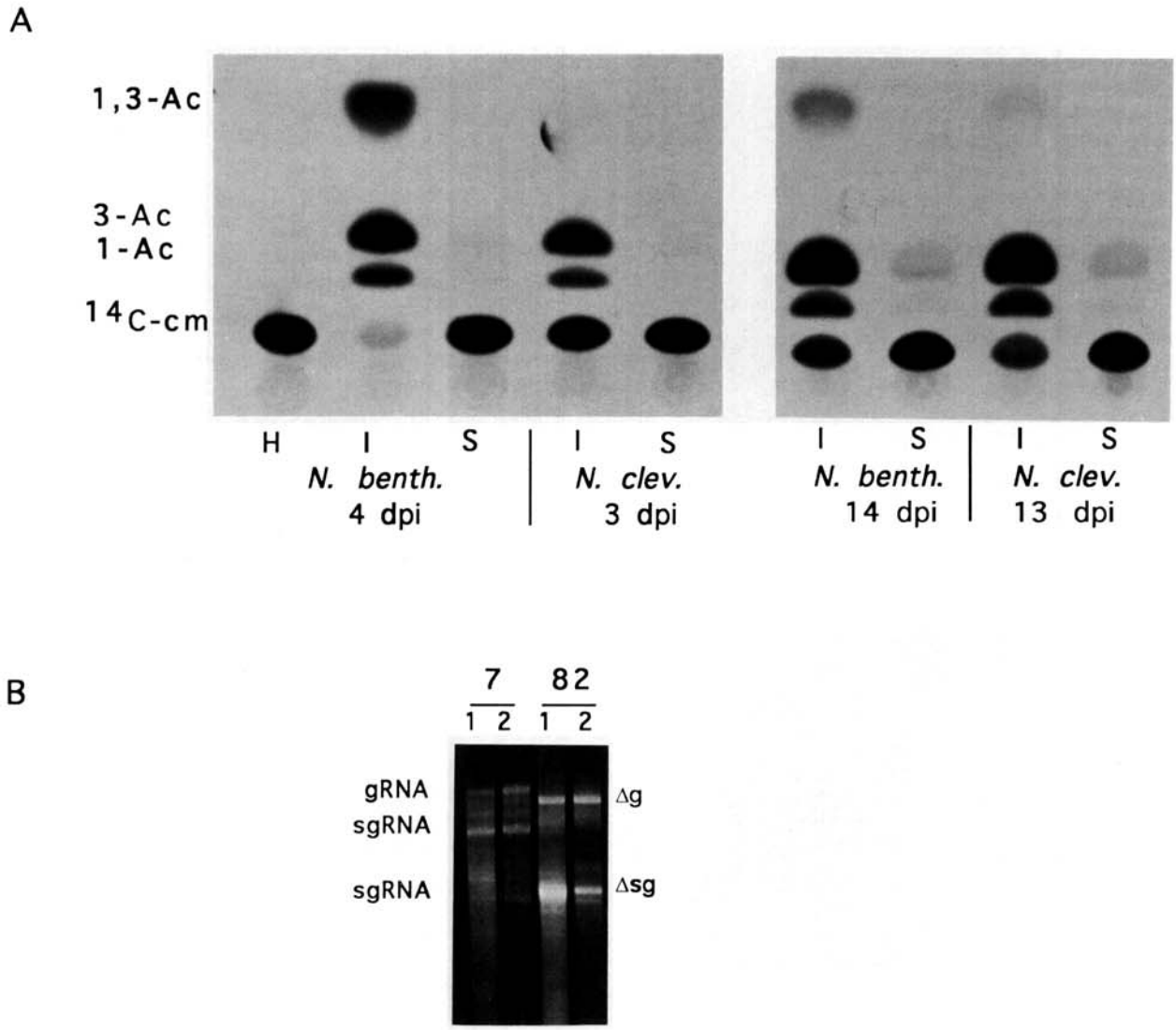


Fig. 8. CAT expression and replication of tomato bushy stunt virus (TBSV) harboring the CAT gene, in plants. **A,** Healthy (H), inoculated (I), or upper systemic leaves (S) from two tobacco hosts, *Nicotiana benthamiana* or *N. clevelandii,* were assayed for CAT activity at different times, as indicated below the lanes. **B,** Ethidium bromide-stained 1% agarose gel with dsRNA from infected *N. benthamiana* plants harvested 3 wk after inoculation with transcripts from pHS7 (7) or pHS82 (82). Since the inoculated leaves had died, the lower (1) and upper (2) systemic leaves were analyzed. The positions of the genomic RNA and sgRNAs are indicated. The delta symbol denotes RNAs with a deletion.

with transcripts from pHS45 (Fig. 9). Transcripts from pHS59, or from pTBSV-100 with a frameshift in the nested p19/p22 ORFs at the *Nco*I site (nt 3886, Fig. 1), failed to elicit local lesions on *C. amaranticolor*. But, these mutants replicated efficiently in protoplasts, thus confirming the role of p19 or p22 in cell-to-cell movement.

C. amaranticolor leaves that were inoculated with pHS45 RNA developed discrete blue spots at sites where local lesions were being formed upon vacuum-infiltration with GUS substrate (Fig. 9). This procedure permitted the visualization of infection foci as early as 1 dpi, when no other signs revealed the presence of the virus. Leaves that were inoculated with transcripts from either pTBSV-100 or pHS82 failed to develop the blue color and remained transparent upon vacuum infiltration and incubation in the GUS substrate solution. These results demonstrate that TBSV is able to replicate and spread to neighboring cells during the first day after inoculation. We also observed that the diameter of the blue lesions increased during the approximately 16-hr incubation, suggesting that the virus replicated and spread at 37° C even after vacuum infiltration of the leaves with GUS substrate.

At 1 dpi, the size (about 0.75 mm) and color intensity of the blue lesions on *C. amaranticolor* were similar to the blue spots that appeared in the systemic host *N. benthamiana* (Figs. 7 and 9). By 2 dpi, the lesion diameter had usually expanded to 1 mm and the first signs of halo formation around the lesions could sometimes be observed after overnight incubation in GUS substrate (data not shown). Lesions resulting from inoculation with pHS45 RNA increased in size until about 4–5 dpi, at which time they still stained blue when exposed to the GUS substrate. By 4 dpi, many lesions had a reduced intensity of blue stain, as illustrated in Figure 9. By 5 dpi typical lesions consisted of an inner blue core, which was often inside transparent tissue bordered on the outside by a brown ring surrounded by a chlorotic halo. Nevertheless, as is apparent in Figure 9, frequent exceptions to this general phenotype were observed. Six days postinoculation, the intensity of blue staining in most lesions was faint or often absent.

DISCUSSION

TBSV replication and gene expression are coat protein-independent events.

This study provides genetic evidence that the capsid protein gene of TBSV is dispensable for infectivity both in protoplasts and plants. The results in protoplasts demonstrate that neither coat protein expression nor the presence of putative *cis*-acting RNA sequences within the coat protein cistron, are required for RNA replication. Moreover, substitution of the coat protein gene with a reporter gene yields viral RNAs of predicted sizes that replicate and express the foreign gene in protoplasts. Evidently the coat protein is not required for regulation of sgRNA promoter activity or for translation of viral mRNAs.

The transient CAT expression data (Fig. 4) suggest that all major TBSV genes are expressed in protoplasts. The gene for p92 must be expressed since it is essential for replication (Scholthof *et al.* 1991a; unpublished). The coat protein cistron is expressed, as indicated by the GUS and CAT activity elicited by RNA from pHS45 and pHS82. Transcription and translation signals for p19 and p22 are also functional since pHS59, pHS91, and pHS87 give rise to RNAs that express the CAT gene. As demonstrated by Rochon and Johnston (1991) for CNV, both overlapping cistrons are very likely translated from the same sg mRNA. Our results show that this process is not affected by partial substitution of the p19 or p22 coding region with the CAT gene. From this evidence, we conclude that viral sequences downstream of the foreign gene insertion site are not involved in the initiation of transcription or translation. The factors and processes involved in the regulation of TBSV gene expression will be discussed in more detail in a future communication. Despite the fact that efficient gene expression occurs in TBSV mutants carrying reporter genes, irrespective of the viral ORF containing the substitution, the level of RNA replication appears to be inversely proportional to the size of the resulting recombinant viral RNA. Transcripts of pHS45 containing the GUS gene of about 1800 nt, replicate less efficiently than wild-type TBSV or TBSV containing an appoximately 800-nt-long CAT gene insert. However, we cannot rule out that some unknown inherent characteristic of the GUS gene may also interfere with replication.

Systemic movement of TBSV occurs in the absence of coat protein but requires expression of the 3′ nested genes.

The infectivity assays on whole plants demonstrate that TBSV RNA can move throughout *N. benthamiana* and *N. clevelandii* plants without the formation of virus particles (Fig. 5). Although the results suggest that the whole coat protein gene is dispensable for systemic movement, the mutants tested in this study contained 72–124 nt of the 5′ terminal coat protein gene sequences. Therefore, one could argue that all the mutants produce a portion of the N-terminal R-domain sequence which could interact with the viral RNA and contribute to the movement phenotype. A mutant of cymbidium ringspot virus (CyRSV), another tombusvirus, in which two amino acids in the shell domain were altered has also been shown to move systemically in *N. benthamiana* but poorly in *N. clevelandii* (Dalmay *et al.* 1992). Coat protein mutants of cucumber necrosis virus, another tombusvirus, also systemically invade *N. clevelandii* (Rochon *et al.* 1991), in a similar manner as TBSV. Only one other group of spherical viruses, the dianthoviruses, have been shown to spread in the absence of the coat protein, but, in this case, long-distance movement occurs only at reduced temperatures and exclusively in *N. benthamiana* (Xiong *et al.* 1993). TBSV mutants in which the coat protein gene is inactivated appear to be less restrictive and they induce systemic infections at normal greenhouse temperatures in *N. benthamiana* as well as in *N. clevelandii*.

Inactivation of p19 and p22 in pHS59 disables even short-distance virus movement, as we also noted in a preliminary report (Scholthof *et al.* 1991a) on wild-type derived mutants containing frameshifts or deletions in the nested genes. These observations agree with studies by Rochon and Johnston (1991) who reported that p21 of CNV (the analogue of the TBSV p22) but not p20 (the

analogue of p19) was essential for infection of plants. Since p22 of TBSV is not required for replication or gene expression, we conclude that this product is very likely a movement protein, which is perhaps functionally analogous to the TMV p30 movement protein (Deom *et al.* 1992). Computer-assisted comparisons of p22 with other viral movement proteins also suggest that p22 has a role in cell-to-cell spread (U. Melcher, personal communication). We speculate that p22 can form a complex with the viral RNA to allow and govern movement through plasmodesmata in a manner similar to that proposed for p30 of TMV (Citovsky and Zambryski 1991). This has also been suggested for analogous proteins involved in movement of other small plus-stranded spherical viruses (Hacker *et al.* 1992; Xiong *et al.* 1993). In accordance with this model, an RNA binding activity has recently been demonstrated for a dianthovirus protein that is required for movement (Osman *et al.* 1992). This model for movement may be applied to many plant viruses, and it fits the observation that TBSV mutants lacking the coat protein gene can undergo movement. Perhaps a nucleoprotein complex of movement proteins can substitute for virions in long-distance movement for some viruses but not for others. Such a model probably depends on the relative stability of the individual complexes formed for each virus. Nonetheless, the coat protein probably has some role in enhancing the efficiency of long-distance movement even in viruses like TBSV. Virion formation by coat protein subunits likely also prevents viral RNA degradation and could be essential for soilborne transmission of TBSV, which is probably important for spread under field conditions. The function of p19 remains unclear; clearly it is not required for replication or gene expression.

Coat protein mutations fail to induce lethal necrosis of systemic hosts.

Systemic tobacco hosts that were infected with coat protein mutants developed obvious symptoms of TBSV infection, but they generally did not succumb to the lethal necrosis associated with infection by wild-type virus. Our protoplast studies indicate that this effect is probably not due to differences in replication or gene expression, but it is possible that the coat protein affects symptom severity directly, as has been suggested to occur with TCV (Heaton *et al.* 1991). The observed change in symptom phenotype elicited by the coat protein mutants may also be due to the slightly impaired ability of coat protein mutants to spread efficiently over long distances, as has also been suggested for TMV coat protein mutants (Dawson and Hilf 1992). The somewhat slower long-distance movement of the virus may enable the plant to activate more effective responses to the invading virus. This could lead to biochemical reactions that circumvent or modulate the host-defense responses that otherwise culminate in a lethal necrosis during the rapid infections that occur with wild-type TBSV. The slower movement of the coat protein mutants may also be responsible for the induction of severe symptoms on the inoculated leaves. A similar interpretation was used to explain the hypersensitive response that was elicited by a coat protein mutant of the tombusvirus CyRSV on inoculated leaves of tobacco hosts (Dalmay *et al.* 1992).

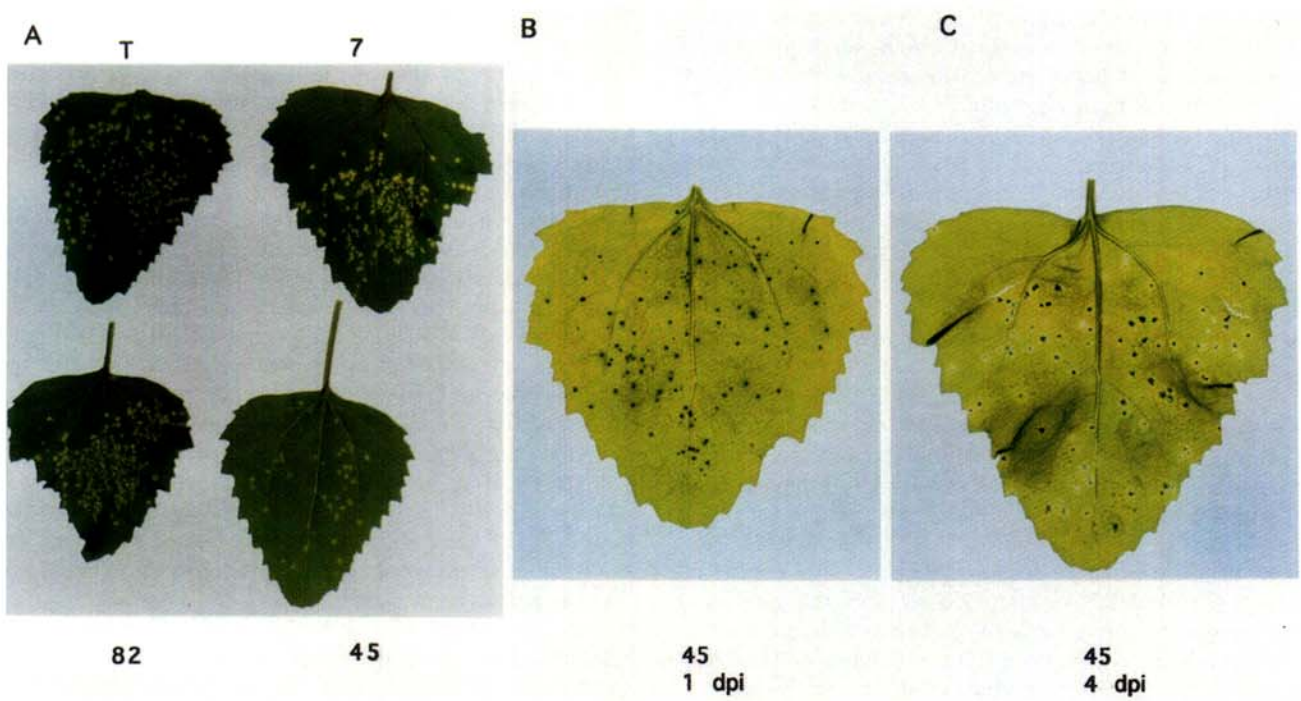


Fig. 9. Elicitation of local lesions on *Chenopodium amaranticolor* by tomato bushy stunt virus (TBSV) and coat protein mutants and visualization of GUS expression within the lesions. **A,** Leaves 5 days after inoculation with transcripts from wild-type pTBSV-100 (T); pHS7 (7); pHS82 (82); or pHS45 (45). **B,** Leaves assayed for GUS activity 1 dpi with pHS45 RNA. **C,** Leaves assayed for GUS activity 4 dpi with pHS45 RNA.

Efficient expression of reporter gene substitutions occurs primarily in inoculated leaves.

Plants that were inoculated with either pHS82 RNA or pHS45 RNA yielded high levels of reporter gene expression in inoculated leaves. However, during the infection cycle the virus effectively deleted the foreign inserts. Consequently the upper leaves became systemically infected with TBSV deletion mutants that were incapable of expressing the reporter genes, but which probably had a selective advantage for replication and rapid systemic movement. The poor levels of reporter gene expression by TBSV in systemically invaded tissue contrasts with similar experiments using rod-shaped viruses. For instance TMV, potato virus Y (PVY), and PVX have been demonstrated to express a foreign gene very efficiently throughout infected plants (Donson *et al.* 1991; Dolja *et al.* 1992; Chapman *et al.* 1992b). Nonetheless, it is apparent that reporter gene expression by TBSV permits the detection and localization of early events in the whole plant when no other signs indicate the presence of the virus. Thus, constructs like pHS45 will be especially useful for studies on cell-to-cell movement and for the identification of different cell types that become infected.

Elicitation of HR occurs in the absence of the coat protein gene and does not prevent early replication and movement of TBSV.

Although the coat protein of some plant viruses is required for elicitation of HR in certain hosts, other gene products can be responsible for this type of reaction (Culver *et al.* 1991; Schoelz *et al.* 1986). Our results demonstrate that expression of a functional coat protein of TBSV is not required for induction of HR on *C. amaranticolor*. At this point we have no information about which of the other TBSV gene products are responsible for HR, symptom expression, or host-range determination.

Transcripts containing GUS gene substitutions for most of the coat protein gene (pHS45) elicit a delayed HR response, which gives rise to small lesions. This corresponds with the delayed appearance of symptoms induced by pHS45 RNA on *N. benthamiana* compared to the timing of symptoms induced by pHS7 RNA, which has a small deletion, resulting in a frameshift in the coat protein gene. These effects might be related to the inhibitory effect of the relatively large GUS gene insert on replication in protoplasts. Transcripts from pHS59, or pTBSV-100 with a deletion or frameshift in the p19/p22 region, replicate in single cells but fail to spread in inoculated leaves. Since neither of these RNAs are able to elicit an HR on *C. amaranticolor,* we conclude that in addition to replication, cell-to-cell spread of TBSV is required for the development of necrotic local lesions.

Twenty-four hours after inoculation of *C. amaranticolor* with pHS45 RNA, the site of infection can be visualized as a small blue lesion upon an *in vivo* GUS assay. At that time no other signs indicate the presence of the virus. Although more accurate measurements are required to establish this firmly, it appears that by 1 dpi the size of blue lesions is similar in the systemic tobacco host and the local lesion *Chenopodium* host. These results suggest that early replication and spread of the virus occur at similar rates in both plants. Presumably, in *C. amaranticolor* the inhibitory effect of HR on virus spread has not taken effect during the first day of infection. A similar observation has been made during comparative studies on the replication and spread of TMV in Samsun versus Samsun NN tobacco which are systemic and local lesion hosts, respectively, for TMV (Fritig *et al.* 1987).

Four to five days postinoculation, the HR was clearly visible in tissue surrounding the blue spots. By this time the HR presumably had limited further movement of TBSV, since the diameter of the blue lesions failed to increase. However, the lesion phenotype exhibited considerable variation even within the same leaf. Sometimes, lesions were completely blue even at 4 dpi, whereas in other lesions a blue ring was occasionally observed surrounding the transparent necrotic lesions. It is likely that the timing of HR differs for different lesions so that several intermediate stages can be observed at 4 dpi on the same leaf. Eventually all lesions developed a chlorotic halo with a brown necrotic ring surrounding the blue center. Over time, GUS activity disappeared from all the lesions (data not shown), presumably because of degradation of cell constituents during necrosis. The biochemical mechanism by which HR in *C. amaranticolor* actually interferes with spread of the virus is not clear (Matthews 1991). Nevertheless, the sequence of events that can be distinguished with the GUS assay in our system are reminiscent of previous studies on the kinetics of local lesion formation in Samsun NN upon inoculation with TMV (Fritig *et al.* 1987). Additional studies using reporter gene expression to localize viral replication at specific time points may provide more details on the timing of the cascade of reactions leading to HR.

MATERIALS AND METHODS

Recombinant plasmids.

Standard molecular biology techniques were employed throughout this investigation (Sambrook *et al.* 1989). The plasmids used in the study were derived from an infectious cDNA clone of TBSV, designated pTBSV-100 (Hearne *et al.* 1990), which is illustrated in Figure 1. The numbering in the following paragraphs refers to the nucleotide positions on this plasmid. DNA from pTBSV-100 was cleaved with *Not*I (nt 2724) to yield a 3′ recessed terminus, which was subsequently filled in with DNA polymerase (Klenow) to create a blunt end. The DNA was also cleaved with *Bal*I (nt 2773) and both blunt ends were ligated. The resulting plasmid pHS7 has a deletion encompassing codons for most of the R domain in the coat protein. As a consequence of the deletion, the 5′ portion of the coat protein gene is fused out of frame with the remainder of the amino acids downstream of the introduced deletion (Fig. 1).

To investigate coat protein gene expression, two plasmids which contain the GUS reporter gene in-frame with the 5′ end of the coat protein gene (pHS8 and pHS45, Fig. 1) were constructed. To allow proper construction of pHS8 and pHS45, a subclone (pHS5) was first generated, which has the GUS gene from pBI121.1 (Jefferson 1987) inserted between the *Bam*HI to *Sac*I sites of pUC119. For construction of pHS8, the GUS gene from pHS5 was

removed with *Bam*HI (filled in) and *Eco*RI, and ligated into pTBSV-100 cleaved with *Bal*I (nt 2773) and *Eco*RI (nt 4034). Consequently, the GUS gene was positioned in-frame with the 5′ end of the coat protein gene, since the 23-nt-long leader sequence of the GUS gene was fused with the 5′ 124 nt of the coat protein gene. To restore the genes for p19 and p22 (Fig. 1), pHS8 was cleaved with *Eco*RI at the 3′ end of the GUS gene, filled-in, and digested with *Sph*I immediately downstream of the 3′ terminal position. This fragment was then replaced with the *Nde*I (nt 3379) (filled-in) to *Sph*I fragment of pTBSV-100 resulting in pHS45.

Additional reporter gene constructs were generated in which portions of ORFs for either the coat protein, p19, or p22 were substituted with the CAT gene (Fig. 1). Plasmid pHS82 has the CAT gene in-frame with the 5′ end of the coat protein gene. This construct was obtained by cleaving pHS45 with *Not*I (nt 2724) and *Sac*I (3′ end of GUS) whose ends were filled in and trimmed back, respectively. Subsequently, the CAT gene was excised with *Sal*I from pKAT and inserted into the linearized pHS45 following a fill-in reaction. The plasmid pKAT is a derivative of pBS9 (a kanamycin-resistant vector, Spratt *et al.* 1986) which contains the CAT gene from pCM-1 (Promega) at the *Sal*I site. The plasmid pHS59 (Fig. 1) has the CAT gene in-frame with the first AUG of p19 and was constructed by replacing a *Nco*I (nt 3886; filled-in) to *Nru*I (nt 4266) fragment of pTBSV-100 with the CAT gene from pKAT with filled-in *Sal*I termini. Plasmid pHS91 is very similar to pHS59, except that the 3′ end of the CAT gene was fused to the *Hpa*I site (nt 4170) instead of the *Nru*I site (Fig. 1). The construct pHS87 contains the CAT gene in-frame with the N-terminal 11 amino acids of p22; this plasmid was constructed by insertion of the CAT gene from pKScCAT (Scholthof *et al.* 1991b), with trimmed back *Sac*I termini, between the *Nco*I (nt 3886; filled in) to *Hpa*I (nt 4170) sites of pTBSV-100. All gene fusions in the plasmids used in this study (Fig. 1) were confirmed by sequence analyses using oligonucleotide primers that annealed in close proximity to the gene fusions.

Bioassays.

Infectious transcripts were generated from pTBSV-100 and its derivatives after linearization of CsCl-purified plasmids with either *Sph*I or *Sma*I, and a portion of the transcription mix was inoculated onto plants (Heaton *et al.* 1989; Hearne *et al.* 1990). *C. amaranticolor* plants were inoculated with 0.5 μg of RNA per leaf and *N. benthamiana* and *N. clevelandii* plants were inoculated with 2–5 μg of RNA per plant (about four leaves). Virus and viral RNA were isolated from 0.2 g (fresh weight) of infected tissue as described by Hillman *et al.* (1985, 1987). One-fifth of the lithium-soluble dsRNA fraction was separated in a 1% agarose gel in TBE. One-tenth of the virus preparation was electrophoresed through a 1% agarose gel in 10 mM Tris and 75 mM glycine (pH 8.0).

For replication and gene expression studies, transient assays were performed in protoplasts from *Cucumis sativus* 'Straight 8' (cucumber), *N. benthamiana* (tobacco), and *Lycopersicon esculentum* 'Pearson' (tomato). Protoplasts were prepared from cotyledons of greenhouse-grown cucumber plants before the first true leaves appeared. True leaves were used to generate protoplasts from *N. benthamiana* or tomato plants which were grown on 0.8% agar (in MS medium) in Magenta boxes (Chicago, IL) placed in a growth chamber (16 hr of 20 W fluorescent plant light, 24° C day temperature, 20° C night temperature) (Jones *et al.* 1990). The lower surface of the leaves was gently rubbed with a 1% Celite solution in 50 mM KH_2PO_4 (pH 7.0) using a cotton-tipped applicator. This process caused slight damage to the leaves, which permitted efficient enzymatic release of protoplasts upon floatation of the tissue on the cell wall-degrading enzyme solution (Jones *et al.* 1990). Further treatments, including incubation, harvesting, and transfection of protoplasts, were performed essentially as described by Jones *et al.* (1990). Transfections were usually carried out with 1–2 μg of transcripts per 2×10^5 protoplasts, but sometimes a lower concentration was used. In general the protoplasts were harvested 16–20 hr posttransfection and RNA was isolated (Jones *et al.* 1990). Approximately one-fifth to one-tenth of the total RNA was electrophoresed through a 1% agarose gel in TBE (Sambrook *et al.* 1989). Subsequently, the gel was subjected to a Northern blot analyses (Jones *et al.* 1990), except that the denaturation/renaturation of the gel was often omitted prior to transfer to Schleicher & Schuell (Keene, NH) Nytran membranes.

Reporter gene expression assays.

Approximately 0.5 g of leaf material was macerated in a mortar with 0.5 ml of GUS extraction buffer (Jefferson 1987). After a 20-sec centrifugation at 10,000 rpm, 50 μl of the supernatant was used for the fluorogenic GUS assay described by Jefferson (1987). For analyses of protoplasts, approximately 1×10^5 cells were disrupted 18 hr posttransfection by vortexing for 20 sec in 100 μl of GUS extraction buffer (Jefferson 1987) and processed as described for the plant extracts. Occasionally samples were stored at −80° C before the assay. To enable standardization of the quantitative data, about one-tenth of each supernatant was used to determine the protein concentration using the Bradford analysis kit (Bio-Rad, Richmond, CA). Histochemical staining of leaves was performed by one or two brief vacuum infiltrations of leaves with GUS substrate buffer (50 mM Na_2PO_4 [pH 7.0], 10 mM EDTA [pH 8.0], 0.5 mM $K_3Fe[CN]_6$, 0.5 mM $K_4Fe[CN]_6 \cdot 3H_2O$, and 0.25 mg/ml X-glucuronic acid, added from a fresh 100 mg/ml DMSO stock solution). The leaves were incubated for about 16 hr at 37° C and 100% humidity in the dark.

For CAT assays, the plant tissue (0.3 g fresh weight) and protoplast samples (about 75,000–100,000) were extracted with GUS extraction buffer as above and 35 μl of the supernatant was processed essentially as described by Gorman *et al.* (1982).

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