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A newly identified role for *Tomato bushy stunt virus* P19 in short distance spread

MASSIMO TURINA,[1] RUSTEM OMAROV, JOHN F. MURPHY†, CYNTHIA BAZALDUA-HERNANDEZ, BÉNÉDICTE DESVOYES[2] AND HERMAN B. SCHOLTHOF*

Department of Plant Pathology and Microbiology, Texas A&M University, College Station, TX 77843, USA and
†Department of Entomology and Plant Pathology, Auburn University, AL 36849, USA

SUMMARY

This study identified a role for the *Tomato bushy stunt virus* (TBSV) *p19* protein (P19) in local lesion expansion on cowpea (*Vigna unguiculata*), and cell-to-cell movement in pepper (*Capsicum annuum*). The contribution to short distance spread in both hosts was strongly influenced by a cluster of charged amino acids between positions 72 and 78 on the 172 amino acid P19. Charged amino acids near this region between positions 43 and 85 were required for long distance spread in pepper. These results indicate that the central domain of P19 plays a key role for its activities in TBSV movement and that additional regions on this protein contribute to virus spread in a host-specific manner.

INTRODUCTION

Tomato bushy stunt virus (TBSV) is an isometric single-stranded plus-sense RNA virus that is the type member of the *Tombusvirus* genus in the family *Tombusviridae* (Martelli *et al.*, 1988; Russo *et al.*, 1994). Previous reverse genetic approaches with an infectious full-length cDNA clone (Fig. 1) demonstrated that the two 5′ proximal open reading frames (ORFs) of TBSV (*p33* and *p92*) are sufficient for replication (Scholthof *et al.*, 1995c). Various studies with other tombusviruses also showed that expression of the coat protein (CP) ORF and the 3′ terminal overlapping *p22* and *p19* genes are not required for replication in various hosts (Dalmay *et al.*, 1992; Dalmay *et al.*, 1993; Rochon *et al.*, 1991). The *p22* protein (P22) is the only viral protein that is required for cell-to-cell movement in all hosts tested thus far (Chu *et al.*, 1999; Scholthof *et al.*, 1995b), whereas the *p19* protein (P19) and CP appear to contribute to long distance spread (Desvoyes and Scholthof, 2002; Qiu *et al.*, 2002; Qu and Morris, 2002; Scholthof *et al.*, 1993, 1995b).

* *Correspondence*: E-mail: herscho@tamu.edu
Present addresses: [1]Istituto di Virologia Vegetale, Torino, Italy, [2]Centro de Biologia Molecular 'Severo Ochoa', Universidad Autonoma de Madrid, Spain.

In the present study, we identified an additional role for P19 in local lesion expansion on the resistant host cowpea (*Vigna unguiculata*), and in movement through pepper (*Capsicum annuum*), a host that is susceptible to a full systemic infection with TBSV. Centrally located residues on the 172 amino acid P19, which were previously shown to be crucial for systemic invasion in spinach (Chu *et al.*, 2000) contribute to the local spread function in cowpea and pepper. Additional amino acids between positions 43 and 85 on the N-terminal half of P19 are required for long distance spread in pepper.

Local spread in cowpea

Inoculations of wild-type TBSV virus, *in vitro*, generated transcripts (Hearne *et al.*, 1990), or *in vivo* transcribed DNA-based constructs (Scholthof, 1999), all lead to the induction of a hypersensitive response-like (HR-like) reaction on inoculated primary and secondary leaves of cowpea. Three days after inoculation, local lesions appeared that remained small and chlorotic under strong light (> 300 μE/s/m^2) and temperature (27 °C). When grown in moderate light (180 μE/s/m^2) and temperature (23 °C) these lesions became enlarged and dark brown.

To analyse the contribution of individual TBSV proteins to the movement and induction of local lesions in cowpea, transcript inoculations were performed using mutants inactive for the synthesis of either wild-type CP (pHS7), P19 (pHS157), or P22 (pHS136) (Fig. 1). The results showed that, as expected, no lesions appeared for the cell-to-cell movement mutant pHS136, and with the exception of a slight delay in local necrosis upon inoculation with RNA from pHS157, no obvious phenotypic changes were observed for this clone. Inoculations of secondary cowpea leaves with pHS157 derived virus inoculum often resulted in the appearance of chlorotic lesions which progressed to necrotic lesions 2–3 days later.

Immunoblot assays for the *p33* protein (P33) or P19 showed that the CP mutant (pHS7) and wild-type TBSV accumulated to similar levels in inoculated cowpea leaves. However, the P33 accumulation was reduced for the *p19*-defective mutant pHS157 (data not shown), although lesions were clearly visible. These

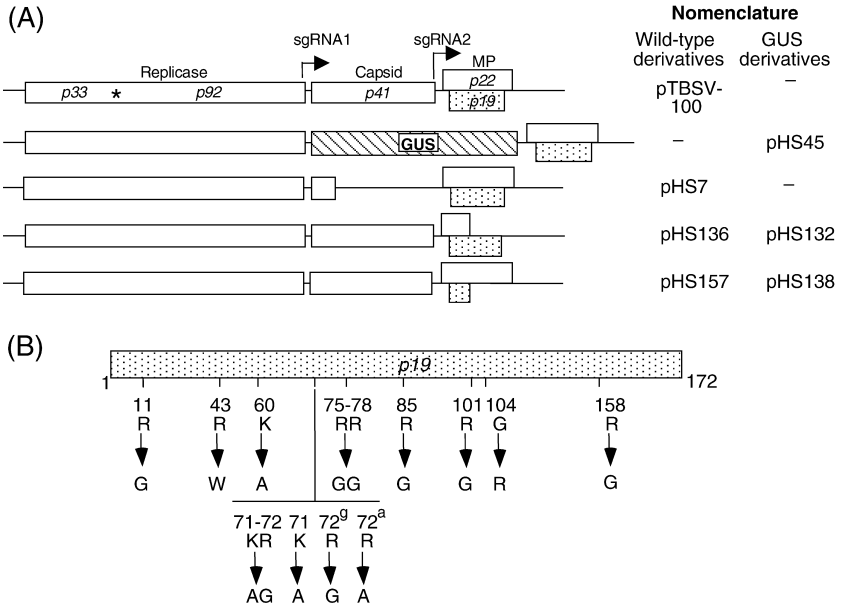

Fig. 1 Schematic diagram of the TBSV genomic organization and the derived *p19* gene mutants. (A) open boxes denote open reading frames (ORFs) that encode proteins for which sizes are given in kDa inside the boxes, and the functions are indicated on top. MP indicates movement proteins, and GUS represents the β-glucuronidase gene. Solid lines represent presumed untranslated sequences, and the transcriptional start-sites of subgenomic RNAs (sgRNA1 and sgRNA2) are indicated by right-angled arrows. The asterisk inside the TBSV genome indicates the relative position of the amber stop codon used for translational read-through of *p33* to yield the *p92* product. The derivative constructs pHS7 through pHS157 were described previously (Scholthof *et al.*, 1993, 1995b), but their relevant features are depicted in the diagram. (B) Amino acid substitution mutants of P19. The position of mutated amino acids on P19 of individual mutants is indicated and denoted by the conventional single-letter notation along the 172 amino acid polypeptide. The construction of the mutants was described previously (Chu *et al.*, 2000). The nomenclature provides the position of the mutation(s) as they appear on the protein; for example for pT19/71–72 the amino acids at positions 71 and 72 of P19 are changed. Throughout the text the prefix 'p' has been omitted when RNA or virus, rather than the plasmid, is the subject of discussion.

results illustrated that the local lesion phenotype was not an accurate indicator for virus spread.

Since the CP did not contribute to local spread, this permitted the utilization of TBSV variants in which the CP gene was substituted for the β-glucuronidase (GUS) gene, to visualize the infection and monitor the contribution of P19 to movement. For this purpose, cowpea leaves were inoculated with transcripts from the following TBSV-GUS constructs (Fig. 1): pHS45 (active for *p22* and *p19* expression), pHS138 (inactive for *p19* expression), or pHS132 (inactive for *p22* expression) (Scholthof *et al.*, 1995b). Results obtained following standard histochemical GUS assays (Jefferson *et al.*, 1987; Scholthof *et al.*, 1993) confirmed the strict requirement of the *p22* gene for cell-to-cell movement (Fig. 2A). In the absence of *p19* expression (pHS138), cell-to-cell movement occurred, but the diameter of the blue foci was substantially reduced compared to those associated with the parental construct pHS45 (Fig. 2A). The same influence of P19 was observed upon inoculation of cowpea with infectious DNA-based constructs which were either active or inactive for expression of *p19* (Scholthof, 1999) (and data not shown).

In a recent report we identified P19 amino acids that were essential for long distance spread in spinach (Chu *et al.*, 2000). (This reference contains the nature of amino acid substitutions that are summarized in Fig. 1B). To elucidate if the suspected local movement function of P19 observed in the present study on cowpea was determined by the same or different amino acids that are essential in spinach, the various P19 amino acid substitution mutants were inoculated on to cowpea. The results showed that most mutants behaved as wild-type, except T19/71–72 (nomenclature derived from plasmid pT19/71–72) and T19/75–78, which caused a 1–2 day delay in the onset of lesions which were generally less necrotic and smaller in diameter. Immunoblot assays revealed that viral proteins consistently failed to accumulate to detectable levels in the small lesions induced by T19/71–72 (not shown).

The above results demonstrate that for TBSV infections on cowpea: (i) P19 contributes to the expansion of local lesions, (ii) the necrogenic P19 (Scholthof *et al.*, 1995a) is not the elicitor of the HR-like reaction as in *Nicotiana tabacum*, but may contribute to the extent of necrosis of the lesions, and (iii) the central region on P19 that is instrumental for systemic invasion of spinach (Chu *et al.*, 2000), also influences the size of the local lesions on inoculated cowpea leaves.

Although the experiments with cowpea were indicating a novel role for P19 in increasing localized infections, due to the resistance response in cowpea it was inconclusive whether the

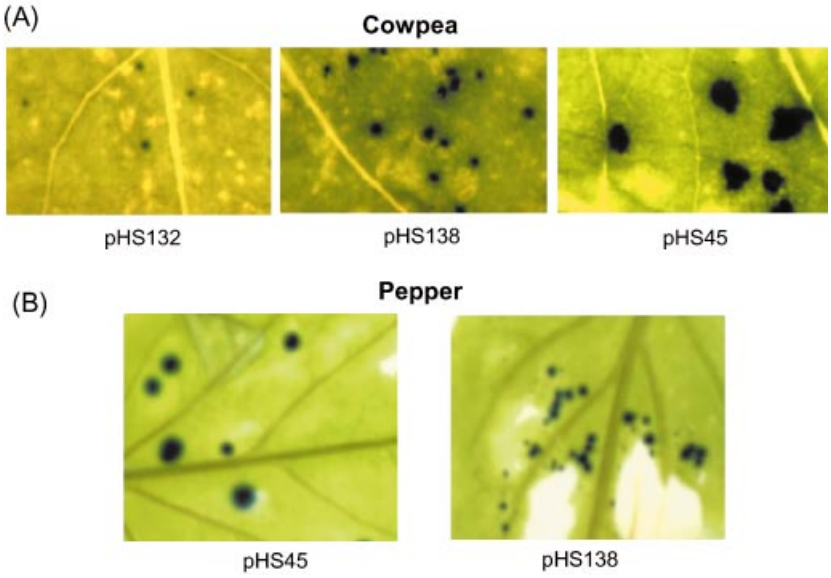

Fig. 2 Histochemical GUS assays on inoculated leaves from (A) cowpea, and (B) pepper, at 3 and 4 days post-inoculation, respectively. Leaves were inoculated with transcripts from the constructs indicated beneath the panels.

observed P19-mediated phenotype was due to effects on viral accumulation, cell-to-cell movement or to differences in plant defence responses. Therefore, the effect of P19 was not further examined in this host because it was deemed necessary to dissect the effect of P19 on viral accumulation, cell-to-cell movement and long distance spread on a host that supports a non-necrogenic P19-dependent systemic infection with TBSV.

Local and long distance spread in pepper

A previous study (Scholthof *et al.*, 1995b) showed that P19 was required for the infection of pepper plants. However, it was not known if P19 was required in pepper for short distance spread, as shown above for cowpea (Fig. 2A), and/or for long distance spread as in spinach (Chu *et al.*, 2000; Scholthof *et al.*, 1995b). It was also unclear if the CP contributed to local spread in pepper. However, it had been established that infections of TBSV on pepper did not cause any substantial necrosis but instead resulted in a systemic chlorosis (Scholthof *et al.*, 1995b).

Recent reports have shown that P19 is an effective suppressor of gene silencing (Qiu *et al.*, 2002; Qu and Morris, 2002; Silhavy *et al.*, 2002; Voinnet *et al.*, 1999). Considering this activity and the critical role of P19 during infection of pepper with TBSV, it was necessary to determine if P19 affected the level of TBSV RNA accumulation in pepper at the cellular level. For this purpose experiments were performed using pepper (*C. annuum* cv. Early California Wonder) protoplasts. As shown in Fig. 3, TBSV genomic RNA did not accumulate to detectable levels at 5 h.p.i. (hours post-inoculation); however, both wild-type TBSV and pHS157 RNAs were detected at 24 h.p.i. The levels of sgRNAs (subgenomic RNAs) were very low, which was consistent with their low level

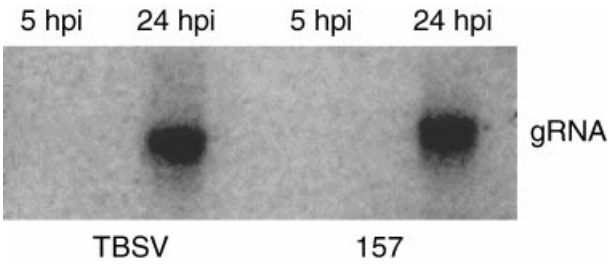

Fig. 3 TBSV RNA accumulation in pepper protoplasts. *Capsicum annuum* L. cv Early California Wonder seed were sown in Magenta boxes containing Pro-Mix soil-less potting medium (Premier Peat, Riviére-du-Loup, Québec, Canada). Protoplast isolation was essentially performed as initially described (Murphy and Kyle, 1994) and modified (Deom *et al.*, 1997). Inoculation of protoplasts was carried out using polyethylene glycol (Deom *et al.*, 1997). TBSV and pHS157 RNA were transcribed as described previously (Scholthof *et al.*, 1993, 1995b). Following transcription, 1 unit of RNase-free DNase was added, incubated for 30 min at 37 °C followed by two cycles of phenol–chloroform extraction. The RNA was precipitated in 0.1 M sodium acetate, pH 5.5, and 2.5 volumes of ice cold absolute ethanol at −20 °C for at least 12 h. Protoplasts (1×10^6 for each treatment) were inoculated using 15 μL of ≈ 0.5 μg RNA/μL. At 5 and 24 h post-inoculation (h.p.i.), 750 000 protoplasts were collected from each treatment and RNA from each sample was isolated using an RNeasy kit according to the manufacturer's instructions (Qiagen, Valencia CA). RNA was denatured in formaldehyde and formamide at 55 °C for 15 min, chilled on ice and then subjected to agarose/formaldehyde gel electrophoresis. Following electrophoresis, the RNAs were transferred to a Nytran membrane using a turbo blotter according to the manufacturer's instructions (Schleicher & Schuell, Keene, NH). The RNA was cross-linked to the membrane using a UV Stratalinker 1800 (Stratagene, La Jolla CA) and hybridized with a TBSV-specific probe as described previously (Scholthof *et al.*, 1993, 1995b). The accumulation of gRNA is shown for 5 and 24 h.p.i.

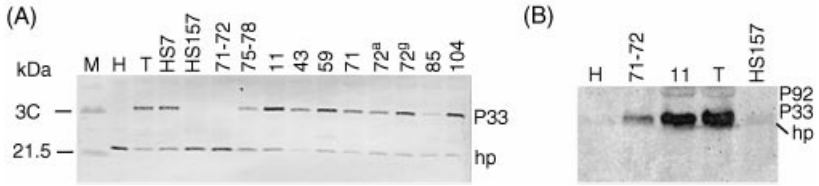

Fig. 4 Immuno-detection of the TBSV *p33* protein (P33) (Scholthof *et al.*, 1995c) in inoculated pepper leaves. (A) Alkaline-phosphatase mediated immunoblot detection of P33 in pepper leaves inoculated with plant sap from *Nicotiana benthamiana* infected with transcripts indicated above the lanes by the numerical designation of the corresponding plasmids. H, healthy; T, TBSV; M, size markers in kDa. The extracts used for the six lanes on the left (H through 75–78) were from leaves inoculated 9 days previously, whereas the other lanes are from leaves 4 days postinoculation. The result shown for lane 104 is also representative of the results obtained with T19/101 and T19/158 (not shown). (B) Tris-tricine gel followed by chemiluminescence-mediated immunoblot-detection of P33 in pepper leaves inoculated 4 days previously with equivalent amounts of virus (based on the intensity of whole virus samples electrophoresed through agarose gels (Scholthof *et al.*, 1993)) obtained from transcript inoculated *N. benthamiana* plants. Dependent on the detection assay, a cross-reaction occurred for antiserum with different host proteins (hp); this is clear in all lanes in (A) and barely discernible in (B) in lanes H and 157. The P92 read-through replicase-associated protein was detected in (B) for lanes T and 11.

of detectable accumulation in pepper plants (R. Omarov and H. Scholthof, unpubl. obs.). These results showed that pHS157 RNA replicated in pepper protoplasts and its overall accumulation was similar to that observed for the wild-type, thus providing evidence that TBSV RNA accumulation at the cellular level is not noticeably affected by P19.

The above findings implied that following inoculation of pepper plants the infection at early stages proceeds very efficiently in the absence of P19 and that subsequent P19-mediated effects occur after the infection of the inoculated cells has been established. To evaluate the contribution of P19 (and CP) to spread in pepper following the establishment of infection at the site of inoculation, leaves were inoculated with extracts containing HS7 (CP mutant) or HS157 (inactive for P19 expression) (Fig. 1). Immunoblot assays for detection of the replicase-associated P33 were used as an indicator of virus accumulation (Chu *et al.*, 2000). The results showed that the accumulation of TBSV was below detectable levels in inoculated pepper leaves in absence of P19 (Fig. 4, lanes HS157). However the CP mutant (HS7) accumulated to similar levels as the wild-type, indicating that CP is dispensable for local spread of TBSV in pepper (Fig. 4, lane HS7). The same results as shown in Fig. 4 for P33 were obtained upon immunoblot detection of P19 (data not shown), but this assay is unsuitable for comparison because HS157 is inactive for *p19* expression.

As for cowpea, the dispensability of CP for local spread in pepper once more permitted the application of the TBSV-GUS constructs. Inoculations of pepper with *in vitro* generated transcripts were most consistent and successful after subjecting plants to dark conditions for 2 days prior to inoculation. Comparative histochemical assays on pepper leaves inoculated with transcripts of pHS45 (expressing *p22* and *p19*) or pHS138 (inactive for *p19* expression) showed that absence of P19 reduced the size of the blue foci (Fig. 2B). This provided evidence that P19 contributes to local spread in pepper leaves.

To compare the genetic requirement of particular P19 regions for spread in pepper to those reported to be essential for spread in spinach (Chu *et al.*, 2000) or local movement in cowpea (see above and Fig. 4), P19 amino acid substitution mutants were compared for infectivity. These inoculations on pepper were carried out with sap extracts or crude virus preparations isolated from transcript inoculated *N. benthamiana* (see legend Fig. 4) in which all P19 mutants readily accumulate (Chu *et al.*, 2000; Scholthof *et al.*, 1995b).

Inoculation of wild-type TBSV and most P19 mutants on pepper resulted in a strong yellow chlorosis on the inoculated leaves. However, inoculations with HS157 and T19/75–78 did not induce any noticeable symptoms, whereas T19/71–72 induced the onset of small necrotic ringspots (data not shown). Alkaline-phosphatase-mediated immunoblot assays for the detection of P19 (not shown) and P33 (Fig. 4A) showed that many of the mutants were able to spread in inoculated leaves similar to wild-type TBSV, although the accumulation for T19/75–78 and T85 was somewhat reduced (Fig. 4A, and data not shown). However, in leaves inoculated with HS157 and T19/71–72 no P33 accumulated to levels detectable with the alkaline-phosphatase-mediated assay (Fig. 4A).

Implementation of sensitive chemiluminescent-mediated assays also did not reveal a detectable level of P33 accumulation for HS157, but did facilitate the detection of trace amounts of this protein in leaves inoculated with T19/71–72 (Fig. 4B). The amount of virus present in the inocula for these experiments were standardized (legend to Fig. 4B and data not shown), eliminating the possibility that the substantial differences between levels of P33 shown in Fig. 4B were due to inoculum concentration variability. The results in Fig. 4B confirmed that T19/71–72 was infectious on pepper and that this mutant was strongly impaired for localized accumulation (i.e. spread). These results also reveal that the onset of ringspots on pepper leaves inoculated with T19/71–72 are not a good indicator of the level of virus spread.

Systemic symptoms of TBSV on pepper were marked by a distinctive yellow mosaic in the upper leaves at approximately 10 days

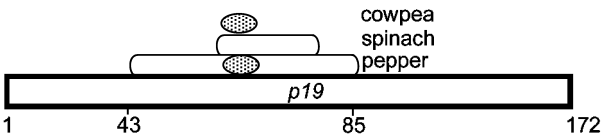

Fig. 5 Schematic diagram depicting the host-specific contribution of P19 regions on spread of TBSV in cowpea, spinach and pepper. The shaded region indicates participation in short distance spread and the open area denotes activities for long distance spread.

post-inoculation. Immunoblot assays readily detected virus proteins in these symptomatic leaves at 2 weeks post-inoculation (Desvoyes and Scholthof, 2002). Systemic symptom development, immunoblots and bioassays on the indicator local lesion host *Chenopodium quinoa* were used to monitor the presence of virus in upper non-inoculated leaves of pepper upon infection of lower leaves with the amino acid substitution *p19* mutants. The summarized results in Fig. 5 show essentially that amino acids in the N-terminal portion of P19 from amino acids 43–85, but outside the core area, are not pivotal for spread in inoculated pepper leaves but contribute to long distance spread in this host.

In summary, the results show that for TBSV infections on pepper: (i) P19 does not affect the level of TBSV RNA accumulation in protoplasts, (ii) P19 contributes to local spread and this activity requires the conservation of its central region, and (iii) P19 is also required for long distance spread and this process is influenced by amino acids upstream and downstream of the central region.

The P19 versatility conundrum

TBSV P19, which is primarily localized to the cytosol (Scholthof *et al*., 1995b), has the capacity to mediate many seemingly different host-specific activities. These include: the induction of a systemic necrosis in several *Nicotiana* species that support a systemic infection (Scholthof *et al*., 1995a), the elicitation of an HR-like reaction in *N. tabacum* (Scholthof *et al*., 1995a), enabling systemic TBSV invasion of spinach and pepper (Chu *et al*., 2000; Scholthof *et al*., 1995b), suppression of gene silencing (Qiu *et al*., 2002; Qu and Morris, 2002; Silhavy *et al*., 2002; Voinnet *et al*., 1999), and as shown in this report, it is required for local spread in cowpea and pepper. Furthermore, P19 needs to be produced at a relatively high abundance to properly exert its biological influence (Scholthof *et al*., 1999).

Inoculation studies with the amino acid substitution mutants in which individual or groups of P19 amino acids have been changed (Fig. 1B), showed that residues in the central region of P19 contribute greatly to local infections in cowpea and pepper. This region was recently shown to be required for *p19*-mediated long distance spread in spinach (Chu *et al*., 2000) and for the suppression of gene silencing (Qiu *et al*., 2002). However, in contrast to what was observed in the study on long distance spread in spinach, mutagenesis of the single amino acid at position 72 from R to G (in pT19/72^{g}) did not noticeably affect the local spread in pepper (Fig. 4A). Intriguingly, however, this substitution did prohibit long distance spread in pepper. The additive contribution of the central amino acids 71 and 72 for local spread in pepper may reflect a direct interaction with host factors but could also indirectly indicate that this region is particularly important for maintaining structural integrity or perhaps for promoting biologically relevant protein–protein interactions.

Further genetic comparisons revealed that the charged amino acids located between positions 43 and the central region (Fig. 5), that are required for long distance spread in pepper, mostly coincide with the amino acids that were recently shown to be crucial for the induction of symptoms in *Nicotiana* species (Chu *et al*., 2000) and a subset was also important for suppression of gene silencing (Qiu *et al*., 2002). These comparisons imply that the central region of P19 is required for all its associated activities and that the stringency of the requirement for maintenance of other amino acids is host-dependent.

The various P19-mediated biological effects could potentially all originate in the biochemical activity of P19 that controls the suppression of post-transcriptional gene silencing (Qiu *et al*., 2002; Qu and Morris, 2002; Silhavy *et al*., 2002; Voinnet *et al*., 1999). In pepper, such a scenario would imply that gene silencing and suppression do not affect events in initially infected cells (Fig. 3), but play a role in subsequent short and long distance spread. However, other than the general requirement for conservation of the central region on P19 there is no strict genetic correlation between any biological activity and suppression of gene silencing (Qiu *et al*., 2002). It is therefore possible that the suppression of gene silencing is an aspect of P19-mediated effects, but other activities may also influence the progression of infection in a host-specific manner.

Despite the absence of clear understanding about the biochemical mechanism of P19 function, combined with previous reports the data in this study provoke some generalizations about the movement of TBSV through plants and the biological contribution of P19. The cell-to-cell movement of TBSV through all its hosts strictly requires the expression of the membrane-bound P22. In some hosts (e.g. cowpea and pepper) local spread of TBSV is assisted by the activity of P19, whereas the CP appears to be dispensable for this local process in plant species studied thus far. Depending on the host, subsequent long distance spread may require the abundant accumulation of P19 (Fig. 4) (Scholthof *et al*., 1999), and the presence of CP generally expedites vascular mediated transport (Desvoyes and Scholthof, 2002; Qu and Morris, 2002). The host-dependent requirements for P19 could reflect the presence of plant-specific pre-existing or defence related symplastic cell-type specific barriers. By analogy with models proposed for other viruses (Blackman *et al*., 1998; Wang *et al*., 1998), it is tempting to speculate that P19 is required for the active penetration of these barriers (perhaps by suppression of silencing defences) to allow a rapid passage of ribonucleoprotein complexes

(i.e. viral RNA and P22). Depending on the host, this activity could extend into vascular loading or unloading events that benefit from association of the complex with CP.

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