Identification of Tomato Bushy Stunt Virus Host-Specific Symptom Determinants by Expression of Individual Genes from a Potato Virus X Vector

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In this study, we analyzed the influence of two nested genes (p19 and p22) of tomato bushy stunt virus (TBSV) on disease symptoms in systemically infected plants and in local lesion hosts. The contribution of individual genes was determined by bioassays with an infectious clone of wild-type TBSV, with p19/p22 mutant derivatives, and by expression of individual TBSV genes from a heterologous potato virus X (PVX) vector. Our results showed that TBSV genes could be expressed at high levels from the PVX vector. The subcellular localization of these proteins as well as the ability of PVX-expressed p22 to trans complement TBSV cell-to-cell movement defective mutants indicate that the exogenously expressed proteins are functionally active. Inoculation studies with TBSV mutants and the PVX derivatives demonstrated that p19 induced a generalized necrosis upon systemic infection of Nicotiana benthamiana and N. clevelandii. In addition, p19 elicited the formation of local necrotic lesions in N. tabacum; however, in N. glutinosa and N. edwardsonii, the local lesion response was activated by p22. These results show that the p19 and p22 proteins of TBSV are important symptom determinants and that closely related plant species may contain different resistance genes that selectively respond to individual TBSV proteins.

INTRODUCTION

The ability of a plant pathogen to establish an infection depends upon many factors that contribute to the compatibility or incompatibility of host–pathogen interactions. In cases of a compatible interaction, the host defense responses may be absent or unable to prevent systemic infections from occurring, or conceivably, the pathogen may be able to actively repress a defense response. However, even compatible interactions differ greatly because various disease symptoms can be caused by the activity of one or more pathogen gene products or by activation of diverse host responses. Thus far, plant virus genes that have been shown to be directly involved in determining compatibility of systemic infections and subsequent symptom development include structural genes, such as the inclusion body protein gene of caulimoviruses (Schoelz and Wintermantel, 1993) and the coat protein genes of some RNA viruses (Culver and Dawson, 1991; Culver et al., 1991; Dawson and Hilf, 1992). However, various combinations of genes, rather than a single gene, often contribute to the local lesion phenotype and to systemic infection and symptom development (Allison et al., 1990; Petty et al., 1990; Dawson and Hilf, 1992; Qui and Schoelz, 1992; Petty et al., 1994).

In incompatible interactions, pathogens fail to establish systemic infections. This failure could be due to a passive mechanism whereby essential host components are missing. In the case of plant–virus interactions, such incompatibility might be due to the inability of a virus to associate with host-specific proteins for replication or movement (Dawson and Hilf, 1992), and in some cases this could lead to apparent immunity (Fraser, 1990). Another defense mechanism also frequently occurs in plant–virus interactions whereby actively regulated host processes that lead to resistance are elicited. In these cases, the plant could recognize one or more specific pathogen products that act as a signal to activate a cascade of defense reactions characterized by a hypersensitive response that culminates in the formation of local (usually necrotic) lesions to arrest the spread of the pathogen. In this scenario, the sacrifice of a small area of tissue limits further spread of the pathogen, thereby protecting the whole plant from systemic invasion and disease. During this response, systemic acquired resistance mechanisms that provide secondary and more generalized protection against other pathogens might be activated (Alexander et al., 1994). Flor (1955) recognized that certain hypersensitive reactions result from specific pathogen race and host cultivar combinations. The current interpretation of this specificity is that in most cases, products from dominant alleles of plant resistance genes recognize products from single dominant avirulence (avr) genes of the pathogen (Keen, 1990). Additional resistance reactions can also be triggered by nonspecific pathogen elicitors that do not appear to...
rely on gene-for-gene interactions (Keen and Staskawicz, 1988; Dixon and Lamb, 1990).

Some examples of combinations of plant and virus genes (or loci) that lead to local lesions or cell necrosis include interactions of the coat protein of tomato mosaic virus (TMV) in *Nicotiana sylvestris* plants containing the *N* gene (Culver et al., 1991), expression of the replicase gene of TMV in *Nicotiana* spp that have the *N* gene (Padgett and Beachy, 1993), the coat protein of potato virus X (PVX) in potato plants with the *Nx* gene (Kavanagh et al., 1992; Santa-Cruz and Baulcombe, 1993), and the movement protein of tomato mosaic tobamovirus on tomatoes with a *tm-2* genetic background (Weber et al., 1993). Other viruses for which component(s) have been shown to elicit a hypersensitive response in certain plant species include the caulimoviruses (Schoelz and Shepherd, 1988), the hordeiviruses (Jackson et al., 1991), and the furoviruses (Jupin et al., 1992). However, in these instances, the corresponding host genes have not been identified. Conversely, a number of resistance genes have been described in various plant species, but the corresponding avirulence determinants remain unclear (Fraser, 1990).

**Tomato bushy stunt virus (TBSV)** is a suitable virus for studies of symptom determinants because of its broad experimental host range and the variety of symptoms it induces on different hosts (Martelli et al., 1988). This small icosahedral virus contains a single copy of a positive sense single-stranded RNA genome of ~4800 nucleotides (Hearne et al., 1990). Five major open reading frames are encoded by the genome of TBSV (Figure 1A). The 5' proximal half of the genomic RNA functions as an mRNA for translation of a 33-kD protein (p33) and a readthrough product of 92 kD (p92), both of which are required for replication (Scholthof et al., 1995a). The 41-kD coat protein gene is located farther downstream, and translation of this protein occurs from a subgenomic mRNA (Hillman et al., 1989). Two small nested genes located near the 3' terminus of the genome are expressed via a second subgenomic mRNA that directs synthesis of a 22-kD protein (p22) and a 19-kD protein (p19) (Hayes et al., 1988). Studies with other tombusviruses indicate that the p22 analog is necessary for systemic movement, and although no function for p19 has been assigned, this gene influences symptom severity (Rochon, 1991; Dalmay et al., 1993). Recently, we have obtained direct evidence that p22 of TBSV is required for cell-to-cell movement, whereas p19 is involved in systemic movement in some hosts but is dispensable in others (H.B. Scholthof, K.-B.G. Scholthof, M. Kikkert, and A.O. Jackson, submitted manuscript).

In recent studies of virus symptom and host range determinants, investigators have increasingly used reverse genetic techniques with biologically active cDNA clones (Boyer and Haenni, 1994), combined with the introduction of genomic exchanges between different virus strains. Gene exchange studies of symptom determinants of the tombusviruses are complicated because the individual members for which infectious clones are available may elicit very similar symptoms on experimental hosts that support a systemic invasion as well as on local lesion hosts (Martelli et al., 1998). Mapping of symptom determinants by mutational analyses of a single virus also has a major disadvantage in that essential genes cannot be inactivated. In addition, some studies are complicated by the fact that the symptom phenotype may be determined by several genes (Allison et al., 1988; Qui and Schoelz, 1992; Petty et al., 1994). Even if mutagenesis of a single gene has been shown to affect symptoms, the results may not conclusively demonstrate whether this is a direct or an indirect effect. For example, inactivation of the TBSV p19 analog in another tombusvirus, cucumber necrosis virus (CNV), results in amelioration of symptoms (Rochon, 1991). Although this suggests a direct role for p19 in symptom induction, the effect could be indirect, because the p19 analog mutant accelerates the generation of defective interfering (DI) RNAs, which also attenuate symptoms.

Because of the interaction of different virus components, it is often difficult to establish firmly the importance and dominance of a single (essential) virus gene in symptom development. To provide such proof, genes have sometimes been expressed in transgenic plants (Baughman et al., 1988; Culver and Dawson, 1991; Goldberg et al., 1991a; Pfizter and Pfizter, 1992). Although this approach has proven extremely valuable, it has some practical and theoretical restrictions, and the technology can thus far only be applied to a limited number of plant species. An additional disadvantage may be that the timing and levels of protein synthesis that occur during a natural infection cannot be duplicated in transgenic plants. This may prevent the efficient activation of certain host responses, as suggested by the results obtained with studies on caulimoviruses. Despite the fact that the gene VI product of these viruses is responsible for symptoms during an infection, transgenic expression of this elicitor in host plants failed to induce symptoms (Goldberg et al., 1991a). Moreover, when the gene under study elicits a lethal host response, additional manipulations might be required to avoid premature death of the transgenic plants. To circumvent some of these difficulties and ambiguities, we have employed an alternative strategy to rapidly identify essential and nonessential TBSV genes that contribute to symptom development, without interference from other viral components, such as DI RNAs. Using this approach, TBSV genes are expressed at high levels from a heterologous virus, PVX (Figure 1B), which is a suitable vector for foreign gene expression in plants (Chapman et al., 1992; Hammond-Kosack et al., 1995). Such studies on the effect of PVX-mediated expression of TBSV proteins on hypersensitive responses and severe systemic symptoms are possible because of the differences in symptoms induced by the two viruses. TBSV elicits the formation of necrotic local lesions or a systemic necrosis on a variety of *Nicotiana* spp that display mild mosaic symptoms when infected with PVX. Using this system, we investigated whether exogenous expression of individual TBSV genes from PVX could elicit TBSV-specific phenotypic host responses.

Our results obtained by mutational analyses of the TBSV genome, combined with exogenous expression of individual
Figure 1. Diagram of TBSV and PVX Genomes, and Derivatives Expressing TBSV p19, p22, and/or p33.

The numerical designations of the plasmids in the pHS series are provided at left. Boxes indicate open reading frames: the stippled box indicates p33, the black box represents p19, and the white box above p19 depicts p22. Short arrows delineate the positions where subgenomic RNA transcripts are initiated. Gene functions are provided above the genes; virus movement is regulated by proteins encoded by the nested genes (NG) of TBSV and triple gene block (TGB) of PVX. The predicted synthesis of p19, p22, or p33 is indicated by (−) or (+) signs at the right.

(A) Genomic organization of pTBSV-100 and pHS derivatives. The sizes of the encoded proteins (p) are given in kilodaltons, and the presence of an amber stop codon is indicated by an asterisk. The relative position of the box depicting the GUS gene indicates the insertion site with respect to the flanking regions of the capsid protein gene in pHS45 and pHS132. During these studies, another plasmid, pHS8, was also used. This plasmid is a derivative of pHS45 with a deletion spanning p19 and p22, as described in detail previously (Scholthof et al., 1993b).

(B) Organization of the genome of the PVX vector pPC2S and its derivatives. The promoter duplication region with downstream cloning sites (oval) permits transcription of an additional subgenomic RNA (middle arrow) for expression of foreign gene inserts. The inserts in pHS143anti and pHS166anti are inverted to reflect their antisense orientation.
genes from the PVX vector, suggest that the coat protein and the replicase genes of TBSV did not contribute significantly to the appearance of disease symptoms in various *Nicotiana* spp. In contrast, the p19 protein appeared to be the main component responsible for eliciting the severe systemic necrosis that occurred during infection of *N. benthamiana* and *N. clevelandii* with TBSV. In addition, p19 was responsible for the onset of local necrotic lesions in *N. tabacum*. The overlapping gene product (p22) failed to elicit an altered symptom phenotype when expressed from PVX in the aforementioned *Nicotiana* spp, but it did elicit a local lesion response in the closely related *N. glutinosa*. The p22 protein also elicited local lesion formation on *N. edwardsii*, which is an amphidiploid hybrid of *N. clevelandii* (systemic host), and *N. glutinosa* (local lesion host), indicating that the resistance response is a dominant trait.

**RESULTS**

**A PVX Vector Can Be Used for High-Level Expression of TBSV Genes**

In a previous investigation, we demonstrated that TBSV p19 and p22 are dispensable for replication, transcription, and gene expression in protoplasts (Scholthof et al., 1993b). In a companion study to this investigation (H.B. Scholthof, K.-B.G. Scholthof, M. Kikkert, and A.O. Jackson, submitted manuscript), we have shown that p22 is required for cell-to-cell movement in all hosts tested. In contrast, p19 is dispensable for systemic infection of *N. benthamiana* and *N. clevelandii*, but it is required for systemic invasion of spinach and pepper. In this study, we focused on the contribution of p19 and p22 as well as p33 in the activation of host responses. Basically, the coat protein was excluded from these experiments because related experiments thus far have failed to identify it as an important symptom determinant (Scholthof et al., 1993b; H.B. Scholthof, K.-B.G. Scholthof, M. Kikkert, and A.O. Jackson, submitted manuscript). Future studies will be directed toward the involvement of the readthrough portion of the replicase (p92) as a possible elicitor of disease phenotypes.

To establish firmly the involvement of individual TBSV genes on symptom induction, they needed to be expressed separately from other TBSV genes. This seemed particularly pertinent because inactivation of the p19 analog in CNV accelerated the spread of recombinant PVX constructs throughout the plants and the accompanying expression of the introduced TBSV genes were conclusively demonstrated by immunoblot assays for the detection of p19, p22, and p33 (Figure 2). These results showed that whether the plants were infected with wild-type TBSV or its p19 mutant (pHS157), p33, p92, and p22 were present in comparable amounts (Figure 2), as was the coat protein (data not shown). However, as predicted, p19 was not expressed in plants systemically infected with pHS157 RNA (TBSV p19 mutant). In addition, the data show that the PVX constructs produced the predicted TBSV proteins. Thus, pHS143 RNA expressed both p19 and p22, pHS158 RNA expressed only p19, pHS159 RNA expressed only p22, and pHS143anti ("anti" indicating insert in the antisense orientation) and pHS160 failed to express either p19 or p22. The pHS142 construct was not included in the results shown in Figure 2; however, it also produced high levels of p19 without the accumulation of p22. Irrespective of the genetic background (TBSV or PVX) that was used for expression of the genes, p19 mostly accumulated in the soluble fraction (S30), whereas p22 was present in highest amounts in the fraction (P30) containing membrane-associated material. Although the results in Figure 2 suggest reduced accumulation of p22 in plants infected with pHS159 (PVX-expressing p22), in comparison with TBSV or pHS143 (PVX-expressing p19 and p22), this reduction was not observed consistently.

Plants infected with RNA from pHS166 (PVX-expressing p33) had p33 levels that were similar to those observed in plants infected with TBSV or virus derived from pHS157 (TBSV p19 mutant). The immunoblot patterns of p33 suggest that the higher mobility forms of p33 represent dimers and trimers that are formed irrespective of the genetic background of the virus. As predicted, pHS166 (PVX-expressing p33) failed to produce the p92 translational readthrough product that is detectable in the lanes with the TBSV constructs. Accumulation of p33 was not observed when pHS166anti (PVX-expressing p33 antisense RNA) was used as the source of inoculum (data not shown).

Immunoblot assays for detection of the coat protein of PVX revealed that transcription of TBSV p19/p22 RNA (pHS160) in the absence of the translation of the corresponding proteins had no substantial effect on PVX capsid accumulation (Figure 2C). However, PVX-mediated translation of p19 and/or p22 proteins from pHS143, pHS158, and pHS159 appeared to interfere with accumulation of the PVX coat protein (Figure 2C). Similarly, RNA analyses of plants infected with pHS143anti that...
Figure 2. Detection of TBSV Proteins Expressed in *N. benthamiana* from TBSV and the PVX Vectors.

Immunoblots of samples from plants infected with the TBSV or PVX derivatives, whose numerical notations are provided above the lanes (H, healthy; 143a, pHS143anti). The nitrocellulose filters were treated with antibodies raised against individual TBSV proteins, as indicated, and either with goat anti-mouse horseradish peroxidase, followed by detection with chemiluminescent substrate and exposure to x-ray film, or with goat anti-rat alkaline phosphatase detection.

(A) Chemiluminescent detection of p19 (top) or p22 (bottom).

(B) Chemiluminescent detection of p33 and p92. The asterisks indicate that the band at this position may represent p33 dimers.

(C) Alkaline phosphatase-mediated detection of the PVX coat protein.

Tissue samples were prepared as described by Scholthof et al. (1994); P30 and S30 consist of either the pelleted (mainly membrane associated) or supernatant (soluble) material obtained after centrifugation at 30,000g. Because of the extraction protocol and protein concentration in the samples, the amount of the S30 fraction that could be resolved on the gels was ~10% of the P30 fraction. Plants were inoculated with TBSV or the p19 mutant (pHS157) transcripts (for p19 and p22) or with infected plant sap (for p33) and assayed at 7 and 4 days postinoculation, respectively. Samples from the PVX-infected plants were harvested 7 days after inoculation with plant sap.
Figure 3. Complementation of TBSV-GUS Constructs Defective for Movement by Exogenous Expression of p22 from a PVX Vector.

The GUS assay was performed with *N. benthamiana* leaves inoculated 5 to 6 days earlier with a 1:1 mixture of transcripts from the TBSV and PVX constructs.

(A) pHs159 (PVX-expressing p22) and pHs45 (TBSV-GUS-expressing p22).

(B) pHs159 (PVX-expressing p22) and pHs132 (TBSV-GUS with inactive p22).

(C) pHS160 (PVX-expressing nontranslatable p22 RNA) and pHs132 (TBSV-GUS with inactive p22).

expressed the p19/p22 RNA in the antisense orientation indicated that comparatively higher levels of genomic RNA accumulated than when derivatives were used that produced the TBSV translational products (data not shown). Thus, with the nontranslatable TBSV inserts in PVX, there was no clear reduction in PVX coat protein accumulation, in contrast with the effects of translatable inserts. However, no clear differences were observed between PVX constructs containing translatable inserts expressing different TBSV proteins.

**TBSV Cell-to-Cell Movement Mutants Can Be Complemented by PVX-Mediated Expression of p22**

The aforementioned experiments demonstrated that the PVX vectors were suitable for the expression of TBSV proteins, but it was not clear whether the exogenously expressed proteins were functionally active. Because *N. benthamiana* supports systemic infections with both PVX and TBSV, and because wildtype PVX does not complement TBSV movement-deficient mutants (data not shown), this plant provided an excellent system to determine whether PVX-mediated expression of p22 could complement movement of TBSV p22 mutants. To easily visualize localized movement of TBSV, we used a TBSV mutant (pHS45) in which the coat protein gene had been replaced by the β-glucuronidase (GUS) gene (Figure 1; Scholthof et al., 1993b). In a separate series of experiments (H.B. Scholthof, K.-B.G. Scholthof, M. Kikkert, and A.O. Jackson, submitted manuscript), we demonstrated that in vivo histochemical assays for GUS activity permit visualization of pHS45 RNA replication and movement and that GUS activity was confined mainly to a single cell for pHS45 derivatives that were unable to express the p22 protein.

For the complementation assays, we first tested the TBSV movement-deficient p22 mutants that expressed GUS in plants that had been inoculated 9 days previously with PVX-expressing p22. Although this approach resulted in some level of complementation, the resulting blue lesions were very diffuse, and we were concerned that this could have been caused by the instability of PVX vectors or by the uncoordinated timing of PVX-mediated p22 expression and TBSV replication. To increase the level and coordination of PVX-mediated p22 expression for complementation of TBSV, additional experiments were performed. During these tests, the PVX vector RNAs were coinoculated with transcripts from the parental TBSV–GUS gene construct, which expresses p22 (pHS45), p22-deficient pHs8 (see legend to Figure 1A), or pHs132, which has a stop codon in the p22 gene (Figure 1A). Coinoculation of the parental TBSV–GUS RNA (pHS45) with either a PVX construct expressing p22 (pHS159; Figure 3A) or with a construct that only expressed nontranslatable RNA (pHS160; not shown) resulted in blue expanded lesions, suggesting that coinoculation with the PVX constructs did not interfere with TBSV replication or spread (Figure 3A). Coinoculation of the p22-deficient TBSV RNA (pHS132) and the PVX vector expressing
Expression of p19 Induces Severe Necrotic Systemic Symptoms in *N. benthamiana*

Even though p19 was dispensable for infection of *Nicotiana* hosts that support systemic infections, inactivation of p19 in the TBSV derivative pH5157 had a major attenuating effect on the lethal apical necrotic symptom phenotype normally elicited in these plants, as shown for *N. benthamiana* in Figure 4A. Three to 4 days after inoculation with transcripts from pH5157 or pTBSV-100, mild chlorotic patterns appeared on the inoculated leaves. Although the leaves inoculated with pH5157 RNA sometimes wilted, they failed to develop the severe necrosis that is characteristic of wild-type TBSV infections. In both cases, systemic symptoms developed in upper leaves at 4 to 5 days postinoculation, but the tissue that was systemically invaded with pH5157 RNA failed to develop the severe apical necrosis associated with wild-type TBSV infections (Figure 4A). Instead, the plants infected with pH5157 RNA displayed mosaic symptoms, dark green islands, leaf distortion, stunted growth, and sporadic necrotic spots (Figure 4A). Moreover, the roots of these plants appeared relatively healthy, and the plants developed flowers and set seed. In contrast, infections with wild-type TBSV typically resulted in a severe apical necrosis, diseased roots, and death of the plant (Figure 4A).

To determine the effects of PVX-mediated expression of p19, *N. benthamiana* plants were inoculated with PVX vectors expressing p19 (pHS142, pH143, and pH5158) or PVX derivatives unable to express p19 (pHS143anti, pH5159, pH5160, pH5166, pH5166anti, and pPC2S). The plants inoculated with the p19-deficient derivatives all displayed the same mild mosaic symptoms without detectable necrosis, as shown in Figure 4B for pH5159 and pH5160. However, plants inoculated with PVX vectors that expressed the p19 protein (pHS143 and pH5158) developed necrotic lesions on the inoculated leaves ~1 week after inoculation. This was followed by an apical necrosis that ultimately led to the death of the plant (Figure 4B). The results obtained with *N. clevelandii* plants were comparable but less dramatic, because PVX constructs that expressed p19 usually failed to cause the death of the plant. Nevertheless, necrosis was always associated with the presence of p19, especially in lower leaves (data not shown). The N-terminal portion of p22, which was translated from pH5143 and pH5158, developed necrotic lesions on the inoculated leaves ~1 week after inoculation. This was followed by an apical necrosis that ultimately led to the death of the plant (Figure 4B). The results obtained with *N. clevelandii* plants were comparable but less dramatic, because PVX constructs that expressed p19 usually failed to cause the death of the plant. Nevertheless, necrosis was always associated with the presence of p19, especially in lower leaves (data not shown). The N-terminal portion of p22, which was translated from pH5143 and pH5158, was not involved in the onset of the severe symptoms, because the PVX derivative pH5142, which is incapable of expressing the N-terminal p22 amino acids, elicited necrosis due to the expression of p19. Similarly, the results with pH5159 and pH5160 revealed that expression of the N-terminal portion of p19 was not sufficient for induction of the necrotic syndrome.

PVX-mediated expression of p22 resulted in small necrotic
spots on *N. clevelandii* plants. This agrees with results observed when this host was inoculated with RNA from pHS157, a TBSV mutant that expresses p22 but not p19 (H.B. Scholthof, K.-B.G. Scholthof, M. Kikkert, and A.O. Jackson, submitted manuscript). Expression of p22 from PVX (pHS159) had no appreciable effect on the mild mosaic symptoms normally observed in *N. benthamiana* plants infected with PVX. However, small scattered bleached spots occasionally appeared, indicating that p22 sometimes has slight effects on the symptom phenotype. In conclusion, our results with the TBSV genomic p19 mutant (pHS157) and the various PVX derivatives (Figure 4) demonstrated that p19 alone, without interaction with other TBSV components, was sufficient to elicit the severe necrotic disease syndrome in *Nicotiana* species that support a systemic infection.

**p19 Induces Necrotic Local Lesions in *N. tabacum***

In contrast with the results from *N. benthamiana* and *N. clevelandii*, *N. tabacum* inoculated with TBSV developed very small, defined necrotic local lesions (Figure 5A) that limited further spread of the virus. During experiments with a TBSV coat protein (p41) deletion mutant or a mutant in which the major portion of p41 was replaced with the chloramphenicol acetyltransferase gene (Scholthof et al., 1993b), we noted that the coat protein was not responsible for the onset of local lesions in *N. tabacum*. However, during simultaneous experiments with the TBSV mutant pHS157 in which p19 was inactivated, *N. tabacum* cv Glurk (carrying the TMV resistance gene *N*) or *N. tabacum* cv Turk (devoid of the *N* gene) failed to develop local necrotic lesions. Instead, enlarged chlorotic lesions initially appeared, often followed by the development of necrotic rings (Figure 5A). These results strongly suggested that p19 can act as an elicitor of a hypersensitive response, but we did not know whether interactions with other viral components contributed to activation of this response.

To determine the role of p19 and other gene products as possible elicitors, we inoculated *N. tabacum* plants with the various PVX derivatives. Control experiments with Chenopodium amaranticolor (*a* local lesion host for PVX) were used to confirm that all inocula were infectious and that insertion of the TBSV gene had not drastically altered the lesion phenotype. *N. tabacum* inoculated with plant sap derived from pHS160, transcribing the nontranslatable p19/p22 RNA, or pHS159, expressing p22, failed to develop necrotic lesions (Figure 5B). In these cases, the inoculated leaves were usually symptomless, but occasionally very mild chlorotic lesions appeared. Similar results were obtained when the inoculum was derived from pPC2S (*the PVX vector*), pHS143anti RNA (PVX-expressing antisense p19/p22 RNA), pHS166 (expressing p33), or pHS168anti (PVX-expressing p33 antisense RNA) (data not shown). However, when the inoculum was derived from pHS143 (PVX-expressing p19 and p22) or pHS158 (expressing only p19), localized water-soaked lesions appeared at ~4 days postinoculation. The lesions became confined and necrotic by 7 days postinoculation. Such lesions formed in inoculated leaves of *N. tabacum* cv Glurk (containing the *N* gene; Figure 5) as well as in the cultivar Turk (devoid of the *N* gene). Inoculum derived from pHS142 (expressing p19) also elicited local lesions on this host (data not shown). Plants inoculated with sap derived from pHS143 (expressing p19 and p22) or pHS158 (PVX-expressing p19 only) sometimes developed a few necrotic local lesions on the leaves immediately above the inoculated leaves. Regardless of the inoculum source, the tobacco plants usually developed mild systemic symptoms characteristic of

![Image](http://example.com/image.png)
Figure 6. Involvement of p22 in the Elicitation of Local Lesions on N. glutinosa Plants.
(A) Leaves inoculated 1 week previously with infected plant sap. Left, wild-type TBSV; right, pH5157 (p19 mutant).
(B) Leaves inoculated 9 days earlier with plant sap virus inoculum from the PVX derivatives. Top left, pH5160 (nontranslatable p19/p22 RNA); top right, pH5143 (p19 and p22); bottom left, pH5158 (only p19); bottom right, pH5159 (only p22).
(C) Symptoms on the upper leaves of N. glutinosa plants 3 weeks after lower leaves were inoculated with virus derived from PVX vectors. Left, pH5158 (p19 only); right, pH5159 (p22 only).

PVX infections. However, the p19-mediated induction of necrotic lesions seemed to have some effect on the spread of PVX, because the systemic mild mosaic symptoms in the upper leaves of these plants appeared 4 to 5 days after systemic symptoms became visible on plants that had been inoculated with derivatives that failed to elicit local lesions. Preliminary RNA analyses of N. tabacum plants infected with the PVX derivatives indicated that the PVX RNA recovered from systemically infected tissue had deletions affecting the TBSV gene insert. This apparent instability of the PVX derivatives in N. tabacum agrees with previously reported findings with PVX vectors in this host (Chapman et al., 1992). Nevertheless, the results demonstrated that expression of TBSV p19 effectively elicited the local lesion response in N. tabacum.

Expression of p22 Elicits a Local Lesion Response in N. glutinosa

As was the case in N. tabacum, inoculation of TBSV onto N. glutinosa plants resulted in the formation of local lesions (Figure 6A). Similarly, TBSV derivatives in which the coat protein was inactivated also produced necrotic local lesions, indicating that the capsid protein was not required for activation of the local lesion response in N. glutinosa (data not shown). However, in contrast with the p19-dependent local lesion formation on N. tabacum plants, activation of local lesions in N. glutinosa was independent of the presence of p19 because inoculum derived from the p19 mutant pH5157 also elicited local lesions.
to form enlarged transparent necrotic areas in the inoculated primary elicitor that is sufficient for the induction of local lesion formation. Apparently, p22 is recognized as the elicitor (pHS143) because they rapidly expanded and merged to become necrotic. However, these lesions differed from those caused by pHS168 RNA, which expressed the TBSV p33 protein, or pHS168anti, expressing p33 antisense RNA. In contrast, N. glutinosa (Figure 6B) and N. edwardsonii plants inoculated with PVX-expressing p19 and p22 (pHS143) developed discrete necrotic lesions surrounded by a darker halo ~5 to 6 days postinoculation. PVX that expressed p19 but not p22 (pHS158) initially elicited chlorotic lesions (Figure 6B) that subsequently became necrotic. However, these lesions differed from those caused by pHS143 because they rapidly expanded and merged to form enlarged transparent necrotic areas in the inoculated leaves. In addition, PVX-mediated expression of p19 also led to a systemic chlorosis and necrosis in the upper leaves (Figure 6C), which sometimes resulted in death of the plants. Thus, although p19 elicited a necrotic response, expression of this protein alone was not sufficient to activate a defined local lesion response.

In contrast, N. glutinosa (Figure 6B) or N. edwardsonii inoculated with PVX derivatives that expressed p22 (pHS143 and pHS159) formed local necrotic lesions. However, these lesions did not prevent the systemic spread of PVX, and the systemic symptoms were interspersed with the formation of local necrotic lesions on the leaves positioned immediately above the inoculated leaves (Figure 6C). The abundance of such lesions varied with each inoculation experiment, but they were usually more prevalent in N. edwardsonii. The young upper leaves of both plant species generally displayed normal PVX-like mild mosaic symptoms.

The results described in this section indicate that p19 is involved in symptom severity in N. glutinosa and N. edwardsonii, but it is not the primary determinant responsible for eliciting local lesion formation. Apparently, p22 is recognized as the primary elicitor that is sufficient for the induction of local necrotic lesions in these two Nicotiana spp. This response most likely involves at least one resistance gene derived from N. glutinosa because a similar response was seen in N. edwardsonii, whose genome is composed of the susceptible N. clevelandii and the resistant N. glutinosa. N. tabacum cv Glurk failed to elicit a necrotic response upon exogenous expression of p22 from PVX. Therefore, we conclude that neither the p19-mediated local lesion response in N. tabacum nor the p22-activated response in N. glutinosa involves the N gene that provides resistance to TMV in N. glutinosa.

DISCUSSION

Expression of TBSV Genes by PVX Gives Rise to High Levels of Active Proteins

In this study, we describe the use of a PVX vector (Chapman et al., 1992) as well as traditional reverse genetic studies for the rapid analysis of the contribution made by individual TBSV genes to phenotypic symptom responses. Immunoblot data from N. benthamiana plants infected with TBSV showed that abolition of p19 expression did not prevent systemic spread or accumulation of TBSV proteins throughout the plant. This confirmed that p19 is not required for systemic infection in this host. Similar immunodetection assays of plants infected with the various PVX vectors showed that p19, p22, and p33 are exogenously expressed at high levels during infection. Further analysis of PVX-mediated expression of p19 and p22 indicated that p19 is present mainly as a soluble protein, whereas a high proportion of p22 is membrane bound. These results agree with companion studies on the localization, distribution, and appearance of p19 and p22 in plants infected with TBSV (Scholthof et al., 1995b; H.B. Scholthof, K.-B.G. Scholthof, M. Kikkert, and A.O. Jackson, submitted manuscript). To determine whether the foreign proteins expressed by PVX were functional, we tested whether exogenously expressed p22 (cell-to-cell movement protein) could complement movement of TBSV p22 mutants that failed to spread from the initially infected cell. The results with TBSV mutants in which the coat protein was replaced by the GUS gene demonstrated that the impaired cell-to-cell movement function of TBSV p22 mutants can be complemented by coinfection with a PVX vector (pHS159) that expresses p22. Complementation was not observed with a PVX construct (pHS160) whose p19/p22 RNA did not yield p19 or p22 translation products. Thus, these data indicate that the TBSV proteins expressed from the PVX vector are biochemically and functionally very similar to their native counterparts expressed from the TBSV genome.

TBSV p19 Induces Lethal Necrosis in Systemically Infected Nicotiana Hosts

The results demonstrated that TBSV p19 is dispensable for systemic infection of N. benthamiana and N. clevelandii. The major difference between infection with wild-type TBSV versus infection with a p19-deficient mutant was the change in symptoms (Table 1). Highly attenuated symptoms appeared in


Table 1. Host Responses Elicited by p19 and p22 Expressed from TBSV or PVX

<table>
<thead>
<tr>
<th>Host</th>
<th>TBSV p19 + p22</th>
<th>TBSV p22</th>
<th>PVX a</th>
<th>PVX p19 + p22</th>
<th>PVX p19</th>
<th>PVX p22</th>
</tr>
</thead>
<tbody>
<tr>
<td>N. benthamiana</td>
<td>N</td>
<td>M, D</td>
<td>M</td>
<td>N</td>
<td>N</td>
<td>M</td>
</tr>
<tr>
<td>N. clevelandii</td>
<td>N</td>
<td>M, D c</td>
<td>M</td>
<td>N</td>
<td>N</td>
<td>M c</td>
</tr>
<tr>
<td>N. tabacum</td>
<td>L</td>
<td>C</td>
<td>M</td>
<td>L</td>
<td>L</td>
<td>M</td>
</tr>
<tr>
<td>N. glutinosa</td>
<td>L</td>
<td>L</td>
<td>M</td>
<td>L</td>
<td>CN</td>
<td>L</td>
</tr>
<tr>
<td>N. edwardsonii</td>
<td>L</td>
<td>L</td>
<td>M</td>
<td>L</td>
<td>CN</td>
<td>L</td>
</tr>
</tbody>
</table>

C, chlorosis; CN, chlorosis and necrosis; D, leaf distortion and stunting; L, necrotic local lesions; M, mosaic symptoms; N, severe systemic necrosis.

These results were obtained with pHS157, which did not express p19. Because p22 is an essential gene, experiments could not be performed with a TBSV derivative devoid of p22.

aPVX (pPC2S) either without TBSV inserts or with inserts not translating p19 or p22.

bAlthough some necrotic symptoms were observed, the lethal apical necrosis syndrome was not induced.

cSome scattered necrotic spots were present.

N. benthamiana and N. clevelandii infected with the p19 mutant. The typical apical necrosis syndrome was absent, and therefore plants survived infection. Although coat protein mutants of TBSV also produce less severe symptoms, infected plants still develop severe necrosis and occasionally die (Scholthof et al., 1993b). The apparently negligible role of the coat protein in symptom production is also consistent with the results obtained in the present study, because the p19 mutant produced levels of coat protein that were comparable to wild-type TBSV infections without eliciting severe symptoms. Our results agree with and extend the observations made with two other tombusviruses, cymbidium ringspot virus and CNV (Rochon, 1991; Dalmay et al., 1992, 1993; McLean et al., 1993). Thus, results from several sources provide evidence that the tombusvirus p19 analog, rather than the coat protein, is involved in the onset of a lethal apical necrosis in Nicotiana hosts that support a systemic infection.

However, previous experiments did not resolve whether p19 is solely responsible for the phenotype or whether p19-mediated necrosis requires interactions of p19 with other viral components. For example, the p19 mutation could have accelerated DI RNA accumulation, as it does in CNV (Rochon, 1991), to yield DI-mediated symptom attenuation. Therefore, to avoid interference with other TBSV components, we performed inoculations with several PVX derivatives to determine the contribution of individual TBSV genes to symptom development. Such investigations are possible because PVX induces only mild mosaic symptoms on hosts that develop severe symptoms when infected with TBSV.

Symptoms typical of a PVX infection appeared when N. benthamiana plants were infected with PVX that expressed either nontranslatable p19/p22 RNA, p19/p22 antisense RNA, p33 or its antisense RNA, or p22 (Table 1). The results obtained with N. clevelandii were very similar, except that in this plant, PVX-mediated expression of p22 led to small necrotic spots on systemically infected upper leaves. In contrast with the results obtained with p22, expression of p19 from PVX resulted in an especially severe systemic necrosis in N. benthamiana that resembled the TBSV disease syndrome (Table 1). In N. clevelandii, PVX-mediated expression of p19 also elicited severe necrotic symptoms, but the plants generally survived the infections. Thus, our results clearly demonstrate that p19 can elicit severe necrotic symptoms in systemically infected Nicotiana hosts. This conclusion agrees with our recent observation that DI-mediated symptom attenuation is related to a dramatic decrease in p19 accumulation in infected N. benthamiana plants (Scholthof et al., 1995b).

TBSV p19 and p22 Individually Induce Local Lesion Formation in Different Hosts

In contrast with the Nicotiana spp that support systemic infections with TBSV, N. tabacum responded by forming small necrotic local lesions. Inoculation experiments with TBSV coat protein mutants indicated that the coat protein was not involved in activation of the lesion response (data not shown). However, TBSV with an inactive p19 (pHS157) failed to induce typical local necrotic lesions; instead enlarged chlorotic infections and concentric necrotic rings appeared. Exogenous expression of p19 from PVX also resulted in the formation of local necrotic lesions on N. tabacum plants. This response was not observed for those PVX constructs expressing nontranslatable p19/p22 RNA, p19/p22 antisense RNA, p33 antisense RNA, p22, or p33. Our results thus demonstrated that in addition to the general necrosis associated with expression of p19 in systemic infections of some Nicotiana hosts (Table 1), the p19 gene is also responsible for the formation of local lesions on N. tabacum plants.

Wild-type TBSV as well as coat protein mutants of the virus also elicited local lesions on N. glutinosa and N. edwardsonii (Table 1). However, in contrast with the situation observed in N. tabacum, the lesion phenotype induced by wild-type TBSV and the p19 mutant (pHS157) on N. glutinosa and N. edwardsonii plants indicates that p19 is not primarily responsible for activating the local lesion response in these plants. This notion
is supported by results obtained with PVX vectors expressing p19 (pHS142 and pHS158), because, despite the fact that these derivatives elicited localized chlorotic lesions on *N. glutinosa* and *N. edwardsonii* plants, the infection foci expanded to form necrotic areas that subsequently developed into a systemic necrosis. This phenotype resembles the p19-mediated necrosis observed in *N. benthamiana* and *N. clevelandii* (Table 1). In contrast, PVX-mediated expression of p22 (pHS143 and pHS159) in *N. glutinosa* and *N. edwardsonii* resulted in the formation of mainly transparent local lesions that were surrounded by darker rings. This suggests that the p22 protein is the primary elicitor for formation of local lesions in these hosts.

Our combined results (Table 1) indicate that both the p19 and p22 proteins of TBSV can serve as distinct elicitors that interact with products of host-specific resistance genes present in closely related *Nicotiana* spp. Perhaps future experiments may identify hosts containing resistance genes specific for recognition of the coat protein or replicase proteins of TBSV. We suspect that all of the TBSV-encoded proteins have the potential to serve as determinants that can activate resistance responses on one or more hosts, because research with TMV has shown that the replicase protein, the coat protein, and the p30 movement protein can individually induce resistance responses in a host-specific manner (Culver et al., 1991; Padgett and Beachy, 1993; Weber et al., 1993).

### Involvement of Host-Specific Factors in Symptom Responses

Protoplasts of various plant species that are either mock-transfected or transfected with TBSV can be maintained for ~3 days; during this time period, p19 expression has no apparent negative effect on survival of the protoplasts (H.B. Scholthof, unpublished results). It is not known at this point whether p19 protein accumulation reaches higher and subsequently more toxic levels in infected tissue cells than in transfected protoplasts. However, it is very likely that the lethal effect of p19 in plants represents a tissue-specific response. This may be similar to results obtained with *NN* tobacco (Otsuki et al., 1972) and *Tm*-2 and *Tm*-2*2* tomato (Motoyoshi and Oshima, 1975, 1977) protoplasts in which TMV replicated without causing cell death.

The p19-mediated activation of the necrotic local lesion response in *N. tabacum* may be distinct from the generalized necrosis induced by p19 in other *Nicotiana* spp. We do not know whether systemic necrosis is induced by p19 directly or indirectly via the activation of host defense responses. However, it is possible that the local lesion response in *N. tabacum* and induction of systemic necrosis in other *Nicotiana* spp are mechanistically similar but that the defense responses are delayed, less effective, or incomplete in these hosts. Consequently, when infections are not confined, the plants become systemically infected and collapse due to generalized necrosis. The differential symptom-inducing properties of p19 resemble to some extent those of the caulimovirus gene VI protein. This protein is a well-documented virus symptom determinant that is able to induce systemic necrosis in some hosts and acts as an elicitor for local lesion formation in other hosts (Schoelz and Shepherd, 1988; Goldberg et al., 1991a).

At this point, we do not know what properties of p19 are responsible for triggering the necrotic reactions in the systemically infected *Nicotiana* hosts. Amino acid sequence analyses (data not shown) indicate potential phosphorylation sites that may be involved in the activation of host responses. For example, the syndrome in *N. benthamiana* or *N. tabacum* infected with PVX-expressing p19 resembles the response that results during infection with a PVX vector that expresses a gene from tomato with a kinase motif that confers sensitivity to the herbicide fenithion (Rommens et al., 1995). We will conduct mutagenesis experiments in attempts to define p19 domains and biochemical activities involved in the induction of symptoms.

We also have little information about the mechanism of p19- and p22-mediated activation of local lesion responses. However, our results demonstrate that both genes participate in activation of defense genes unique to different hosts. These hypersensitive responses and their host specificity are compatible with a model whereby different hosts express different resistance genes that recognize distinct avirulence determinants (Keen, 1990). Although the p19-mediated local lesion response in *N. tabacum* could possibly be due to nonspecific interactions, p22 appears to behave like an avirulence determinant that is recognized by *N. glutinosa* derivatives but not by the close relatives *N. tabacum*, *N. benthamiana*, and *N. clevelandii*. This characteristic is typical of gene-for-gene interactions. Because *N. edwardsonii* is an amphidiploid of *N. glutinosa* and *N. clevelandii* (Christie, 1969), our results suggest that the local lesion response is determined by a dominant host gene. The interaction of p19 and p22 with putative resistance genes in the different *Nicotiana* spp must be distinct from the *N* gene–mediated response of *N. glutinosa* against TMV, because p22 does not elicit local lesions in *N. tabacum* species carrying the *N* gene, whereas p19 activates local lesions in isogenic lines irrespective of the presence of the *N* gene.

### Activation of Local Lesions by PVX Vectors Expressing p19 or p22 Affects Virus Spread

Local lesions elicited by TBSV on *N. tabacum*, *N. glutinosa*, and *N. edwardsonii* plants effectively prevented virus spread. Although the local lesions that were induced by PVX-mediated expression of p19 or p22 delayed the onset of systemic symptoms, they did not prevent a systemic infection by the vector. During the course of infection with the PVX vectors, p19- and p22-induced necrotic lesions appeared on the systemically invaded leaves, which were positioned immediately above the inoculated leaves, and a PVX-like mild mosaic developed on the remaining upper leaves. This result suggests that the local lesions elicited in response to the exogenously expressed TBSV proteins are not fully capable of preventing an infection...
with PVX. It is also possible that insufficient levels or delayed timing of p19 and p22 expression by the PVX vector permits a fraction of the population to establish infections in upper leaves. Moreover, the pattern of symptom development is very likely affected by the relative instability of PVX vectors in certain hosts (Chapman et al., 1992). This results in deletions of the inserts during replication to reconstitute the wild-type PVX virus. Because our results also suggest that expression of foreign proteins interferes with PVX accumulation, it is likely that derivatives that have deleted the foreign gene will rapidly dominate the population. We are planning experiments to determine whether the inability of the local lesions to prevent PVX spread effectively is due to virus-specific differences in host responses, to insufficient levels or uncoordinated timing of protein expression, or to rapid deletion of the foreign TBSV genes.

Although the elicitation of local lesions on N. tabacum and N. glutinosa plants by PVX-expressing p19 or p22, respectively, did not prevent the spread of the vector, the necrotic responses in the secondary leaves were confined to a limited number of distinct lesions and a systemic necrosis did not develop. This indicates that a few derivatives expressing the heterologous genes escaped and caused a limited number of hypersensitive lesions interspersed with the systemic mosaic resulting from the derivatives that had lost p19 or p22. However, this is apparently not the case when PVX is used to express the avr9 gene from Cladosporium fulvum in resistant tomato plants, because a systemic necrosis ensues (Hammond-Kosack et al., 1995). This course of events resembles the generalized necrosis induced by PVX-expressing TBSV p19 in several Nicotiana hosts used in our studies. As discussed by Hammond-Kosack et al. (1995), several interpretations can be used to explain why virus-mediated expression of a fungal elicitor fails to limit virus spread. However, our results showed that PVX-mediated expression of elicitor proteins from another virus can induce responses that mimic the original local lesion response, even though the hypersensitive reaction fails to prevent the spread of the vector. Our cumulative data indicate that an effective local lesion response that is capable of inhibiting pathogen egress from infection foci not only requires qualitative responses but may also involve spatial, temporal, and quantitative regulatory events.

**Plant Virus Vectors as Tools in Plant Biology**

The results of this study indicate that the use of virus vectors, such as PVX, in investigations of host–pathogen interactions may provide valuable tools to accelerate the characterization of avr genes and their association with host proteins. A particular advantage of PVX for expression of TBSV genes is that the vector induces only mild symptoms on many hosts so that exacerbated host responses can be readily visualized. Furthermore, exogenous expression of pathogen-derived genes in a nonhost using a virus vector that can infect this host may be used to identify avr genes and can possibly reveal new sources of masked resistance genes.

Apart from the relative ease and speed of generating the desired virus construct versus the laborious efforts involved in producing transgenic plants, virus vectors have several other advantages. These include high levels of gene expression and coordinated expression in an environment that avoids a nuclear phase or dependence on host cell division. Coordinated expression rather than constitutive expression may be particularly important for functional studies, such as virus movement, because the proteins required for these functions may have a narrow window of activity (Deom et al., 1990; Holt and Beachy, 1991). Although the relative instability of the inserted genes may be a disadvantage for certain experimental analyses, it ensures that in the unlikely case of escape from containment facilities, the recombinant viruses will be outcompeted rapidly by their wild-type progeny.

We believe that plant virus vectors, such as PVX (Chapman et al., 1992) and other vector systems based on TBSV (Scholthof et al., 1993b), tobacco etch virus (Dolja et al., 1992), geminiviruses (Stanley, 1993), and TMV (Donson et al., 1991; Hamamoto et al., 1993), form an attractive pool of alternative vectors for analysis of genes in plants. Despite the focus of this study, the use of plant virus vector systems does not need to be limited to studies of plant–microbe interactions but can be employed for general use in plant biology, in particular when transient expression of a gene of interest can elicit a specific phenotype.

**METHODS**

**General Protocols**

Standard molecular biology techniques used during this investigation involved minor modifications of the methods described by Sambrook et al. (1989). Bioassays with plants and protoplasts, virus or RNA analyses, and reporter gene expression studies were performed as described previously (Scholthof et al., 1993b). Previously reported procedures (Scholthof et al., 1994) were employed to overexpress glutathione S-transferase (GST) fusion proteins of p19 and p22 in Escherichia coli. After overexpression, the GST fusion proteins were purified by SDS-PAGE 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 p19- and p22-specific 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., 1993; Scholthof et al., 1994). Rat antiserum raised against potato virus X (PVX) capsid protein was generously provided by D.C. Baulcombe (Sainsbury Laboratory, Norwich, UK), and the procedure for obtaining p33-specific antiserum has been described previously (Scholthof et al., 1995a).

Protein extraction and fractionation from plants and immunoblot analyses were performed essentially as described by Scholthof et al. (1994). Unfractionated samples were obtained by grinding 200 to 500 mg of infected tissue in two volumes (w/v) of Tris-EDTA, mixed with one-third volume of 5× sample buffer (Scholthof et al., 1994), boiled for 5 min, and frozen at -80°C. Before use, the samples were boiled again for 5 min, briefly centrifuged at maximum speed in a microcentrifuge, and...
kept on ice before loading 20 μL on a 12% SDS–polyacylamide gel. Additional details involving individual experiments are described in the legend of Figure 2 and in the text.

The templates used for in vitro transcription were prepared by digestion of CsCl-purified DNA with Smal (pTB5V-100 derivatives), by digestion with Spal followed by trimming (derivatives containing the β-glucuronidase [GUS] gene), or by digestion with Spel (PVX derivatives). The transcripts from PVX derivatives were capped (Chapman et al., 1992) using a cap analog (Pharmacia), whereas the tomato bushy stunt virus (TBSV) derivatives remained uncapped. Plant sap inoculation experiments were performed with two to three infected leaves that were macerated in ~5 mL of inoculation buffer (50 mM KH2PO4, pH 7.0, 1% Cetilite).

Recombinant Plasmids

The TBSV plasmids used in this study were derived from an infectious full-length cDNA clone, designated pTB5V-100 (Hearne et al., 1990), that is illustrated in Figure 1. The numbering refers to the nucleotide positions on this plasmid, and the constructs used for the bioassays are schematically diagrammed in Figure 1. The construction of pH57, pH85, and pH545 was described previously (Scholthof et al., 1993b).

For several cloning purposes, an intermediate plasmid (pHS24) was used. It contains the Stul (nucleotide 1059) to SalI (nucleotide 4500) fragment of pTB5V-100 inserted into the Smal to SalI sites of pBS9, which is a kanamycin-resistant vector (Spratt et al., 1986). To obtain GST fusion products, plasmids pH511 and pH513 were generated by digestion of pH524 with Ncol (nucleotide 3886) or PflMl (nucleotide 3849), respectively, and SalI (nucleotide 4500), followed by treatment with the Klenow fragment of DNA Polymerase I to generate blunt-ended termini. Subsequently, the fragments containing the p19 or p22 genes were inserted in frame into pGEX2T (Pharmacia) that had been digested with Smal to create pH511 or EcoRI (nucleotide 4034; filled in) to create pH513. The correct fusion of the reading frames was confirmed by sequencing.

Plasmid pH5132 was constructed using a 5′ oligonucleotide spanning a region from nucleotide 4031 to 4060 with a one-base alteration of a T residue (nucleotide 4058) to an A residue, which introduced a premature stop codon in p22 but left p19 intact (Figure 1A). This oligonucleotide was used as a 5′ primer in a polymerase chain reaction (PCR), together with a 3′ primer farther downstream that overlapped the region around the Spal site at nucleotide 4805 (Knorr et al., 1991) of the pH549 template (Scholthof et al., 1993a). This PCR product was cloned into pBS9 before it was used to replace the equivalent in pH512, which is a derivative of pH545 that has the unique SalI site (nucleotide 4500) converted to a PvuI site. Use of the latter construct enabled screening for clones in which the EcoRI to Spal fragment was replaced. In addition to the restoration of the SalI site, these recombinant plasmids could also be checked for the formation of a new AvrII site that was introduced as a consequence of the one-base alteration in p22.

For construction pH5136 (H.B. Scholthof, K.-B.G. Scholthof, M. Kikkert, and A.O. Jackson, submitted manuscript), a similar strategy was followed using an oligonucleotide with a single-base alteration of a C residue (nucleotide 4044) to a T residue, which introduced a premature stop codon in p19 while leaving p22 intact (Figure 1A). The PCR product was blunt ended and ligated into the Smal site of a kanamycin resistance vector designated pKAN2, which is pBS9 (Spratt et al., 1986), with the multiple cloning site of pUC119. Recombinant plasmids with the appropriate inserts were selected. Subsequently, the EcoRI to PvuI fragment (with the mutation) from the pKAN2 intermediate was used to replace the corresponding fragment of pH5132. Subsequent sequence analyses confirmed that the predicted mutations were introduced in the correct context. To generate the same specific mutations in a wild-type TBSV background rather than in the GUS gene–containing derivatives, the internal BamHI (nucleotide 2440) to NcoI fragment of pH5132 and pH5138 was replaced with the counterpart of pH524 to produce pH5136 and pH5137, respectively.

The PVX vector used in these studies (pPC25) is a derivative of pGC3 (Chapman et al., 1992) in which the GUS gene was replaced by unique sites that allow convenient cloning. This vector was generously provided by S. Chapman and D. Baulcombe (Sainsbury Laboratory, Norwich, UK). For our study, the TBSV genes were engineered into the EcoRV site of the PVX vector (nucleotide 5677 for pPC25) to generate subgenomic mRNAs that permit translation to initiate at the start codon of the inserted gene. These plasmids are diagrammed in Figure 1B. For construction of pH5142-20 (pH5142 in Figure 1), the filled-in NcoI (nucleotide 3886) to SalI fragment of pH524 was inserted into the PVX vector to permit translation of only p19. To generate pH5143, pH5154, or pH5159, the PmiI (nucleotide 3849; trimmed-back) to SalI (filled-in) fragment of pH524, pH5132, or pH5138 was inserted into pPC25. This resulted in mRNAs that translated p22 and p19 (pHS143, p19 only [pHS158], or p22 only [pHS159]. An additional construct, pH5160, was made using the NcoI to SalI fragment of pH5138 to provide a p19/p22 mRNA unable to express the p19 or p22 proteins. To generate pH5168 for the expression of TBSV p33 from the PVX genome, a DNA fragment was amplified by PCR using a 5′ oligonucleotide encompassing the p33 start codon and a 3′ oligonucleotide containing its amber stop codon. The resulting fragment was treated with the Klenow fragment and ligated directly into the EcoRV site of pPC25. During most of the cloning experiments, plasmids were also selected that contained the insert in the opposite or antisense orientation. These are referred to in the text with the added notation "anti," as in pH5143anti and pH5168anti in Figure 1.

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