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Evidence that the Potyvirus P1 Proteinase Functions in *trans* as an Accessory Factor for Genome Amplification

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The tobacco etch potyvirus (TEV) polyprotein is proteolytically processed by three viral proteinases (NIa, HC-Pro, and P1). While the NIa and HC-Pro proteinases each provide multiple functions essential for viral infectivity, the role of the P1 proteinase beyond its autoproteolytic activity is understood poorly. To determine if P1 is necessary for genome amplification and/or virus movement from cell to cell, a mutant lacking the entire P1 coding region (ΔP1 mutant) was produced with a modified TEV strain (TEV-GUS) expressing β-glucuronidase (GUS) as a reporter, and its replication and movement phenotypes were assayed in tobacco protoplasts and plants. The ΔP1 mutant accumulated in protoplasts to approximately 2 to 3% the level of parental TEV-GUS, indicating that the P1 protein may contribute to but is not strictly required for viral RNA amplification. The ΔP1 mutant was capable of cell-to-cell and systemic (leaf-to-leaf) movement in plants but at reduced rates compared with parental virus. This is in contrast to the S256A mutant, which encodes a processing-defective P1 proteinase and which was nonviable in plants. Both ΔP1 and S256A mutants were complemented by P1 proteinase expressed in a transgenic host. In transgenic protoplasts, genome amplification of the ΔP1 mutant relative to parental virus was stimulated five- to sixfold. In transgenic plants, the level of accumulation of the ΔP1 mutant was stimulated, although the rate of cell-to-cell movement was the same as in nontransgenic plants. Also, the S256A mutant was capable of replication and systemic infection in P1-expressing transgenic plants. These data suggest that, in addition to providing essential processing activity, the P1 proteinase functions in *trans* to stimulate genome amplification.

The genome of tobacco etch virus (TEV), a member of the *Potyviridae* family of positive-strand RNA plant viruses, encodes a single large polyprotein that undergoes proteolytic processing catalyzed by three virus-encoded proteinases (26). Two potyviral proteinases, the nuclear inclusion-a proteinase (NIa) and the helper component proteinase (HC-Pro), are multifunctional proteins containing C-terminal proteolytic domains and N-terminal domains that serve other roles during the replicative cycle (49). The NIa proteinase resembles the picornavirus 3C proteinase (1, 24) and catalyzes most cleavage reactions of the viral polyprotein (9, 11, 16, 27, 34). The N-terminal half of NIa functions as the genome-linked protein (VPg [43, 48, 51]), which is necessary for RNA replication. The HC-Pro proteinase is a cysteine-type enzyme that autoproteolytically cleaves at a C-terminal site (10, 45). The N-terminal region of HC-Pro contains sequences necessary for transmission of the virus by aphids, efficient RNA replication, and systemic movement through plants (6, 7, 18, 22, 39, 52).

The P1 proteinase is derived from the N-terminal region of the viral polyprotein, catalyzes autoproteolytic cleavage between itself and HC-Pro, and exhibits single-strand RNA binding activity in vitro (8, 55, 56). The P1 proteinase is the least-conserved protein among potyviruses (24, 53), ranging in size from 30 kDa to 63 kDa. The C-terminal proteolytic domain resembles chymotrypsinlike serine proteinases, with His-214, Asp-223, and Ser-256 forming the catalytic triad (55). In a recent study, the N-terminal half of P1 was shown to be dispensable for proteolysis as well as genome amplification, cell-to-cell movement, and systemic infection (54). Proteolytic separation of P1 and HC-Pro, but not P1 proteolytic activity per se, was shown to be essential for infectivity in plants. Nonviable TEV mutants encoding processing-defective P1 proteinases were restored by second-site mutations that introduced a NIa cleavage site between P1 and HC-Pro (54). Separation of P1 and HC-Pro could be required to stimulate their nonproteolytic activities or for proper subcellular localization of one or both proteins.

If the P1 proteinase is a multifunctional protein similar to NIa and HC-Pro, the additional nonproteolytic functions are not understood. It was suggested that P1 functions as a movement protein involved in cell-to-cell transport of virus in plants (5, 8, 24, 37, 42, 49, 57). Despite extensive speculation in the literature, however, experimental evidence for a movement function of P1 protein is lacking. In the present study, the putative nonproteolytic function of P1 was addressed by mutational and complementation analyses. The results indicate that the P1 protein plays little, if any, role in virus movement but rather provides an activity that stimulates genome amplification in *trans.*

MATERIALS AND METHODS

Plasmids, strains, and plants. All plasmids were prepared with *Escherichia coli* HB101. The TEV nucleotide numbering and protein sequence numbering were as described in reference 1. Complementary DNA representing the TEV genome was the same as that used in previous studies (23). *Nicotiana tabacum* cv. "Xanthi nc" (tobacco) was the host for all experiments.

The plasmids pTEV7DAN-G↓H and pTEV7DAN-GUS contain an SP6 promoter, cDNA representing the complete TEV genome, and the β-glucuronidase (GUS) coding sequence inserted between the P1 and HC-Pro coding regions (15, 23). The pTEV7DAN-G↓H plasmid contains the sequence encoding Glu-Asn-Leu-Tyr-Phe-Gln-Ser, which is recognized and cleaved by the NIa proteinase (12, 25), between the GUS and HC-Pro coding regions. The ΔP1 mutant, which lacks the entire P1 coding sequence, was prepared by digestion of pTEV7DAN-G↓H with *Nco*I at sites flanking each end of the P1 sequence, followed by religation and transformation. The S256A mutant contains a three-nucleotide mutation in pTEV7DAN-GUS, resulting in the replacement of Ala for Ser-256 at the active site of the P1 proteinase. The VNN mutant contains a mutation resulting in substitution of Val-Asn-Asn for the conserved Gly-Asp-Asp motif

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within the NIb polymerase. Both the S256A and VNN mutants were characterized previously (15, 54).

Transgenic plants were prepared by *Agrobacterium tumefaciens*-mediated transformation techniques. The 0027-1031-1B5 line was generated by introduction of sequences (TEV nucleotides 12 to 2681) coding for modified P1 and HC-Pro and a 10-kDa segment of the P3 protein. The modification in P1 resulted from a nine-nucleotide insertion encoding Thr-Met-Ala after nucleotide 615 in the nonproteolytic domain. This insertion was shown to have no effect on P1 function (54). The TEV sequence was first inserted into the intermediate vector pRTL2, which contains the cauliflower mosaic virus 35S promoter and terminator (14). The expression cassette was transferred to the binary vector pGA482 (2). The recombinant plasmid was introduced into *A. tumefaciens* LBA4404 (19), and transgenic plants were generated by the leaf-disk transformation method (36). Transgenic plants from the F_2 generation were used for these experiments.

In vitro transcription, in vitro translation, and protoplast inoculation. All transcripts were synthesized with the bacteriophage SP6 RNA polymerase (Ambion, Inc.). Full-length, m^7GpppG-capped TEV-GUS or mutant transcripts were prepared from *Bgl*II-linearized plasmids and were concentrated fivefold by precipitation in 2 M LiCl (23). Protoplasts were prepared from tobacco and inoculated with transcripts by the polyethylene glycol-mediated transfection method as previously described (13, 44).

For in vitro translation analysis, SP6 RNA transcripts were produced from *Bst*EII-linearized plasmids. Because *Bst*EII cleaves within the HC-Pro coding sequence, the resulting transcripts encoded P1, GUS, and a 14-kDa segment of HC-Pro. The transcripts were translated in wheat germ extract (Promega, Inc.) in the presence of [^{35}S]methionine (DuPont-NEN). Proteolytic processing reactions with purified NIa proteinase were performed as reported in references 46 and 54. Translation products were analyzed by sodium dodecyl sulfate (SDS)–12.5% polyacrylamide gel electrophoresis and autoradiography.

Plant inoculations and preparation of inoculum. Leaves of tobacco plants were mechanically inoculated with full-length transcripts with carborundum as an abrasive. Inocula for further experiments were prepared by grinding TEV-GUS- and ΔP1 mutant-infected leaves in 2 volumes of TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 7.6]).

Assays for virus infection. Protoplasts were harvested at 24, 48, and 72 h postinoculation (hpi) by low-speed centrifugation and resuspended in lysis buffer (40 mM sodium phosphate, 10 mM EDTA, 0.1% Triton X-100, 0.1% sodium lauryl sarcosine, and 0.07% β-mercaptoethanol [pH 7.0]). GUS activity was assayed with the fluorometric substrate 4-methylumbelliferyl-β-D-glucuronide, as described previously (13, 38). Activity values (picomoles of substrate cleaved per minute per 10^5 protoplasts) were calculated from replicates of contemporaneous samples and were compared statistically with a two-sample *t* test with the StatView 4.0 program (Abacus Concepts, Inc.). The relative GUS activities from mutant-inoculated protoplasts at 48 and 72 hpi were calculated individually as a percentage of the average GUS activities from parental TEV-GUS-infected protoplasts within the same experiment.

Virus infection in plants was assayed by several methods. Inoculated leaves were harvested 24, 48, and 72 hpi and analyzed for GUS activity in situ with the histochemical substrate 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid, cyclohexylammonium salt (X-Gluc), by the vacuum infiltration technique (23). The diameters of individual infection foci (numbers of epidermal cells) were measured microscopically. GUS activity was assayed quantitatively in upper, noninoculated leaves by the fluorometric assay, with extracts prepared by grinding leaves in 5 volumes of lysis buffer. Activity values were calculated (picomoles of substrate cleaved per minute per microgram of protein) and compared as described above. Reverse transcriptase-PCR (RT-PCR) analysis was conducted with crude virus extracts from upper, noninoculated leaves at 14 days postinfection as described previously (22, 54). For analysis of the ΔP1 mutant, the first-strand RT-PCR primer was complementary to GUS nucleotides 387 to 407, and the second-strand primer corresponded to TEV nucleotides 123 to 142 in the 5′ noncoding region. For analysis of the S256A mutant, the same first-strand primer was used, but the second-strand primer corresponded to TEV nucleotides 523 to 538.

RESULTS

In vitro translation of parental TEV-GUS and ΔP1 mutant transcripts. The ΔP1 mutant was prepared by deleting the entire P1 coding sequence from the TEV-GUS parental plasmid, pTEV7DAN-G ↓ H (Fig. 1A). Both TEV-GUS and ΔP1 mutant genomes encoded a NIa cleavage site between the GUS and HC-Pro regions of the polyprotein. To verify the translation and processing products of parental and ΔP1 mutant viruses, RNA transcripts were produced with *Bst*EII-linearized plasmid DNA and translated in a wheat germ extract. Transcripts from the parental plasmid were predicted to encode a 117-kDa P1/GUS/partial HC-Pro polyprotein, while transcripts from the ΔP1 mutant were predicted to encode an 82-kDa GUS/partial HC-Pro protein. In the absence of NIa proteinase, translation of parental TEV-GUS transcripts resulted in production of the processed 35-kDa P1 protein and the 82-kDa GUS/partial HC-Pro (Fig. 1B). Translation of ΔP1 transcripts resulted only in accumulation of the GUS/partial HC-Pro product. In the presence of NIa proteinase, the 82-kDa GUS/partial HC-Pro product encoded by both parental and mutant transcripts was processed to form the 68-kDa GUS protein (Fig. 1B).

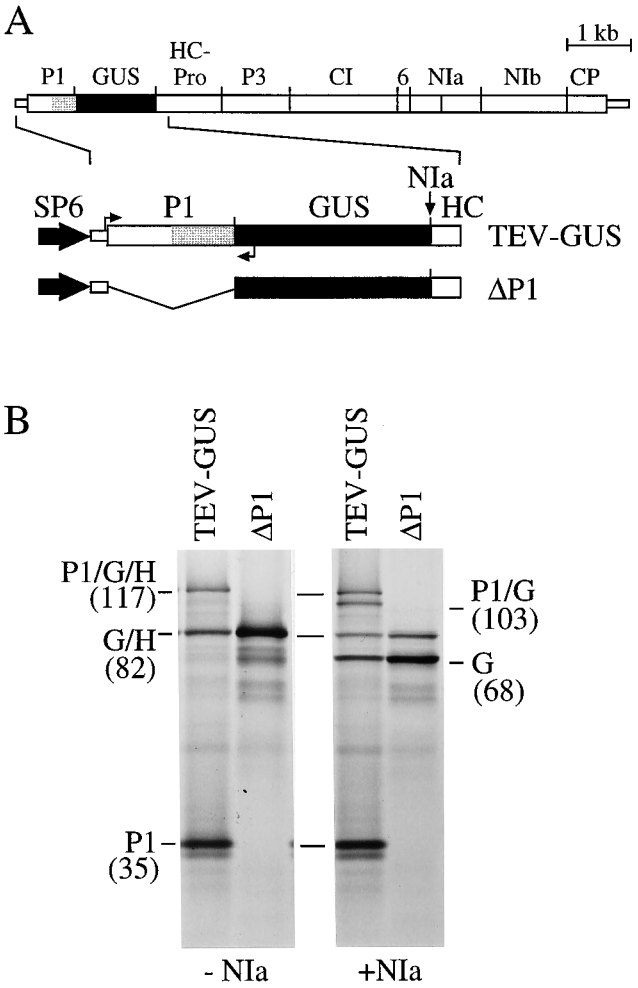

FIG. 1. In vitro translation of TEV-GUS and ΔP1 mutant transcripts. (A) Diagrammatic representation of *Bst*EII-linearized plasmids used for preparation of transcripts. The boxes indicate the TEV and GUS coding sequences. The stippled region indicates the coding sequence for the P1 proteolytic domain. The bent arrows indicate the positions corresponding to oligonucleotides used as primers for RT-PCR mixtures. The sequence coding for the introduced NIa cleavage site is indicated by the arrow between the GUS and HC-Pro regions. (B) [^{35}S]methionine-labeled translation products from TEV-GUS and ΔP1 mutant transcripts were analyzed by SDS–12.5% polyacrylamide gel electrophoresis and autoradiography. Products derived from TEV-GUS and ΔP1 transcripts in the absence (left) and presence (right) of NIa proteinase are shown. The molecular masses (in kilodaltons) of the translation and processing products are indicated in parentheses. Abbreviations: P1/G/H, P1/GUS/partial HC-Pro polyprotein; P1/G, P1/GUS polyprotein; G/H, GUS/partial HC-Pro polyprotein; G, GUS.

Infectivity of the ΔP1 mutant in protoplasts. The GUS reporter has been used as a sensitive, quantitative tool to analyze and compare genome amplification rates of parental TEV-GUS and mutant genomes (15, 20, 47). In one recent study, deletion of the N-terminal half of P1 had only modest effects on amplification in protoplasts and infectivity in plants (54). To

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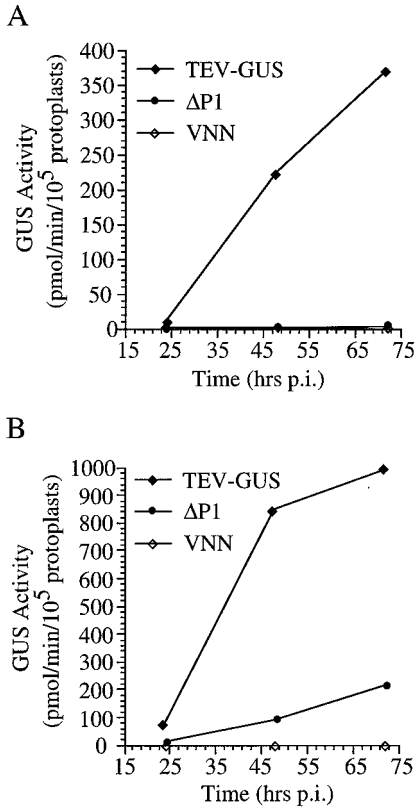

FIG. 2. Amplification of TEV-GUS and the ΔP1 mutant in nontransgenic (A) and P1/HC-Pro-expressing transgenic (B) protoplasts. GUS activity was measured at 24, 48, and 72 hpi (hrs p.i.). The VNN mutant was an amplification-negative control.

determine the effect of deleting the entire P1 sequence on genome amplification, protoplasts were inoculated with full-length parental and ΔP1 mutant genomes, and GUS activity was measured at 24, 48, and 72 hpi. As a negative control, protoplasts were inoculated with replication-defective VNN mutant transcripts, which encode an inactive NIb polymerase (15, 40). This control serves to distinguish between GUS activity from amplified genomes and activity only from translation of input transcripts. Comparisons of absolute levels of GUS activity stimulated by parental TEV-GUS and the ΔP1 mutant were conducted only between contemporaneous samples with the same preparations of protoplasts.

A slow but consistent increase in GUS activity was detected in ΔP1 mutant-infected cells over the 72-h time course (Fig. 2A). In multiple experiments, ΔP1 mutant accumulated to approximately 2 to 3% the level of parental TEV-GUS at 48 and 72 hpi (Table 1). Although debilitated, the ΔP1 mutant was significantly different from the replication-defective VNN mutant ($P = 0.0039$ at 72 hpi [Table 1]). These data suggest that efficient genome amplification requires the P1 protein and/or P1 coding sequence.

Cell-to-cell and long-distance transport of the ΔP1 mutant in plants. If the P1 protein is required for cell-to-cell movement of TEV, then inoculation of plants with the ΔP1 mutant should result in infections restricted to initially infected cells. Plants were inoculated with parental TEV-GUS or the ΔP1 mutant, and infection foci were visualized by infiltration with a GUS histochemical substrate at 24, 48, and 72 hpi. This technique was employed successfully to analyze several different

TABLE 1. Relative amplification of TEV-GUS and the ΔP1 mutant in P1/HC-Pro-expressing transgenic and nontransgenic protoplasts

Source of protoplasts	Virus	Relative amplification (±SE) at[a]:	
		48 hpi	72 hpi
Nontransgenic[b]	TEV-GUS	100	100
	ΔP1	3.2 ± 1.5	2.0 ± 0.5
	VNN	0.3 ± 0.1	0.1 ± 0.1
Transgenic[c]	TEV-GUS	100	100
	ΔP1	15.4 ± 2.0	12.7 ± 2.0
	VNN	0.4 ± 0.1	0.2 ± 0.2

[a] Relative amplification of each virus was calculated with the formula (GUS activity in sample virus-infected cells/GUS activity in TEV-GUS-infected cells) × 100.
[b] Mean values were derived with $n = 15$.
[c] Mean values were derived with $n = 9$.

TEV-GUS mutants with defects in cell-to-cell or systemic movement (20, 21, 54). The diameters (in epidermal cells) of infection foci were measured microscopically, and the cell-to-cell movement rates between 48 and 72 hpi were calculated on the basis of focus expansion. Two results were clear from this analysis. First, the ΔP1 mutant was not restricted to initially infected cells, because infection foci averaging 8.9 epidermal cells were measured at 72 hpi (Table 2). Second, the rate of cell-to-cell movement of the ΔP1 mutant was approximately 40% the rate of parental TEV-GUS movement (Table 2). In general, the intensity of the blue histochemical reaction was lower in ΔP1 mutant foci than in TEV-GUS foci, a possible indication of depressed accumulation levels at the single-cell level, as was seen in the protoplast experiments.

Systemic movement and accumulation of the ΔP1 mutant were measured by GUS activity assay with extracts from upper, noninoculated leaves. Virus was detected in 60% of plants inoculated with the ΔP1 mutant (Table 3). The ΔP1 mutant accumulated to approximately 2% the level of TEV-GUS (Fig. 3A). The identity of the virus in ΔP1 mutant-inoculated plants was verified by RT-PCR analysis. The RT-PCR primers (Fig. 1A) were predicted to amplify DNA fragments of 1346 and 407 bp from TEV-GUS and ΔP1 mutant genomes, respectively. RT-PCR products corresponding to these predicted sizes were observed with extracts from the respective plants (Fig. 3B). Furthermore, nucleotide sequence analysis of the RT-PCR products from two independent ΔP1 mutant-infected plants confirmed that progeny virus in systemic leaves lacked the entire P1 coding sequence (data not shown). These results indicate that the P1 protein is not required for cell-to-cell or systemic movement.

Complementation of P1 mutants in transgenic cells. The ΔP1 mutant exhibited partially debilitated genome amplification and slow cell-to-cell movement phenotypes. In contrast, the S256A mutant, which encodes a processing-defective P1 proteinase, was shown to be nonviable in a previous study (54). The ΔP1 and S256A mutant phenotypes may be due to defects in *cis*-acting or *trans*-acting functions. A *cis*-acting function could be one in which the RNA sequence itself was active, in which the P1 protein acted together with other proteins synthesized within the same polyprotein, or in which the P1 protein was active preferentially in association with the RNA serving as the translational template. To distinguish between *cis*- and *trans*-acting defects in the P1 mutants, a complementation assay was performed in which transgenic plants or protoplasts expressing the P1 protein were inoculated. The trans-

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TABLE 2. Cell-to-cell movement of TEV-GUS and the ΔP1 mutant in P1/HC-Pro-expressing transgenic and nontransgenic plants

Plant	Virus	Infection focus diam (±SE) at[a]:			Rate of cell-to-cell movement (cells/h)[b]
		24 hpi	48 hpi	72 hpi	
Nontransgenic	TEV-GUS	1.3 ± 0.1	6.7 ± 0.2	17.8 ± 0.3	0.46
	ΔP1	—[c]	4.5 ± 0.6	8.9 ± 0.4	0.18
Transgenic	TEV-GUS	1.2 ± 0.1	5.4 ± 0.2	13.2 ± 0.5	0.33
	ΔP1	—	2.8 ± 0.2	6.3 ± 0.5	0.14

[a] Infection focus diameter (number of epidermal cells) was measured microscopically. The *n* value for each mean ranged between 14 and 88.
[b] The rate of cell-to-cell movement was calculated between 48 and 72 hpi.
[c] —, no infection foci were identified.

genic plants encoded both P1 and HC-Pro, the latter of which accumulated to approximately 10% the level of HC-Pro in TEV-GUS-infected plants (18). *trans*-Active functions of two other TEV proteins have been demonstrated by this approach (20, 21, 40).

Amplification of the TEV-GUS and ΔP1 mutant genomes in transgenic and nontransgenic protoplasts was analyzed in parallel. The ΔP1 mutant was amplified to a level approximately 12 to 15% that of TEV-GUS over the 72-h time course in transgenic cells, compared with 2 to 3% in nontransgenic cells. This represented a five- to sixfold relative enhancement of ΔP1 mutant accumulation (Fig. 2 and Table 1). The VNN mutant transcripts failed to stimulate GUS activity in either transgenic or nontransgenic cells (Table 2).

Cell-to-cell movement and systemic movement were assayed in transgenic plants inoculated with TEV-GUS and the ΔP1 mutant. No relative stimulation of the ΔP1 cell-to-cell movement rate was observed in transgenic plants. In fact, the rates of movement of both parental TEV-GUS and the ΔP1 mutant were slightly slower in transgenic plants than in nontransgenic plants (Table 2). Accumulation of the ΔP1 mutant in systemic leaves, however, was significantly increased in transgenic plants compared with nontransgenic plants ($P = 0.0001$), reaching approximately 40% the level of TEV-GUS (Fig. 3A). RT-PCR analysis was conducted to verify that the increased virus accumulation in ΔP1 mutant-inoculated transgenic plants was actually due to the ΔP1 mutant rather than to a recombinant virus that had acquired the P1 coding sequence from the transgenic mRNA. Only the short, ΔP1-specific RT-PCR product was identified after reactions with extracts from transgenic plants (Fig. 3B), indicating that the stimulatory effect in transgenic plants was due to P1 supplied in *trans* rather than to recombination.

The S256A mutant was also tested in transgenic plants. Systemic infection was assayed by immunoblot analysis with anti-capsid serum and by RT-PCR. The GUS reporter was not used in these experiments because of the enzyme-inactivating effect of fusing P1 to the N terminus of GUS (54), as is the case with the S256A mutant. The S256A mutant failed to infect nontransgenic plants. However, this mutant infected 90% of the transgenic plants inoculated (Table 3). Viral RNA was detected in virion preparations from TEV-GUS and S256A mutant-infected transgenic leaves (Fig. 4). Nucleotide sequence analysis of RT-PCR products from two independent S256A mutant-infected leaves revealed that the proteinase-inactivating mutation was retained in the progeny virus (data not shown.) These data indicate that P1 protein supplied in *trans* can stimulate or rescue two types of P1-defective mutants.

TABLE 3. Systemic infection of TEV-GUS and P1 mutants in P1/HC-Pro-expressing transgenic and nontransgenic plants

Plant[a]	Virus	No. infected/ no. inoculated[b]	Symptom appearance (days postinfection)
Expt 1			
Nontransgenic	TEV-GUS	10/10	4
	ΔP1	6/10	7
Transgenic	TEV-GUS	9/10	5
	ΔP1	8/10[c]	5
Expt 2			
Nontransgenic	TEV-GUS	4/5	6
	S256A	0/10	
Transgenic	TEV-GUS	5/5	6
	S256A	9/10[c]	8–9

[a] Data from two independent experiments are shown. Inocula for the first experiment were derived from TEV-GUS- and ΔP1 mutant-infected plants. Inocula for the second experiment were RNA transcripts.
[b] Infected plants were identified by appearance of symptoms and GUS activity (first experiment) or by immunoblot analysis with anti-capsid serum (second experiment), in upper leaves. Infection of several plants was also confirmed by RT-PCR analysis.
[c] The sequences of RT-PCR products from two ΔP1 mutant- and two S256A mutant-infected transgenic plants were determined in order to confirm the presence of the respective deletion and point mutations.

DISCUSSION

Deletion of the entire P1 coding sequence from TEV-GUS considerably reduced genome amplification. The reduced amplification phenotype, however, had only minor effects on cell-to-cell and long-distance transport. These data indicate that TEV can establish a systemic infection despite substantially debilitated replication at the cellular level. The extensive speculation about a direct movement function of the P1 protein (see the introduction) appears now to have little basis, although a movement-enhancing function cannot be excluded.

In contrast with the ΔP1 mutant, the S256A mutant was shown previously (54) and in this study to be nonviable in plants. The S256A mutant encodes a proteolytically nonfunctional P1 proteinase. Insertion of a cleavage site recognized by a heterologous proteinase (NIa) between P1 and HC-Pro restored infectivity to the S256A mutant, strongly suggesting that proteolytic separation of P1 from HC-Pro is a critical event (54). Those experiments also revealed that the P1 proteolytic activity is not required beyond its role in P1/HC-Pro processing. Comparison of the infection phenotypes of the ΔP1 and S256A mutants further suggests that lack of proteolytic separation of P1 and HC-Pro is more detrimental than deletion of the P1 sequence altogether. These data can be rationalized by proposing that P1 exerts a negative effect on one or more

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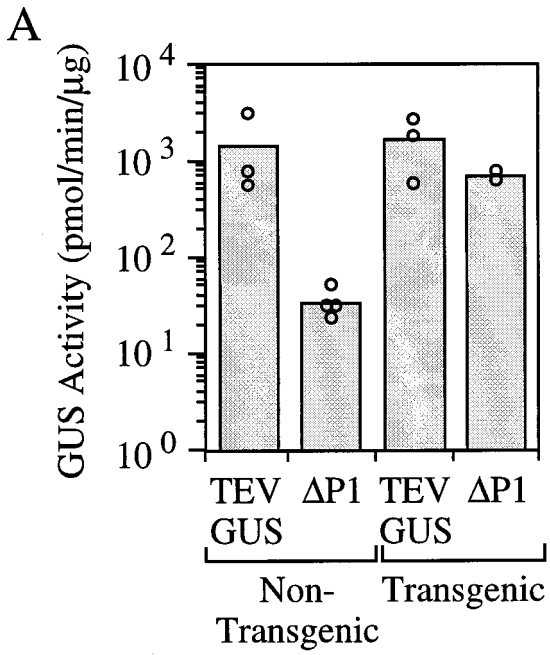

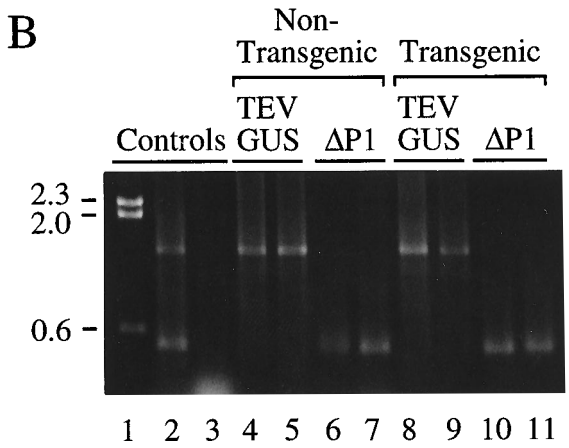

FIG. 3. Systemic infection of TEV-GUS and the ΔP1 mutant in nontransgenic and P1/HC-Pro-expressing transgenic plants. (A) Virus accumulation was measured indirectly by GUS activity assay with extracts from the second leaf above the inoculated leaf in sets of plants at 10 days postinfection. Individual samples are indicated by open circles. (B) RT-PCR analysis with extracts from virus-infected plants. The positions corresponding to RT-PCR primer sites are indicated in Fig. 1A. Lanes: 1, DNA size standards; 2 to 11, RT-PCR products from combined extracts from TEV-GUS- and ΔP1 mutant-infected transgenic plants (lane 2), a noninfected, nontransgenic plant (lane 3), TEV-GUS-infected nontransgenic plants (lanes 4 and 5), ΔP1 mutant-infected nontransgenic plants (lanes 6 and 7), TEV-GUS-infected transgenic plants (lanes 8 and 9), and ΔP1 mutant-infected transgenic plants (lanes 10 and 11). Sizes (in kilobase pairs) of DNA standards are indicated at the left.

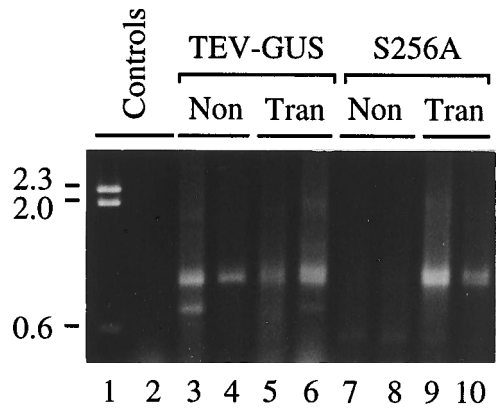

FIG. 4. RT-PCR products from extracts of nontransgenic (Non) and P1/HC-Pro-expressing transgenic (Tran) plants inoculated with TEV-GUS or the S256A mutant. Lanes: 1, DNA size standards; 2 to 10, RT-PCR products from a noninfected, nontransgenic plant (lane 2), TEV-GUS-infected nontransgenic plants (lanes 3 and 4), TEV-GUS-infected transgenic plants (lanes 5 and 6), S256A mutant-inoculated nontransgenic plants (lanes 7 and 8), and S256A mutant-infected transgenic plants (lanes 9 and 10). Sizes (in kilobase pairs) of DNA standards are indicated at the left. Note that the primer set used for these reactions was different from the set used for Fig. 3B, resulting in shorter parental RT-PCR products.

HC-Pro activities when both proteins are linked within the same polyprotein. HC-Pro is known to be necessary for efficient genome replication and long-distance movement within plants (6, 7, 18, 39).

The suppressed amplification phenotype of the ΔP1 mutant in protoplasts was stimulated five- to sixfold in transgenic cells expressing P1 and HC-Pro. It is unlikely that this activity resulted from the transgenic HC-Pro, because several amplification-depressed TEV mutants with mutations affecting HC-Pro were not stimulated in the same cells (unpublished observations). These data suggest that the P1 protein functions as a *trans*-acting accessory or regulatory factor to stimulate genome amplification. The P1 protein might function directly in the RNA replication process by interacting with enzymatic components of replication complexes or with RNA sequences. On the other hand, P1 may function indirectly in genome amplification by stimulating translation of viral RNA. On the basis of genetic data, such a role was proposed for the 2A proteinase of picornaviruses (32, 41). The RNA binding activity of P1 (8) may function in this proposed accessory role. The fact that amplification of the ΔP1 mutant was not rescued to parental virus levels may have been due to relatively low levels of P1 supplied by the transgenic cells. Alternatively, the RNA sequence lacking in the ΔP1 mutant may possess *cis*-acting features necessary for optimal genome amplification.

In addition to stimulating RNA amplification at the cellular level, the transgenic protein also stimulated systemic infection of both the ΔP1 and S256A mutants in whole plants. In the case of ΔP1, the increased accumulation was probably due to increased amplification within each infected cell, similar to the effect measured in protoplasts, rather than to more rapid movement. In the case of the S256A mutant, complementation may have resulted from the activities of both P1 and HC-Pro supplied by the transgenic plants.

Which part of P1 is necessary for the *trans*-active accessory function? Deletion of the nonproteolytic N-terminal domain resulted in viruses that were amplified at approximately 20% the level of parental virus (54). Deletion of the entire P1 sequence resulted in a further reduction of amplification to 2 to 3% the level of parental virus. These combined data could reflect a requirement for both proteolytic and nonproteolytic domains in the proposed transactivation function. An emerging theme with proteinases encoded by positive-strand RNA viruses is their multifunctional nature. The picornavirus 2A and 3C proteinases function as RNA-binding proteins active in either translation or RNA replication (3, 4, 32, 41). The alphavirus nsP2 protein possesses both proteinase- and helicaselike domains and plays a specific role in subgenomic RNA synthesis (29, 31, 33, 35, 50). The alphavirus capsid protein, which forms a T=4 structure (17), contains RNA-binding and chymo-

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trypsinlike proteinase domains (28, 30). In addition, the P1, HC-Pro, and NIa proteinases of potyviruses each contain multiple domains involved in proteolytic, RNA replication, intercellular movement, or insect transmission functions (26).

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