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Tomato Bushy Stunt Virus Spread Is Regulated by Two Nested Genes That Function in Cell-to-Cell Movement and Host-Dependent Systemic Invasion

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We have investigated the importance of two small nested genes (p19 and p22) located near the 3′ end of the genome of tomato bushy stunt virus (TBSV) for infectivity in several hosts. Our results show that both genes are dispensable for replication and transcription and that the p19 gene encodes a soluble protein, whereas the p22 gene specifies a membrane-associated protein. Assays using TBSV derivatives that have the β-glucuronidase gene substituted for the capsid protein gene demonstrate that p22 is required for cell-to-cell movement in all plants tested. Mutations inactivating p19 ameliorate the severe necrotic systemic symptoms elicited by wild-type TBSV in *Nicotiana benthamiana* and *Nicotiana clevelandii*, but p19 does not obviously affect movement in these hosts. However, in some local lesion hosts p19 influences the lesion diameter, which suggests that it has an auxiliary host-dependent role in movement. This notion is supported by the observation that p19 is required for long-distance spread of TBSV in spinach and for systemic infection of pepper plants. Thus, movement of TBSV is regulated by two nested genes; p22 governs cell-to-cell movement and p19 has a host-specific role in systemic invasion. © 1995 Academic Press, Inc.

INTRODUCTION

A particularly important requirement for plant virus infections involves the ability of a virus to spread systemically throughout the host (Atabekov and Dorokhov, 1984; Dawson and Hilf, 1992; Hull, 1989). Movement of plant viruses is an active process that can be divided into two broadly defined phases: cell-to-cell movement and long-distance transport. At least two strategies that permit effective cell-to-cell movement have evolved, and they both require modification of plasmodesmata to enable passage of macromolecules from one cell to another (Citovsky and Zambryski, 1993; Lucas and Gilbertson, 1994). One strategy permits transport of modified ribonucleoprotein complexes from cell-to-cell via plasmodesmata whose size exclusion limits have been modified by the movement protein. Another mechanism involves transport of whole virus particles through virus-induced tubules that traverse the plasmodesmata (van Lent *et al.*, 1991). Other mechanisms may also exist because the coat protein of potyviruses is involved in cell-to-cell movement, but this might not require virion formation (Dolja *et al.*, 1994, 1995).

No models are yet available to describe biochemical events mediating long-distance movement. However, with only a few exceptions (Petty and Jackson, 1990), the coat protein is essential for efficient long-distance transport of plant viruses, because even in the rare cases where the coat protein gene is partially or wholly dispensable for systemic infection, the time required for symptom development is often reduced in its absence (Dalmay *et al.*, 1992; Dolja *et al.*, 1994; Flasinski *et al.*, 1995; Hamilton and Baulcombe, 1989; Hilf and Dawson, 1993; Qui and Schoelz, 1992; Rao and Grantham, 1995; Scholthof *et al.*, 1993b; Taliansky and Garcia-Arenal, 1995; Xiong *et al.*, 1993). Long-distance movement can also be influenced by genes other than the cell-to-cell movement protein or the coat protein. In this regard, a recent study with tobacco mosaic virus (TMV) indicates that nonstructural components may assist phloem-dependent spread (Nelson *et al.*, 1993). In addition, long-distance transport of barley stripe mosaic virus can depend on the relative form and abundance of the expressed replicase proteins (Jackson *et al.*, 1991; Petty *et al.*, 1990). In the case of cauliflower mosaic virus (CaMV), the inclusion body protein, which functions as a posttranscriptional transactivator, is also involved in long-distance movement (Schoelz *et al.*, 1991). Moreover, long-distance movement of the potyvirus tobacco etch virus requires the helper component proteinase that is also essential for aphid transmission (Cronin *et al.*, 1995). Host-specific components involved in long-distance spread have not been identified yet, but recent investigations on movement of CaMV and turnip crinkle virus in several hosts suggest that host factors have important roles in systemic movement processes (Leisner *et al.*, 1993; Schoelz and Wintermantel, 1993; Simon *et al.*, 1992).

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Tomato bushy stunt virus (TBSV), the type member of the tombusvirus genus in the Tombusviridae (Francki *et al.*, 1991; Russo *et al.*, 1994) is a worldwide distributed soilborne root pathogen (Gerik *et al.*, 1990; Martelli *et al.*, 1988). TBSV replicates to high titers in a variety of plants and the experimental host-range includes over 100 dicotyledonous and monocotyledonous plant species in 20 different families (Martelli *et al.*, 1988). The ability of tombusviruses, and TBSV in particular, to be successful under very diverse conditions does not appear to be constrained by its relatively small genome size and compact organization (Grieco *et al.*, 1989; Hearne *et al.*, 1990; Rochon and Tremaine, 1989; Tavazza *et al.*, 1994). Genetic analyses of the genome of the cherry strain of TBSV revealed a positive-sense single-stranded RNA genome (Hearne *et al.*, 1990) that contains five major open reading frames (Fig. 1). The ca. 4800-nucleotide (nt) genomic RNA functions as a mRNA for the translation of two 5′-proximal genes that encode a 33-kDa protein (p33) and a readthrough product of 92 kDa (p92), which are both required for replication (Scholthof *et al.*, 1995b). The coat protein gene is located further downstream, and translation of this 41-kDa protein occurs from a subgenomic mRNA (Hillman *et al.*, 1989). The coat protein of tombusviruses is not required for infectivity in *Nicotiana benthamiana* or *Nicotiana clevelandii*, but mutants lacking a functional coat protein require a longer time to induce systemic symptoms, presumably because the rate of systemic spread is reduced (Dalmay *et al.*, 1992; McLean *et al.*, 1993; Scholthof *et al.*, 1993b). Two small nested genes, located near the 3′ terminus of the tombusvirus genome, are translated from a second subgenomic RNA (Hillman *et al.*, 1989; Rochon and Johnston, 1991) to yield two proteins (Hayes *et al.*, 1988; Johnston and Rochon, 1990) that are 22 kDa (p22) and 19 kDa (p19) in TBSV infections. Previous studies with TBSV and two other tombusviruses, cucumber necrosis virus (CNV) and cymbidium ringspot virus (CymRSV), with very similar genome organizations, suggest that p22, or its analog, is involved in movement (Dalmay *et al.*, 1993; Rochon and Johnston, 1991; Russo *et al.*, 1994; Scholthof *et al.*, 1993b). Thus far, the role of p19 in the infection process has remained unclear. However, p19 is an important symptom determinant (Dalmay *et al.*, 1993; Scholthof *et al.*, 1995a) and inactivation of the analogous protein in CNV accelerates *de novo* synthesis of defective interfering (DI) RNAs (Rochon, 1991).

The ability of TBSV to establish infections in numerous plant families suggests that the virus possesses virulence determinants that distinguish it from other small RNA viruses with more restricted host-ranges. Since plant virus movement seems to be a critical host-range determinant, we have focused on identification of factors that influence TBSV (cherry strain) cell-to-cell movement and long-distance spread in different hosts. The present study characterizes the 3′ nested genes and their encoded p19 and p22 proteins and provides functional analyses of these genes in selected members of different plant families.

MATERIALS AND METHODS

General

Molecular biology techniques used during this investigation followed standard protocols provided by the suppliers of reagents or by Sambrook *et al.* (1989). Bioassays with plants and protoplasts, virus or RNA analysis, and reporter gene expression studies were performed as described previously (Scholthof *et al.*, 1993b). The cucumber, spinach, and pepper plants used in this study were *Cucumis sativus* "Straight 8," *Spinacia oleracea* "Marathon," and *Capsicum annuum* "Early California Wonder," respectively. Protein extraction and fractionation from plants, purification of proteins from *Escherichia coli*, immunization of mice, and immunoblot assays were performed essentially as described by Scholthof *et al.* (1994). Additional details are provided in the figure legends and corresponding text.

The templates used for *in vitro* transcription were prepared by digestion of CsCl-purified DNA with *Sma*I for pTBSV-100 derivatives or *Sph*I, followed by treatment of the 3′ overhang with DNA Polymerase I Klenow fragment (Klenow treatment) for TBSV derivatives containing the β-glucuronidase (GUS) gene.

Recombinant plasmids

The plasmids used in this investigation were derived from pTBSV-100 (Fig. 1), an infectious full-length cDNA clone of the cherry strain of TBSV (Hearne *et al.*, 1990). The numbering throughout the present report refers to the nucleotide positions on this plasmid, and the constructs used for bioassays are schematically diagrammed in Fig. 1. The construction of the plasmids pHS7, pHS8, and pHS45 was described previously (Scholthof *et al.*, 1993b).

Plasmid pHS24 was constructed to provide a progenitor for many other constructs. This plasmid contains the *Stu*I (position 1059) to *Sal*I (position 4500) fragment of pTBSV-100 inserted into the *Sma*I to *Sal*I sites of pBS9, a kanamycin-resistant vector (Spratt *et al.*, 1986). To obtain glutathione S–transferase (GST) fusion products for antiserum production, the plasmids pHS111 and pHS113 were generated by digestion of pHS24 with *Nco*I (position 3886) or *Pfl*MI (position 3849), respectively, and *Sal*I (position 4500), followed by treatment with Klenow to generate blunt-end termini. Subsequently, the fragments containing the p19 or p22 genes were inserted in frame into pGEX-2T (Pharmacia, Piscataway, NJ) that had been digested with *Sma*I to create pHS111, or *Eco*RI (Klenow treated) to create pHS113. The correct fusion of the reading frames was confirmed by sequencing. Procedures previously described (Scholthof *et al.*, 1994) were em-

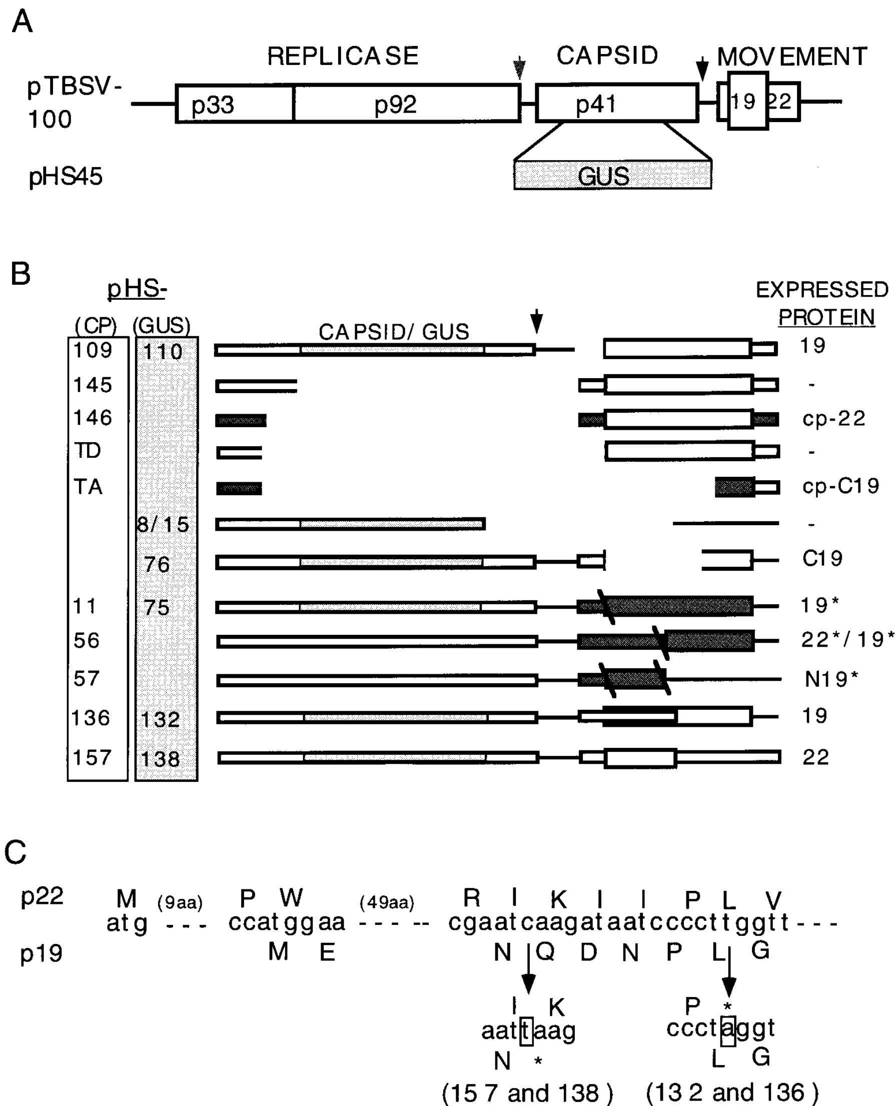


FIG. 1. Schematic diagram of TBSV and derived mutants. (A) Organization of the ca. 4800-nt single-stranded plus-sense genomic RNA of TBSV, based on the sequence and genetic data obtained with the biologically active cDNA of pTBSV-100 (Hearne *et al.*, 1990; Scholthof *et al.*, 1993b, 1995b). Open boxes denote ORFs that encode proteins whose sizes are given in kDa inside the boxes and whose associated functions are provided above. Solid lines represent presumed untranslated sequences. Arrows indicate the transcriptional start sites of the two sgRNAs. The diagonal lines and shaded box depict the part of the coat protein gene that is replaced with the GUS gene in pHS45. (B) Mutants used in this study. Only the relevant portion of the genome, from the 5′ end of the coat protein gene (on the left) to the 3′ end of p22, is shown. The numerical designations of mutants in the pHS series and the letter codes for pTA and pTD are given on the left. The plasmids in the "(CP)" column have a mutation in a TBSV background that contains the coat protein gene, whereas the mutants in the shaded "(GUS)" column were derived from pHS45 with the GUS gene substitution (shaded box). Some mutations were engineered in both CP and GUS backgrounds. The pHS15 plasmid is a pHS8 derivative that is replication deficient due to a deletion in p92, similar to that in pHS9 described by Scholthof *et al.* (1995b). Symbols are mostly as in (A), with the addition of gaps that represent deletions, single diagonal lines that indicate sites where the addition of four nucleotides introduced a translational frameshift, and dark shaded boxes that depict fusion of different genes into the same translational ORF. The expected p19 and p22 derivative proteins are given to the right: cp, coat protein; C, carboxyl-terminal end; N, amino-terminal end; asterisks (*) indicate p19/p22 fusions; minus signs (−) indicate lack of p19 or p22 proteins. (C) Detailed map of the p19 and p22 coding regions that were changed by introduction of site-specific mutations. Conversion of a C to a T in pHS157 and pHS138 creates a premature stop codon (*) in p19, whereas the substitution of a T to an A in pHS132 and pHS136 introduces a translational stop codon in p22.

ployed to overexpress the fusion proteins from pHS111 and pHS113 in *E. coli.* After overexpression, the GST fusion proteins were purified by PAGE, electroeluted, and injected into female Swiss Webster mice. Tail bleeds of the mice were obtained during the injection regimen to monitor for high-titer production of antibodies. Selected mice were injected with T-180 sarcoma cells and the ascites fluid was collected for use in serological assays as described previously (Goldberg *et al.*, 1991; Scholthof *et al.*, 1994).

Plasmid pTA was generated by digestion of pTBSV-100 with *Not*I (position 2724) and *Nru*I (position 4266), followed by Klenow treatment and ligation. The deletion removed essentially all of the coat protein gene and over

half of the p19 and p22 genes. Translation of the sgRNA from this mutant could potentially lead to a chimeric protein in which the N-terminal 26 amino acids of the coat protein are fused to the carboxyl 27 amino acids of p19. The plasmid pTD was made upon digestion of pTBSV-100 with *Not*I (position 2724) and *Nco*I (position 3886), followed by Klenow treatment and ligation. This plasmid also had most of the coat protein gene deleted so that its 5′ end was fused with the p19/p22 sequence at the *Nco*I site, but out of frame with either p19 or p22. As a consequence of the deletion, the 5′ end of p22 was removed in pTD, but the p19 ORF remained intact. However, the construction eliminated sequences that are required for transcription of sgRNAs that could translate p19. Plasmid pHS145 was produced by digestion of pTBSV-100 with *Msc*I (position 2773) and *Pfl*MI (position 3849, Klenow treated). The resulting construct had the 5′ 123 nucleotides of the coat protein gene fused to the p22 RNA sequence in a configuration that could code for 41 N-terminal amino acids of the coat protein, plus 3 additional amino acids encoded by codons that overlap but are not in frame with the very 5′ end of p22. Plasmid pHS146 was constructed by digestion of pTBSV-100 with *Not*I (position 2724) and *Pfl*MI (position 3849) followed by Klenow treatment and religation. This yielded a translational fusion of the first 26 codons of the coat protein gene to the start codon of p22. As with pHS145, the coding regions for the p19 and p22 proteins remained intact, but the small sgRNA used for their translation could not be transcribed. In pHS109 and pHS110, the DNA of pTBSV-100 and pHS45, respectively, was digested with *Pfl*MI (position 3849, Klenow treated) and *Nco*I (position 3886, Klenow treated) and ligated. This produced derivatives in which p22 was inactivated through deletion of 33 nucleotides at its 5′ terminus, so that the first AUG on the resulting sgRNA was the start codon for p19. In pHS11 and pHS75, the *Nco*I site (position 3886) of pTBSV-100 and pHS45, respectively, was Klenow treated followed by ligation to generate a translational reading frame consisting of a fusion of the 33 nucleotides at the 5′ of p22 to the entire p19 ORF. The plasmids pHS56 and pHS57 were obtained by digestion and subsequent Klenow treatment at the *Eco*RI site (position 4034) of pTBSV-100 and pHS11, respectively. In pHS56, this caused a fusion of the ORF for the N-terminal 61 amino acids of p22 to the carboxyl 123 amino acids of p19. Since in pHS11, the immediate 5′ end of p22 was already fused to p19, this additional manipulation at the *Eco*RI site, to create pHS57, introduced a stop codon at position 4038 leading to premature translational termination of the p22/p19 fusion product. For construction of pHS76, pHS45 was digested with *Nco*I (position 3886, Klenow treated) and *Hpa*I (position 4170), and the ends were ligated. This internal deletion and subsequent ligation resulted in an in-frame fusion of the AUG of p19 to its 3′ half, and in p22, it introduced a stop codon after the first 10 amino acid codons. Construction of the plasmids pHS132, pHS136, pHS138, and pHS157, which contain mutations that eliminate expression of either p19 or p22 (Fig. 1), was described previously (Scholthof *et al.*, 1995a).

RESULTS

Inactivation of p19 and p22 does not affect replication and transcription

Three different classes of mutations that affected the p19 and p22 nested genes were introduced into the infectious cDNA clone pTBSV-100 (Fig. 1). The first set of mutants contained deletions that prohibited expression of the coat protein, p19, and p22 in pHS145, pHS146, pTD, and pTA, whereas the small deletion in pHS109 only affected the p22 coding region. Since these deletions potentially could have affected important secondary or tertiary structural elements of the RNA genome, a second set of mutants was generated to introduce less substantial modifications. In this set, the coding region of p19 and p22 was altered by introducing four nucleotides at various sites to yield the frameshift mutants pHS11, pHS56, and pHS57. A third set of mutants contained site-specific mutations. In pHS157, a premature stop codon was introduced into the p19 gene that truncated the protein after translation of 52 out of 172 amino acids without interfering with synthesis of p22. Similarly, in pHS136, a stop codon was introduced that terminated translation of p22 after 67 of the 189 amino acids without affecting synthesis of p19.

Transfection of protoplasts with transcripts from pTBSV-100 (wild type), pHS57 (p19/p22 mutant), pHS157 (p19 mutant), or pHS136 (p22 mutant) (Fig. 1) followed by immunoblot assays (Fig. 2A) confirmed that the predicted proteins were expressed from each mutant. In addition, these data showed that the mutations eliminated the accumulation of the target gene product without interfering with the accumulation of the protein encoded by the other nested gene. Expression of p19 in protoplasts (Fig. 2A) was consistently and easily detectable, in contrast to the very low signal for p22. Additional immunoblot assays (data not shown) also demonstrated that viral RNAs derived from pHS11, pHS56, and pHS146 (Fig. 1) produced the expected translational fusion proteins. The predicted pTA and pHS57 short fusion products consisting only of portions of p19 or p22 (Fig. 1) were not detected, perhaps because of instability, their small size, or poor recognition by the antisera.

The results of RNA blot analyses show that none of the p19 and/or p22 mutations eliminated genomic RNA (gRNA) replication or production of subgenomic RNAs (sgRNAs) in *N. benthamiana* protoplasts (Fig. 2B). Similar experiments with protoplasts from cucumber and tomato confirmed that p19 and p22 were dispensable for replication and transcription (data not shown). Although levels of RNA accumulation sometimes varied for different mutants, we have thus far failed to observe a significant

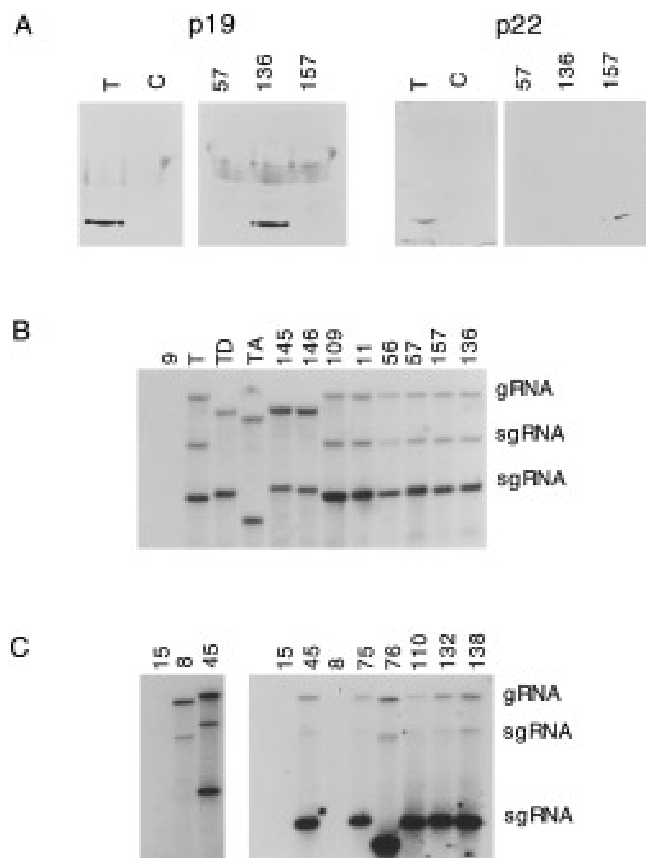


FIG. 2. Effect of p19 and p22 mutations on TBSV replication. (A) Immunoblot analyses of total protein extracts from *N. benthamiana* protoplasts transfected with RNA from the plasmids in the pHS series (Fig. 1), whose numerical designations are provided above the lanes. Lanes designated T show results of inoculation with wild-type TBSV transcripts derived from pTBSV-100. Control (C) plasmids were TBSV derivatives (pHS9 or pHS97) whose RNAs fail to replicate (Scholthof *et al.,* 1993b, 1995b). The panels show the immunoblots after treatment with p19 (left)- or p22 (right)-specific antibodies, followed by a chemiluminescent assay. (B) Hybridization blot of RNA from *N. benthamiana* protoplasts after transfection with transcripts from pHS9 (9, negative control; Scholthof *et al.,* 1995b), pTBSV-100 (T), pTD (TD), pTA (TA), and other mutants in the pHS series that were derived from pTBSV-100. (C) Replication of RNA from the GUS gene substitution pHS derivatives in protoplasts from cucumber (left) and *N. benthamiana* (right). Differences in replication usually corresponded very closely with differences in GUS expression levels. The hybridization probes in (B) and in the right panel of (C) consisted of randomly primed DNA fragments from a plasmid that contains the 3′ end of the TBSV genome from position 4500 to the 3′ end. The blot in the left panel in (C) was hybridized with pHS49 (Scholthof *et al.,* 1993a), which contains all sequences downstream of position 3781 in addition to ca. 400 nt at the 5′ end of the genome which composes the 5′ end of DI molecules (Knorr *et al.,* 1991).

and consistent quantitative effect. The sizes of the gRNA and sgRNAs produced by TBSV in protoplasts were as predicted. Since the hybridization probe was designed to measure equimolar amounts of the 3′ end of the RNAs, differences in intensity of the bands indicate that the molar accumulation of the 3′ proximal p19/p22 sgRNA exceeded that of the coat protein sgRNA or the gRNA. This is in agreement with our studies on TBSV RNA accumulation in plants (Scholthof *et al.,* 1995c) and previous investigations with a different tombusvirus, artichoke mottle crinkle virus (Tavazza *et al.,* 1994).

As shown in Fig. 2B, analyses of protoplasts transfected with transcripts from pTD, pTA, pHS145, and pHS146 revealed that all deletions within the region upstream of the p22 start codon abolished transcription of the small sgRNA used for translation of p19 and p22. The sgRNA present in those lanes corresponded in size to the predicted truncated version of the large coat protein sgRNA. Deletion of sequences immediately downstream of the start codon for p22, as in pHS109, had no obvious effect on accumulation of the smaller sgRNA. These results confirmed that the p19 and p22 proteins are not required for replication and that they are also not necessary for transcription of sgRNAs. In addition to the dispensability of the p19 and p22 proteins, RNA sequences within the 5′ half of the nested genes were also dispensable for replication and transcription.

The p19 protein is mostly soluble whereas p22 fractionates with host membranes

To investigate the accumulation and distribution of p19 and p22 in infected plants, *N. benthamiana* tissue was fractionated at different days postinoculation (dpi) with TBSV. Immunoblot assays (Fig. 3) revealed that p19 could be detected by 2 dpi in inoculated leaves and by 3 dpi in systemically invaded leaves. Analyses of the protein distribution in the cell fractions indicated that p19 was predominantly present in the S30 fraction, which consists primarily of soluble proteins. In contrast to p19, the majority of the p22 protein copurified with a fraction (P30) that was enriched for membrane-associated proteins, and p22 also appeared to accumulate slightly later than p19 because it was not detected in inoculated leaves until 3 dpi, and 5 dpi in systemically infected leaves (Fig. 3). As was observed for total extracts of protoplasts (Fig. 2A), the signal intensities of the immunoblots were higher for p19 in the S30 fraction than for p22 in the P30 fraction (Fig. 3). This effect is even more dramatic when taken into account that due to constraints imposed by the isolation procedure, the S30 lanes contained about 10-fold less of the total fraction than the lanes loaded with the other fractions.

Mutations inactivating p22 abolish cell-to-cell movement

To determine requirements for cell-to-cell movement, transcripts from deletion and frameshift constructs in which both the p19 and the p22 ORFs were inactivated (Fig. 1) were inoculated onto *N. benthamiana* (a host supporting systemic infection) and *Chenopodium amaranticolor* (a local lesion host). None of the RNAs with mutations that affected both p19 and p22 were able to establish systemic infections, and they also failed to elicit local lesions. More specific analyses showed that muta-

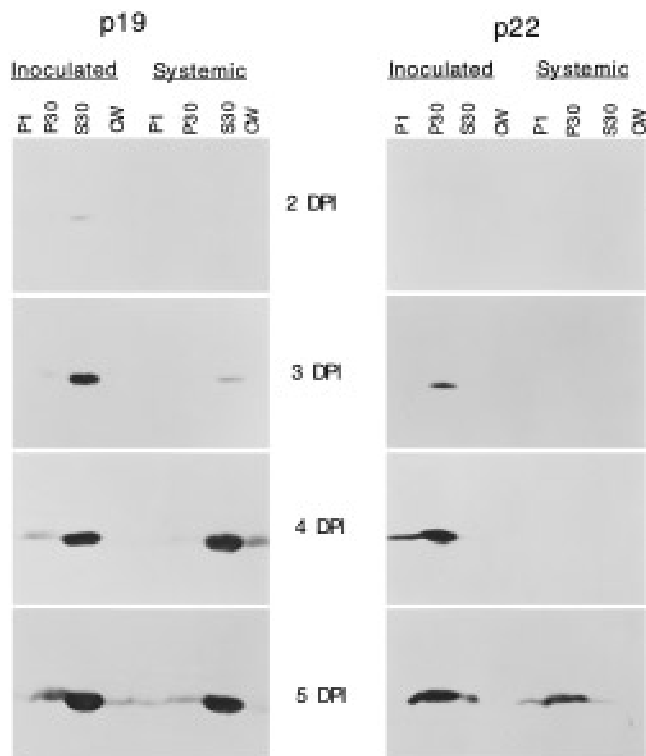


FIG. 3. Immunodetection, localization, and temporal accumulation of TBSV p19 and p22. Proteins were extracted from inoculated (Inoculated) or systemically invaded (Systemic) leaves of *N. benthamiana* plants at 2 to 5 days postinoculation (dpi). Immunoassays were performed with antibodies against p19 (left) or p22 (right). Only the relevant portions of the exposed film are shown. The proteins migrated at the expected position compared to the relative positions of size markers (not shown). Note that the S30 (30,000-g supernatant) lane was loaded with only about 10% of the relative amounts of the fractions loaded in the P30 (30,000-g pellet), P1 (5000-g pellet), and CW (cell wall) lanes.

tion of p19, in pHS157, did not impair the ability of TBSV to establish local lesions, as will be discussed in more detail in the following sections. However, RNA from pHS136, which expresses a truncated p22 derivative, failed to induce lesions (not shown). These experiments suggested that p22 was required for virus spread from the primary sites of infection to neighboring cells.

In order to obtain more detailed information concerning the cell-to-cell movement function of p22, and the dispensability of p19, mutations similar to those described for the wild-type pTBSV-100 were introduced into a virus background (pHS45) in which the coat protein gene was replaced by the GUS gene (Fig. 1) (Scholthof *et al.,* 1993b). In preliminary experiments with pHS45, infections within single cells could be detected as early as 15 hr postinoculation (hpi) in leaves with the *in vivo* histochemical GUS assay, and the infection foci subsequently spread throughout the inoculated leaf (data not shown). These experiments also revealed that inactivation of both p19 and p22 (as in pHS8) or p22 alone (as in pHS110) resulted in localization of staining primarily to single cells. However, in contrast to the observations with the p19/p22 deletion mutants in the wild-type background (Fig. 2B), some of the deletions in the p19/p22 region of the GUS substitution derivatives, such as in pHS8 RNA (Fig. 1), had unpredictable and inconsistent effects on replication. This could possibly have been influenced by the host because the negative effects of the deletion in pHS8 were less dramatic in cucumber than in *N. benthamiana* (Fig. 2C). Moreover, for unknown reasons, RNA from other GUS substitution derivatives (pHS75, pHS76, and pHS110) appeared to be less infectious, since fewer infected cells were observed than with the parental RNA from pHS45 (data not shown).

While the preliminary experiments with the GUS gene substitution derivatives supported the notion that p22 is required for cell-to-cell movement, they did not provide conclusive proof since the lower infectivity of some of the p19/p22 mutants could potentially have interfered with efficient movement. However, RNA from the parental clone (pHS45) and mutants with the site-specific changes within p19 (pHS138) and p22 (pHS132) (Fig. 1) replicated to similar levels in protoplasts (Fig. 2C). Although the number of infection foci per leaf varied considerably in plant experiments, consistent differences were not observed in the infectivity of RNAs from pHS45, pHS132, or pHS138. Inoculation of *Chenopodium quinoa* (a very susceptible local lesion host) with RNA from these three GUS derivatives resulted in infected cells that could be visualized within 16 hpi (Fig. 4). GUS assays with leaves sampled later than 20 hpi demonstrated that progeny RNAs from the parental GUS derivative (pHS45) and from the p19 mutant (pHS138) were able to spread from the initially infected epidermal cells to adjacent epidermal cells and to underlying mesophyll cells. These infection foci expanded rapidly with or without p19 expression (Fig. 4) and, by 3 days after inoculation, lesions were visible that encompassed hundreds of cells (data not shown). Beyond this time point, the lesions became necrotic and further expansion was restricted. These results showed that p19 is not normally required for localized cell-to-cell spread.

In contrast to the p19 mutant (pHS138), the p22 mutant (pHS132) failed to move from the initially infected epidermal cell to neighboring cells (Fig. 4). Results very similar to those shown in Fig. 4 for *C. quinoa* were obtained with another local lesion host, *C. amaranticolor,* and with hosts such as *N. benthamiana* and spinach that support systemic TBSV infections. The infection foci induced by pHS132 RNA normally appeared as dark blue stained individual epidermal cells (Fig. 4). However, sometimes a diffuse blue area was visible around several cells, which appeared to result from collapse of the initially infected epidermal cells, and subsequent release of GUS enzyme or its product, from these cells. Thus, the data above provide evidence that p22 is required for efficient cell-to-cell movement of TBSV and that p19 is dispensable for this process. This effect was observed in several plant species belonging to different families, regardless of

whether local lesions or a systemic infection occurred. Therefore, we conclude that p22 represents the cell-to-cell movement protein of TBSV.

p19 is dispensable for systemic infection of *N. benthamiana* and *N. clevelandii*

Since the evidence suggested that p19 has no primary role in replication, transcription of sgRNAs, or cell-to-cell movement, we evaluated the effects of p19 inactivation on infection of whole plants, using two *Nicotiana* hosts that support systemic TBSV infections. Inoculation of *N. benthamiana* or *N. clevelandii* with the p19 mutant (pHS157) resulted in systemic mosaic symptoms that appeared at the same time (4 to 6 dpi) as with wild-type TBSV. The major difference we observed was that infection with pHS157 RNA failed to induce the pronounced necrosis that normally leads to death of these host plants in wild-type TBSV infections (Fig. 5A). Previously, we showed that coat protein mutants also attenuate symptoms but, in contrast to the p19 mutant, inactivation of the coat protein gene normally delayed the onset of systemic symptoms. In addition, the capsid mutants still elicited severe necrosis (Scholthof *et al.*, 1993b), especially when very young plants (third or fourth leaf stage) were inoculated (data not shown). The concentration of viral specific dsRNA isolated from *N. clevelandii* leaves and roots infected with RNA from pHS157 (p19 mutant) was readily detectable in ethidium bromide-stained agarose gels, and the concentration appeared similar to that of dsRNA isolated from wild-type TBSV-infected plants (Fig. 5B). In addition, agarose gel electrophoresis of partially purified virus particles demonstrated that the distribution, concentration, and gel migration patterns of virus from leaves and roots was not obviously affected by inactivation of p19 (data not shown).

To determine whether the systemic infection induced by the p19 mutant (pHS157) was caused by an *in planta* mutation that could have (partially) restored p19 activity, an immunoblot analysis was performed on extracts from infected plants. Initially these assays were performed with plants that had been inoculated with transcripts, and the data confirmed that p19 was not detectable in plants inoculated with pHS157 RNA (data not shown). To test for possible low-frequency reversion events that could have contributed to systemic movement, infected plants were used as inoculum during four subsequent passage experiments and again immunoblot assays were performed (Fig. 5C). These results demonstrated that p22 accumulated to similar levels in plants infected with wild-type TBSV or the p19 mutant (pHS157). The same results were observed for accumulation of the replicase proteins (p33 and p92) and the coat protein (data not shown). However, p19 was not detected in plants infected with the p19 mutant (pHS157). Inactivation of the p19 analog in CNV resulted in the acceleration of DI accumulation in passaged plants (Rochon, 1991). We have thus far failed to detect substantial accumulation of DIs in plants inoculated with pHS157 transcripts (Fig. 5B), but we have not yet monitored the effect of p19 mutation on DI accumulation in serial passages. Nevertheless, the available data show that the p19 mutation was maintained *in vivo* and that p19 is not required for establishment of systemic infections in *N. benthamiana* or *N. clevelandii.*

p19 has variable effects on the local lesion phenotype in several hosts

N. benthamiana and *N. clevelandii* are two experimental hosts that are susceptible to many plant viruses and they often support infections with virus mutants that fail to infect more restrictive hosts. Therefore, we inoculated less permissive TBSV hosts to obtain supplementary information about the role of p19. To investigate whether the function of p19 could possibly be host dependent, we first monitored its effect on localized infections in several plant species (Fig. 6). Inoculation of the p19 mutant (pHS157) onto *Nicotiana edwardsonii* resulted in the appearance of local necrotic lesions that were similar to those elicited by wild-type TBSV (Fig. 6A) and the coat protein mutants (data not shown). Similar results were obtained for *C. amaranticolor* (data not shown), although the onset of lesions on leaves inoculated with the p19 mutant (pHS157) was occasionally slightly delayed. Other hosts in which the local lesion phenotype was not greatly affected by inactivation of p19 included *Gomphrena globosa, Datura metel,* or young *Nicotiana glutinosa* plants (data not shown). In contrast, inactivation of p19 resulted in local lesions in cucumber, spinach, and *C. quinoa* that were mostly chlorotic, rather than the necrotic lesions induced by wild-type TBSV (Figs. 6B–D). In addition to the decreased levels of necrosis, the local lesions elicited on *C. quinoa* (Fig. 6D) and on older *N. glutinosa* plants (data not shown) were smaller in diameter than those elicited by wild-type TBSV. The latter observations suggested that p19 could have an auxiliary role in movement in some members of the Chenopodiaceae and Solanaceae.

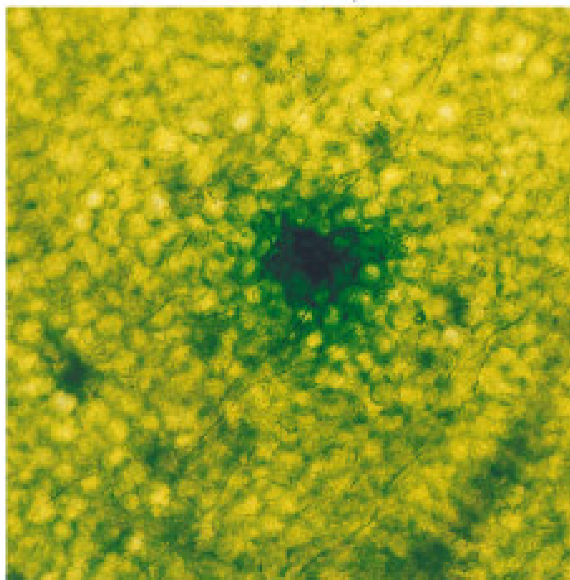
p19 is required for systemic invasion of pepper and spinach

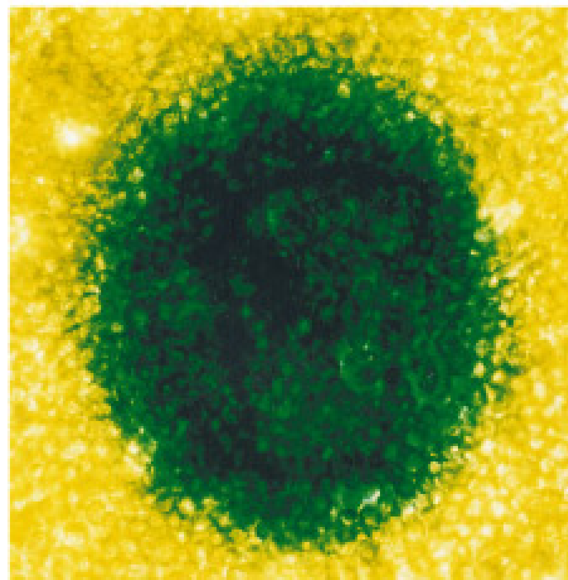
To further evaluate the possible role of p19 in movement of TBSV in selected members of the Chenopodiaceae and Solanaceae, we tested its requirement on spin-

FIG. 4. Visualization of TBSV cell-to-cell movement in *Chenopodium quinoa* by *in vivo* histochemical staining of GUS gene expression. Movement over time was monitored in leaves inoculated with RNA from pHS45 (top), pHS138 (middle), and pHS132 (bottom) by GUS assays at 16 hr (left) or 40 hr (right) after inoculation. Pictures were taken at 100× magnification on a Zeiss Axiovert 35 inverted light microscope.

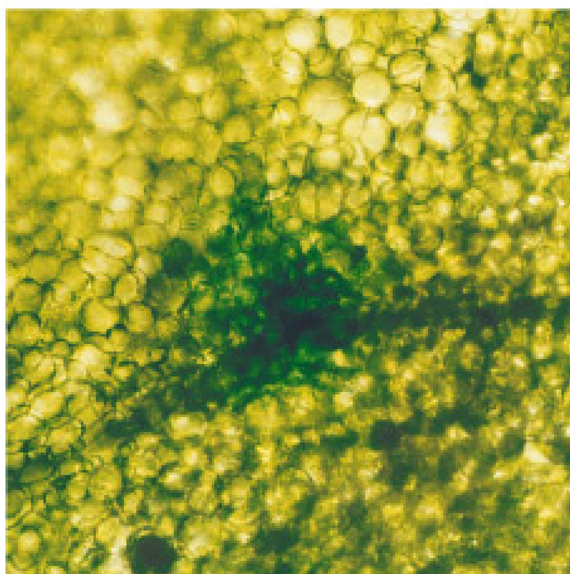
16 hpi | 40hpi

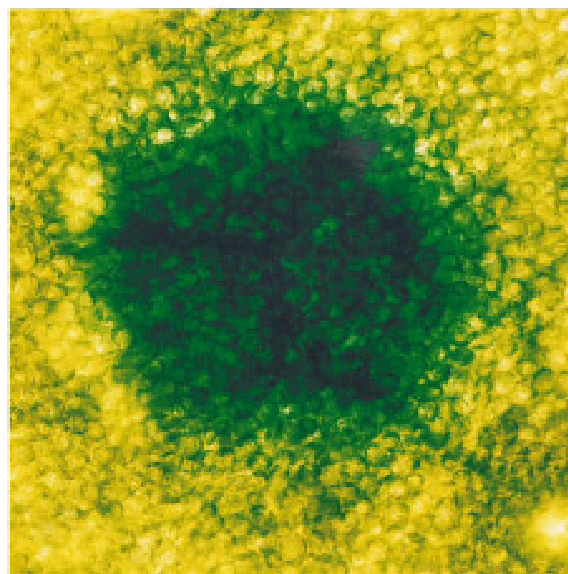
pHS45



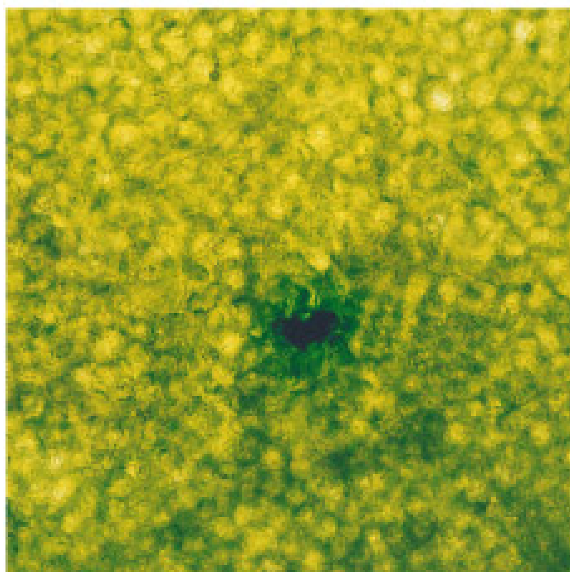


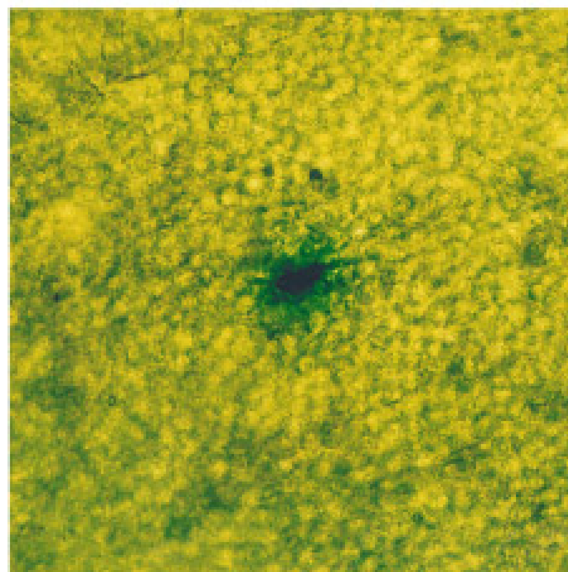
pHS138





pHS132





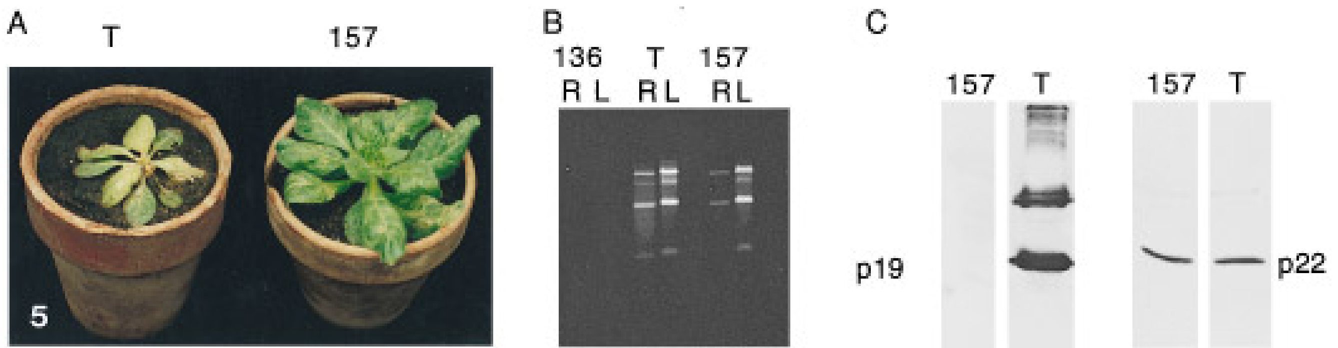


FIG. 5. Effect of p19 on systemic spread and symptom development in *Nicotiana* species that support systemic infections of TBSV. (A) *N. clevelandii* plants inoculated 2 weeks previously with plant homogenates containing TBSV (left) or pHS157 (p19) mutant inoculum (right). (B) Ethidium bromide-stained agarose gel showing TBSV-specific LiCl-soluble dsRNAs from leaves (L) or roots (R) of *N. clevelandii* plants inoculated 1 week previously with transcripts from pTBSV-100 (T), pHS136 (136), or pHS157 (157). (C) Immunoblot detection of p19 (left) and p22 (right) in tissue from *N. benthamiana* plants 4 days after inoculation with the fourth passage of TBSV (T) or pHS157 (157). The intense band above p19 has a relative mobility of ca. 40 kDa and is thought to represent a p19 dimer. In companion experiments, extracts from healthy plants failed to react with either the p19 or the p22 antisera.

FIG. 7. Effects of p19 on systemic spread and symptom development in spinach and pepper. (A) Spinach plants 1 month after inoculation with plant homogenates containing virus from TBSV (left) or pHS157 (right) and (B) pepper plants 2 weeks postinoculation (as for spinach). (C) GUS assay of a spinach leaf 4 days after inoculation with transcripts from pHS138. (D) Immunoblot assay for detection of coat protein in uninoculated plants (H) or in inoculated (I) leaves of pepper and spinach plants that were inoculated 2 weeks previously with plant homogenates containing virus from TBSV (T) or pHS157 (157). Lanes designated (S) were loaded with extracts from potentially systemically infected leaves located above the primary inoculated leaves. Samples from spinach plants infected with wild-type TBSV were omitted because of the extensive necrosis of leaves.

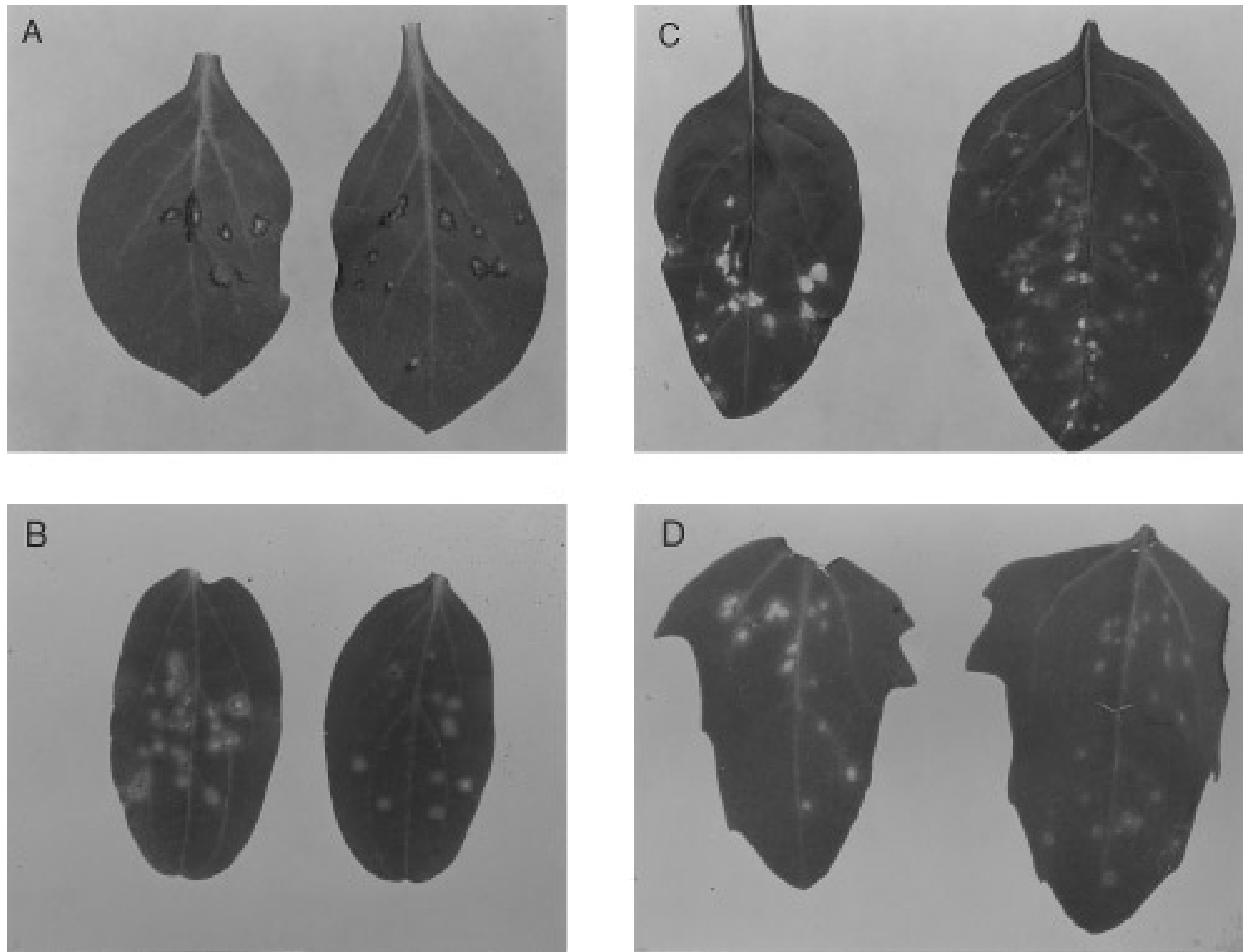


FIG. 6. Effect of p19 inactivation on the local lesion phenotype. Leaves were inoculated with plant homogenates containing either wild-type TBSV (left) or virus derived from pHS157 (right). (A) *N. edwardsonii* 5 dpi, (B) cucumber 10 dpi, (C) spinach 8 dpi, and (D) *C. quinoa* 5 dpi.

ach and pepper, both of which support systemic infections with TBSV. Since we failed to infect pepper upon inoculation with *in vitro* generated transcripts, a homogenate from infected *Nicotiana* plants was used for inoculations. Spinach was very susceptible to both the plant homogenates and to RNA inoculations, and the results were similar using either source of inoculum.

Spinach plants inoculated with wild-type TBSV developed a pronounced deformation of systemically infected leaves, followed by a severe necrosis that often culminated in plant death (Fig. 7A). The localized symptoms that were induced by the p19 mutant (pHS157) in spinach (Fig. 6C) suggested that p19 was not required for replication or cell-to-cell movement in this host. This was confirmed with the GUS gene derivative of the p19 mutant (pHS138), which produced blue lesions (Fig. 7C) similar in size to those induced by the parental GUS derivative (pHS45) (data not shown). This demonstrated that the p19 mutant replicated and spread to neighboring cells in inoculated spinach leaves. However, in contrast to the systemic infections occurring in *N. benthamiana* and *N. clevelandii,* spinach plants inoculated with pHS157 failed to display systemic symptoms in the upper leaves (Fig. 7A), although some chlorotic lesions were occasionally observed on older leaves positioned immediately above the inoculated leaves. An immunoblot of infected spinach plants (Fig. 7D) demonstrated that the p19 mutation prevented long-distance spread because coat protein failed to accumulate in the upper leaves, although this protein was easily detectable in the inoculated leaves. In addition, spinach plants inoculated with the p19 mutant and healthy spinach plants showed similar seasonal variations in flowering and setting of seed. Although the accumulation of symptom-attenuating DIs was shown to be accelerated by a mutated p19 analog in CNV (Rochon, 1991), the failure of the TBSV p19 mutant to establish systemic infections in spinach is not likely to be due to a DI-mediated effect. This conclusion is based on our observation that co-inoculation of TBSV RNA and transcripts from an infectious DI clone (B10; Knorr *et al.,* 1991) gave rise to attenuated, but fully systemic, symptoms in spinach (data not shown).

Inoculation of pepper plants with wild-type TBSV resulted in systemic symptoms that appeared ca. 1 week after inoculation. These symptoms included chlorosis, leaf curling, and some necrosis (Fig. 7B). The symptoms

induced by the p19 mutant on the inoculated pepper leaves (Fig. 7B) were not as consistent, but chlorotic areas were occasionally observed. However, in contrast to the observation in spinach, the p19 mutant failed to accumulate to detectable levels in the inoculated pepper leaves (Fig. 7D). Similarly, TBSV RNA could not be detected in pepper leaves inoculated with the p19 mutant (pHS157), as determined by dsRNA analysis or hybridization for detection of single-stranded RNA (data not shown). More refined cellular analyses of replication of the p19 mutant in pepper have thus far failed because we have not been able to transfect pepper protoplasts. Moreover, since pepper plants were not susceptible to inoculation with *in vitro* generated transcripts, utilization of the GUS gene derivatives for studies of cell-to-cell movement was not possible. Nevertheless, as was the case with spinach, the pepper plants inoculated with the p19 mutant displayed no abnormal symptoms on the upper uninoculated leaves, they flowered normally, and bore fruit. Additional immunoblot assays confirmed that the p19 mutant (pHS157) did not accumulate in the upper leaves (Fig. 7D). We have not yet determined if the failure of the p19 mutant to infect pepper involves the appearance and accumulation of DIs.

We were also able to obtain evidence that the p19 mutant (pHS157) failed to invade the upper uninoculated spinach and pepper leaves, even after prolonged periods of infection. In these experiments, homogenates from the upper leaves of spinach or pepper plants whose lower leaves had been inoculated 5 weeks previously were used to inoculate the sensitive indicator plants *N. benthamiana* and *C. amaranticolor.* With inoculum from the upper leaves of spinach and pepper infected with wild-type TBSV, the indicator plants became infected. However, no signs of infection were observed on indicator plants rubbed with extracts obtained from the upper leaves of plants inoculated with the p19 mutant. Based on these results, we conclude that p19 is required for systemic invasion of spinach and pepper by TBSV.

DISCUSSION

TBSV p19 and p22 are dispensable for replication and transcription

In this study we have characterized the function of the 3′ nested TBSV genes p19 and p22. The mutagenesis assays show that small mutations affecting the p19 and/or p22 ORFs, as well as large deletions, do not have obvious effects on replication or transcription. The deletion analyses also indicate that regulatory sequences governing transcription of the p19/p22 sgRNA are located upstream of the translation initiation site of p22. Together with results from a previous report (Scholthof *et al.*, 1993b), we have now shown that at least 70% of the p19/p22 coding region (from nucleotide positions 3849 to 4266) can be deleted without inactivating replication or transcriptional activity. CymRSV mutants with extensive deletions of the nested gene sequences replicated and transcribed very poorly (Dalmay *et al.*, 1993), suggesting that essential sequences reside within the nested genes of CymRSV. If such sequences exist on the TBSV genome, they must be located downstream of position 4266 at the 3′ end of the nested gene deletion in pTA (Fig. 1), because mutants with sequences deleted upstream of that position replicate and transcribe efficiently.

Although the coat protein gene of TBSV is dispensable for infection (Scholthof *et al.*, 1993b), derivatives containing GUS substitutions for the coat protein gene replicate less efficiently than the wild-type virus or mutants containing deletions that inactivate the coat protein gene. In addition, our results show that deletions in the p19/p22 region of the GUS gene derivatives have variable and unpredictable effects on the level of replication that are not observed in the wild-type TBSV background. These effects could reflect introduction of secondary structural changes within the RNA that may have subtle effects on replication of mutants containing the GUS gene that are not particularly evident in mutants lacking the reporter gene.

Bioassays with gene-specific mutants and subsequent immunoblot analyses with antibodies raised against p19 or p22 demonstrate that both proteins accumulate early during infection of plants or protoplasts, and time course studies suggest that p19 can be detected 1 to 2 days earlier in plants than p22. Fractionation studies demonstrate that p19 is predominantly a soluble protein, whereas p22 is mostly present in the fraction enriched for membrane-associated proteins. Although p19 can be detected earlier and is perhaps expressed at higher levels than p22, at this point we cannot make firm conclusions about quantitative differences in the abundance of p19 and p22 because variations in antibody specificity may influence the levels of detection.

p22 is required for cell-to-cell movement in plants

A substantial amount of evidence is accumulating that many plant RNA virus replicase complexes as well as proteins involved in cell-to-cell movement are associated with membranes and that the latter also interact with cell-wall components (Deom *et al.*, 1992). Our results show that p22 is mostly present in the membrane fraction and is not required for replication in protoplasts. Both these observations are consistent with an involvement of p22 in virus movement. Previous infectivity studies with TBSV, CNV, and CymRSV have shown that mutants predicted not to express p19 and p22, or p22 alone, fail to establish systemic infections (Dalmay *et al.*, 1993; Rochon and Johnston, 1991; Scholthof *et al.*, 1993b). Our infectivity assays also show that p22 is required for the onset of lesions in local lesion hosts. TBSV mutants that do not produce the p19/p22 sgRNA, or mutants in which p22 is fused out of frame (pHS145) or in frame (pHS146) with the 5′ end of the coat protein gene, also fail to

establish infections. This indicates that the translational fusion inactivates p22 and that production of the small sgRNA is essential for spread of the infection.

Although the data obtained prior to the present experiments showed that TBSV p22, or its analog in other tombusviruses, is required for establishment of systemic infections, the direct involvement of p22 in cell-to-cell movement per se had not been demonstrated previously. In particular, the effects on cell-to-cell movement versus long-distance spread have not been clearly differentiated, and the possible involvement of p19 in these processes has not been previously elucidated. To obtain additional information about the roles of the nested genes in invasion of various host plants, we employed a TBSV vector (pHS45) expressing the GUS gene to permit infection foci to be visualized by a blue color reaction during the initial stages of localized infection (Scholthof *et al.*, 1993b). Between 16 to 40 hr postinoculation, expansion of the blue infection focus occurred both in local lesion hosts (*C. quinoa* and *C. amaranticolor*) and in hosts that support systemic infections (*N. benthamiana* and spinach). During these time course experiments, inactivation of p19 (pHS138) had no obvious effect on localized movement in any of these hosts, suggesting that p19 is not required for cell-to-cell movement. However, in the absence of p22 (pHS132), infections remained confined to a single epidermal cell. Therefore, our data conclusively demonstrate that the membrane-bound protein p22 is essential for cell-to-cell movement of TBSV in all plant species tested. This suggests that tombusviruses employ a cell-to-cell movement strategy that is more similar to that of tobamoviruses (Deom *et al.*, 1992) and dianthoviruses (Giesman-Cookmeyer and Lommel, 1993) than that of comoviruses (van Lent *et al.*, 1991), caulimoviruses (Perbal *et al.*, 1993; Thomas and Maule, 1995) or potyviruses (Dolja *et al.*, 1994, 1995). Moreover, the broad experimental host range of TBSV (Martelli *et al.*, 1988) provides suggestive evidence that common host factors involved in cell-to-cell movement of this virus are present in many different plant species.

p19 is required for systemic invasion of some hosts

The present study demonstrates that p19 is not required for replication or cell-to-cell movement of TBSV. In addition, infectivity assays show that inactivation of p19 does not have obvious effects on either the timing of symptom appearance or the initial accumulation of virus and viral RNA in *N. benthamiana* and *N. clevelandii.* The role of p19 in eliciting symptoms has been examined in more detail in a separate communication (Scholthof *et al.*, 1995a). The ability of the TBSV p19 mutant, and equivalent mutants of CNV and CymRSV (Dalmay *et al.*, 1993; Rochon, 1991; Russo *et al.*, 1994), to systemically invade *N. benthamiana* or *N. clevelandii* may be related to a "universal" host anomaly that is particularly evident in *N. benthamiana* (Dawson and Hilf, 1992; Rushing *et al.*, 1987; Selling *et al.*, 1990). Because this suggests that *N. benthamiana* imposes fewer restraints on virus transport than other plants, we decided to test the requirement of p19 for infection of other TBSV hosts. During inoculation tests on various plants, we observed that p19 is dispensable for the formation of local lesions on *G. globosa, N. edwardsonii, D. metel, C. amaranticolor, C. quinoa,* cucumber, and spinach. However, a notable difference was that in the absence of p19, the degree of local lesion necrosis was reduced in cucumber and spinach and that comparatively smaller local lesions were formed on relatively old *N. glutinosa* plants and on *C. quinoa.* However, the rates of cell-to-cell movement during the early stages of *C. quinoa* infection appeared not to be affected by inactivation of p19, as assessed by the GUS histochemical assay. These observations suggested that, despite its dispensability for cell-to-cell movement, p19 may have an auxiliary effect on the rate of virus invasion in some members of the Solanaceae and Chenopodiaceae.

To further evaluate the possible role of p19 in virus spread, we decided to test the requirement of p19 in spinach and pepper which are members of the Chenopodiaceae and Solanaceae, respectively, which both become systemically infected with TBSV. Our results demonstrated that p19 is not required for cell-to-cell movement in spinach, but that it is essential for the accumulation of virus in the upper uninoculated spinach leaves. The expression of p19 is also required for a systemic infection of pepper, with the added effect that in this host, inactivation of p19 also reduces the accumulation of TBSV in inoculated pepper leaves to amounts below the levels of detection. Therefore, we conclude that the requirement of p19 for establishing systemic TBSV infections is host dependent. In the "permissive" *Nicotiana* hosts, p19 is dispensable. But, in other less permissive hosts such as spinach, p19 appears to be required for long-distance vascular transport, and in pepper, it also influences the effectiveness of localized infections. Future experiments will be carried out to determine whether these differences are strictly correlated with taxonomic characteristics of these hosts or if variations exist within host species or cultivars of spinach and pepper.

Possible mechanism(s) of p19-mediated systemic spread

Our observations in spinach confirm the notion that cell-to-cell movement and long-distance or phloem-associated spread may be implemented via different processes that require different viral factors (Atabekov and Taliansky, 1990; Barker and Harrison, 1982; Nelson *et al.*, 1993). Our results also imply that different host factors regulate short-distance versus long-distance movement of TBSV. This may be related to anatomical differences between connections of different cell types and the differential effect of movement proteins on these conductive

elements (Ding *et al.*, 1992; Kempers *et al.*, 1993). The observation that virus resistance can be active at the level of long-distance transport (Goodrick *et al.*, 1991) also suggests that this type of resistance results from host-mediated interactions that differ from those involved in cell-to-cell spread. Moreover, studies on long-distance spread of CaMV in *Arabidopsis* also indicate that developmentally regulated host factors may be involved in governing vascular spread (Leisner *et al.*, 1993).

The biochemical activities whereby the mostly soluble p19 gene product promotes long-distance movement in certain hosts are unclear. Nevertheless, the ability of p19, whether expressed from TBSV or from a heterologous virus vector, to mediate induction of severe necrosis or tissue collapse in permissive hosts (Scholthof *et al.*, 1995a) may provide some clues about its role in host-dependent long-distance movement. Perhaps p19 induces intracellular and intercellular "leakage" that interferes with normal cellular events in very susceptible hosts and causes severe vascular dysfunction when exacerbated by stress-related responses. In less permissive hosts, the putative "leakage" inducing activity of p19 could actually be required for penetration of barriers that would otherwise block systemic invasion. The existence of barriers that may limit entry of vascular parenchyma cells was also hypothesized to be responsible for the delay in spread of the masked strain of TMV (Ding *et al.*, 1995). It is also possible that the p19 activity interferes with defense responses that prohibit virus accumulation in the inoculated leaves, as may be the case in pepper. As discussed in more detail previously (Scholthof *et al.*, 1995c), the necrosis-inducing activity of p19 (Scholthof *et al.*, 1995a) may also facilitate virus dispersal from plant debris into the soil. It is therefore possible that the combined effects of p19 activity contribute to a number of facets in the epidemiology of TBSV that collectively serve to increase the efficiency of TBSV traversal through host populations.

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