Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 11919–11923, December 1993
Agricultural Sciences

Mutational analysis of the helper component-proteinase gene of a potyvirus: Effects of amino acid substitutions, deletions, and gene replacement on virulence and aphid transmissibility

(chimeric plant virus/insect transmission)

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Communicated by Robert J. Shepherd, September 7, 1993 (received for review June 5, 1993)

ABSTRACT **We have previously provided evidence that amino acid substitutions within the N-terminal portion of the helper component-proteinase (HC-Pro) from tobacco vein mottling virus (TVMV), in particular at Lys-307, not only affect the aphid transmission activity of HC-Pro but also have a significant effect on TVMV virulence. In the present study amino acids which differ in their charge properties were substituted at position 307. A highly basic residue was required to retain helper component activity and virulence. Deletion and insertion mutagenesis in the 5′ terminus of the HC-Pro gene suggested that this RNA domain may be an essential element for TVMV infectivity. Replacement of the TVMV HC-Pro gene with that from another potyvirus, zucchini yellow mosaic virus, maintained infectivity and aphid transmissibility of the chimeric virus, although symptoms were attenuated. Our results suggest that, in addition to its importance in aphid transmission, the HC-Pro gene may be of general importance in regulating virulence of potyviruses, possibly by interaction of these sequences with the host.**

Tobacco vein mottling virus (TVMV) is a well-studied member of the potyvirus group of plant viruses transmitted in nature by specific vectors, the aphids. The TVMV genome comprises a single-stranded positive-sense RNA of approximately 9400 bases with a coding capacity for a single polyprotein of about 340 kDa. Amino acids 257–713 of the TVMV polyprotein (1) represent a nonstructural protein, the helper component-proteinase (HC-Pro) (2), which mediates virus transmission by aphids (3). Previous studies have established that mutations within the 5′-terminal portion of the TVMV HC-Pro gene not only affect the aphid transmission activity of HC-Pro but also have a significant effect on virulence of the virus (4). In the present study we have used several approaches to further understand how sequences within the 5′ terminus of the HC-Pro gene affect these properties.

Comparisons of deduced amino acid sequence have suggested that lack of aphid transmission activity of HC-Pro is correlated with a Lys-to-Glu amino acid change within a block of conserved sequence of the N terminus in potato virus C (PVC) (5) and in a non-aphid-transmissible strain of zucchini yellow mosaic virus (ZYMV) (6). By site-directed mutagenesis studies (4), we have provided direct evidence for the deleterious effect of a Glu residue at this position in TVMV HC-Pro (residue 307) on aphid transmission activity. On the basis of these studies, we proposed that loss of aphid transmission activity resulted from the charge difference engendered by the change from Lys (highly basic) to Glu (highly acidic) at position 307. In the present study we substituted amino acid residues with differing charge properties at position 307 to test this hypothesis.

The N terminus of all potyviral HC-Pros, including that of TVMV, contains a His and several Cys residues that are conserved and have been postulated to form a "zinc finger-like" motif (7). Cys-rich motifs of viral proteins are known to form tertiary structures having functional roles (8). To test whether these His and Cys residues have a role in TVMV helper component activity as well as in virulence, we replaced His and Cys residues with Ser residues in the present study.

While our mutational studies on TVMV HC-Pro (4) have suggested a role for its N terminus in maintaining the normal virus phenotype, Dolja *et al.* (9) have reported that in the case of another potyvirus, tobacco etch virus (TEV), deletions in the N terminus of HC-Pro were tolerated. Such deletions originated spontaneously when a β-glucuronidase (GUS) coding sequence was inserted near the 5′ terminus of the TEV HC-Pro coding sequence (9). This suggests that the 5′ terminus of the TEV HC-Pro gene is not vital for virus survival. To further investigate this apparent discrepancy between TVMV and TEV, we inserted a GUS coding sequence near the 5′ terminus of the TVMV HC-Pro-encoding region. We also introduced specific deletions in the TVMV HC-Pro gene 5′-terminal region as well as a deletion that removes almost the entire HC-Pro gene.

Given the apparent importance of the TVMV HC-Pro gene/gene product in symptomatology and virulence, we substituted HC-Pro from another potyvirus, ZYMV, which differs from TVMV in its effect on *Nicotiana* species, into the TVMV genome. The effect of the heterologous HC-Pro on virus infectivity and virulence as well as on helper component activity was then determined.

MATERIALS AND METHODS

Construction of TVMV Genome-Length Plasmids Representing Amino Acid Substitutions in HC-Pro. Individual site-directed mutations corresponding to amino acid positions 280 (His to Ser), 282 (Cys to Ser), 291 (Cys to Ser), 305 (Cys to Ser), 307 (Lys to Lys with a codon change, or to His, Gln, or Arg), and 310 (Cys to Ser) in the TVMV polyprotein were created in the present study essentially according to Kunkel *et al.* (10). The Lys-to-Glu mutation at position 307 had been created previously (4). Full-length pTVMV-WT [pXBS7 of Domier *et al.* (11)] containing the mutation(s) was subse-

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Abbreviations: TVMV, tobacco vein mottling virus; ZYMV, zucchini yellow mosaic virus; PVC, potato virus C; TEV, tobacco etch virus; HC-Pro, helper component-proteinase; GUS, β-glucuronidase; p.i., post inoculation.

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quently reconstructed as described by Atreya *et al.* (4). Mutations were confirmed by sequencing the double-stranded plasmid DNA. Plasmids representing nucleotide changes corresponding to amino acid substitutions at residue 307 are designated pTVMV-307K_{AAG}, -307H, -307Q, and -307R, and the Ser substitutions as pTVMV-280S, -282S, -291S, -305S, and -310S. The positions and the nature of the amino acid changes in the TVMV polyprotein are illustrated in Fig. 1.

Construction and Transcription of Genome-Length Plasmids Containing HC-Pro Deletions, a GUS Insertion, and a ZYMV HC-Pro Substitution. Defined deletions were made in the plasmid PB1933 (4), producing a partial HC-Pro gene that would result in a truncated gene product with a deletion of 15 or 75 amino acid residues. Another deletion of 452 residues, representing almost the entire HC-Pro, was targeted in the plasmid pES6 (4). The deletions were generated by altering the coding sequences for the dipeptides Asp-Ile (DI), Ala-Ile (AI), Arg-Ile (RI), and Val-Gly (VG) at positions 260–261, 275–276, 335–336, and 712–713 of the polyprotein to *Eco*RV restriction sites by an inverse PCR as previously described (13), followed by *Eco*RV digestion and ligation of appropriate PCR products (Fig. 2*A*). The *Eco*RV enzyme digest resulted in cleaving the nucleotide sequence between the codons for the Asp-Ile (DI) dipeptide so that the reading frame was unaltered. The cloned PCR products were sequenced to confirm the specific deletions as well as to search for any potential artifacts introduced during the PCR. Genome-length plasmids containing the specific deletions with no other sequence alterations were created by using pPB1933 or pES6 as described (4).

A DNA fragment representing the GUS coding region was engineered by PCR to have *Sma* I and *Pvu* II sites at the 5′ and 3′ ends, respectively. The PCR product was then digested with *Sma* I and *Pvu* II and inserted into an *Eco*RV-digested pPB1933 in which an *Eco*RV site had been created earlier, without changing the amino acid sequence at position 260–261; this manipulation added a Gly residue to the N terminus of GUS (Fig. 2*B*).

A chimeric viral RNA encoding a transmission-functional ZYMV HC-Pro (which has a Lys in the same context as that of Lys-307 of TVMV) was created in the TVMV background by substituting a cDNA copy of the ZYMV HC-Pro gene into the TVMV genomic cDNA-containing plasmid pTVMVΔ452HC-Pro. Compatible ends containing *Eco*RV sites [recognition sequence codes for the dipeptide Asp-Ile (DI)] were generated by PCR, using the ZYMV HC-Pro gene cDNA-containing plasmid. The PCR fragment was digested and inserted into pTVMVΔ452HC-Pro partially digested with *Eco*RV (there are two such sites in this plasmid) in such a way that in terms of amino acid sequence, ZYMV HC-Pro residues begin at the sixth residue from the N terminus of the hybrid protein. This manipulation does not alter the P1 and HC-Pro protease processing sites, although it adds a tripeptide (Gly-Asp-Ile) sequence to the N terminus of the P3 protein (Fig. 2*C*).

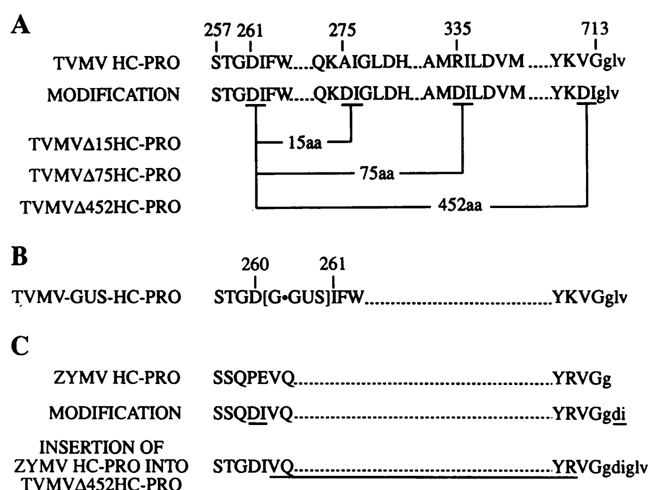

FIG. 2. Diagrammatic representation of deletions, GUS insertion, and ZYMV HC-Pro substitution in the TVMV polyprotein. The underlined amino acid residues (DI) represent the corresponding *Eco*RV hexanucleotide recognition sequences in the gene. Broken lines represent intermediate amino acid residues not involved in the manipulations. (*A*) TVMV HC-Pro wild-type sequence. Lowercase letters represent amino acids in the adjoining P3 protein. The numbers above the sequence represent the position of the first amino acid of the dipeptide for which corresponding nucleotides were changed to create *Eco*RV sites. The locations of the resultant 15-, 75-, and 452-residue deletions are shown below. (*B*) Position of the GUS insertion (shown in parentheses) into TVMV HC-Pro. The GUS N terminus has a Gly (G) residue as a result of the manipulation. (*C*) Substitution of the ZYMV HC-Pro sequences into the TVMV polyprotein as a result of the corresponding gene manipulations, details of which are described in the text. The underlined sequence in the hybrid represents sequences originating from the ZYMV HC-Pro. Note that a GDI (Gly-Asp-Ile) tripeptide was added to the N terminus of the P3 protein as a result of the manipulation.

Transcription reactions from linearized plasmids and plant inoculations were as described (14). Products of the transcription reaction were stored at −70°C until use. Tobacco (*Nicotiana tabacum* cv. Kentucky 14) was inoculated unless otherwise noted. Plants were assayed 7–12 days postinoculation (p.i.) by immunodiffusion, immunoblot analysis of SDS/PAGE-separated proteins, and ELISA (15, 22), to detect the presence of TVMV. Sequencing of PCR-amplified virus-specific RNA from plants inoculated either mechanically or by aphid transmission was carried out according to Atreya *et al.* (16).

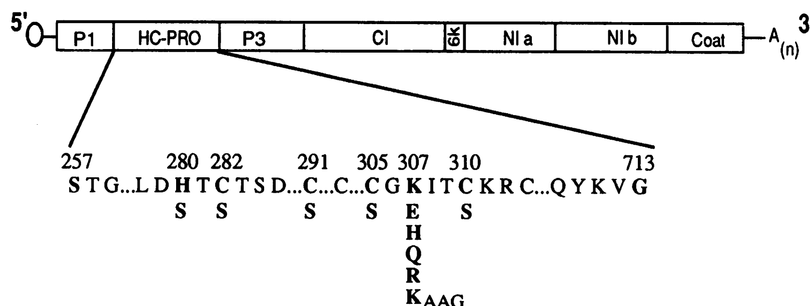

FIG. 1. Genetic map of TVMV, showing the positions of the site-directed mutations in HC-Pro tested in this study. The single open reading frame is depicted by the elongated box, and vertical lines in the box delineate individual processed proteins as described (12). The open circle at the 5′ end represents the viral genome-linked protein (VPg). The numbers above the amino acids (in single-letter code) indicate their location in the TVMV polyprotein and the single letters below the sequence show the identity of the substituted residue at that position. K_{AAG} represents the codon change for Lys.

Assay of Helper Component Activity. For each mutant, at least three aphid transmission experiments were conducted, using infected plants as the source of virus and HC-Pro. Three or more additional transmission experiments were done with partially purified HC-Pro preparations. HC-Pro protein was isolated from the infected plants by following a protocol which preserves helper component activity (3). Purified virus was mixed with the HC-Pro, and transmission assays were conducted as described previously (15). In the case of mutants that produced low amounts of HC-Pro, highly concentrated protein was used for transmission assays and the relative HC-Pro concentrations used in the assay mixture were determined by quantitative Western blot analyses as described before (4). Procedures for rearing and handling aphids and assay plants have been described (17), and transmission assays were carried out as described previously (16).

RESULTS

Effect of Mutations at Amino Acid Position 307. In a previous investigation (4), a change from Lys to Glu at position 307 in the TVMV polyprotein abolished HC-Pro transmission activity and also reduced virus accumulation and symptom expression over a 4-week period p.i. Further mutations at this position (Table 1), made in the present study, showed that changing the highly basic Lys to His (slightly basic) or Gln (neutral) destroyed the transmission activity of HC-Pro and resulted in attenuated symptom phenotype and reduced virus accumulation (Table 1) in a manner similar to the TVMV-307E mutation. Since the virus levels decreased rapidly in plants infected with TVMV-307H and TVMV-307Q, HC-Pro was isolated, concentrated, and tested for transmission activity by *in vitro* acquisition assays (4) to rule out the possibility that lack of transmission from the mutant transcript-infected leaves was due to low levels of virus and/or HC-Pro. As shown in Fig. 3, HC-Pro isolated from TVMV-307H- and -307Q-infected plants did not have helper component activity even when tested at concentrations higher than those at which HC-Pro from TVMV-infected plants had high activity.

In contrast, when the Lys residue was changed to Arg, which is also a highly positively charged residue, or when the codon for the Lys at this position was changed, the mutants retained helper component transmission activity and the wild-type virus phenotype (Table 1).

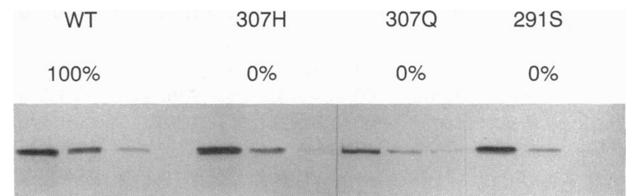

FIG. 3. Immunoblot showing the comparative amounts of HC-Pro used in aphid transmission assays. HC-Pro isolated from leaves infected with the TVMV-307H, TVMV-307Q and TVMV-291S mutants was suspended in a minimum volume of buffer, and a series of dilutions (1:1, 1:5, and 1:10) were compared with dilutions (1:5, 1:10, and 1:20) of TVMV wild-type (WT) HC-Pro. The blot was probed with anti-TVMV HC-Pro, and the band in each lane reflects the amount of partially purified HC-Pro added to purified virus for aphid transmission tests. TVMV-WT HC-Pro mediated 100% transmission at all the concentrations depicted, while HC-Pro from the mutants did not mediate transmission at any of the concentrations depicted.

Substitution of Ser for His and Cys Residues in the Putative Zinc-Finger Motif of TVMV HC-Pro. Individual substitutions of Ser residues in place of His-280, Cys-282, Cys-291, and Cys-310 in TVMV HC-Pro had profound effects on virulence. The mutant transcript containing the His-to-Ser substitution (TVMV-280S) was noninfectious, while in plants inoculated with TVMV-282S or TVMV-310S transcripts, virus could not be detected by ELISA or PCR at periods up to 4 weeks p.i. Plants inoculated with TVMV-291S behaved in a manner similar to TVMV-307H and -307Q; the virus titer decreased significantly between 1 and 4 weeks p.i. (Table 1), and HC-Pro isolated and concentrated from plants infected with the 291S mutant did not support virus transmission by aphids (Fig. 3). We were unable to create a Cys-to-Ser change at amino acid position 313 despite eight separate site-specific mutagenesis reactions done at different times, and thus the importance of Cys-313 remains unknown. The mutation of Cys to Ser at position 305 (TVMV-305S) had no effect on HC-Pro activity; virus accumulation and symptom expression were similar to the wild type.

Stability of Mutants. Between 5 and 6 weeks p.i., plants infected with TVMV-307E, which previously were asymptomatic, began to develop blotches on newly emerging leaves. This was followed by the development of typical TVMV symptoms on subsequently emerging leaves. Virus accumulation in these leaves, as estimated by ELISA, was similar to that of the wild type, and aphids readily transmitted TVMV from these leaves (data not shown). Nucleotide

Table 1. Effect of amino acid substitutions in HC-Pro on TVMV phenotype

Position and nature of amino acid	Aphid transmission, %		Symptom expression (1–4 wk p.i.)	Relative conc. of virus*		Mutation reverted (5–6 wk p.i.)
	From infected plant	With isolated HC-Pro		wk 1	wk 4	
Wild type	70	90	Wild-type	1.00	1.00	—
307K†	90	NT	Wild-type	0.80	1.07	No
307E‡	0	0	None	0.33	0.02	Yes
307Q	0	0	None	0.29	0.02	No
307H	0	0	None	0.25	0.02	No
307R	90	80	Wild-type	0.96	1.08	No
280S	NA	NA	None	ND	ND	No
282S	NA	NA	NA	ND	ND	Yes
291S	0	0	None	0.28	0.02	No
305S	70	70	Wild-type	1.07	1.04	No
310S	NA	NA	None	ND	ND	Yes

NT, not tested, since transmission from infected plant is high; NA, not applicable, since infection was not detected (for details refer to appropriate text); ND, not detectable.

*Tested by ELISA from systemically infected tissue; values represent the ratio of mutant to wild type at each time period.

†Lysine with codon changed from AAA to AAG.

‡This mutant was from Atreya *et al.* (4).

sequencing revealed that the Glu substitution at position 307 had reverted to the wild-type Lys in the symptomatic areas. The reversions occurred in each of over 30 plants from five different experiments. No reversion to wild-type RNA sequence or symptoms was found to occur with the 307Q or 307H mutants held for a similar length of time (Table 1).

In the case of the Ser for Cys or His substitutions, reversions similar to those described for the 307E mutation occurred with the 282S and 310S mutants, while the other mutants did not appear to revert (Table 1), as determined by reverse transcriptase/PCR sequencing of viral RNA from plants 6 weeks after inoculation.

TVMV Transcripts with a GUS Fusion or Deletions in HC-Pro. Transcripts which encoded the GUS fusion near the N terminus of HC-Pro as well as deletions of 15, 75, or 452 amino acids in the HC-Pro (Fig. 2) were unable to infect tobacco (cvs. Kentucky 14 and Xanthi) or *Nicotiana benthamiana*, despite extensive testing. These TVMV transcripts were also inoculated onto transgenic burley tobacco plants which express the first three genes of TVMV (15) to test whether the transgenic HC-Pro gene or its product would complement the TVMV transcripts with deletions in the HC-Pro gene. Even after 6 weeks, the plants inoculated with these transcripts were negative for the presence of TVMV, as determined by ELISA. These experiments were repeated at least three times, and the negative results were consistent. The failure of the transgenic tobacco plants expressing the HC-Pro gene and gene product to complement the deletion mutants could be due to an insufficient level or location of expression. Alternatively, the HC-Pro gene sequences must be present in cis for infection to occur.

Substitution of ZYMV HC-Pro in TVMV. To test whether the infectivity and aphid transmissibility of TVMV could be maintained by providing a heterologous HC-Pro gene, a cDNA copy of the ZYMV HC-Pro was substituted into the TVMV genome. The chimeric HC-Pro contained N-terminal residues from TVMV to maintain the TVMV P1 proteolytic junction (Fig. 2*B*). The resultant transcripts readily infected *N. benthamiana*, although the symptoms produced were less severe than those produced by TVMV (Fig. 4).

The chimeric nature of the virus was verified by PCR. Total RNA was isolated from uninfected, TVMV-infected, and chimera-infected *N. benthamiana* plants and from ZYMV-infected zucchini. A primer specific to the 3′ end of the ZYMV HC-Pro gene was used to synthesize cDNAs from 1 μg of total RNA isolated from infected and control plants. PCR was then performed on each sample, using this 3′ primer and a primer specific to a sequence at the 5′ end of the TVMV HC-Pro gene. A chimera-specific PCR DNA fragment of the expected size (1.3 kb) was amplified only from RNAs isolated from plants infected with the TVMV-ZYMV chimera (Fig. 5). Immunoblot analysis of chimera-infected *N. benthamiana* leaf tissue demonstrated the normal processing of ZYMV HC-Pro from the TVMV polyprotein, and helper component from chimera-infected plants was able to support virus transmission by aphids (data not shown).

FIG. 4. Symptoms produced by TVMV and the TVMV-ZYMV chimera on *N. benthamiana* plants. On the left is a TVMV-infected plant showing severe symptoms, leaf distortion being most prominent. On the right is a chimera-infected plant with attenuated symptoms.

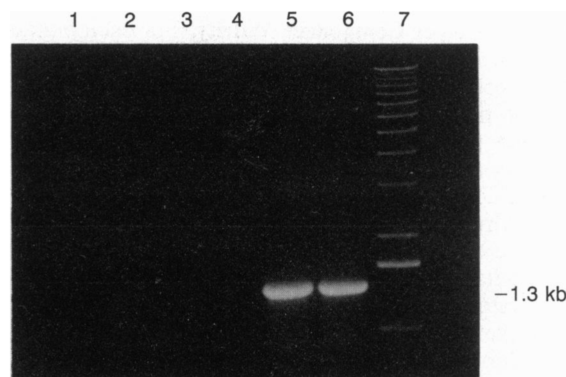

FIG. 5. PCR amplification profile demonstrating the chimeric nature of TVMV-ZYMV RNA isolated from infected plants. Lane 1, *N. benthamiana* (uninfected); lane 2, zucchini (uninfected); lane 3, *N. benthamiana* (TVMV); lane 4, zucchini (ZYMV); lane 5, *N. benthamiana* (chimera); lane 6, *N. tabacum* (chimera); and lane 7, DNA size markers.

The chimeric transcripts themselves did not infect tobacco, but tobacco could be infected by inoculation with sap from *N. benthamiana* plants infected with the chimeric virus (Fig. 5, lane 6). Tobacco infected with the chimeric virus displayed an attenuated symptom phenotype and reduced virus accumulation similar to that previously described for some of the HC-Pro mutations.

DISCUSSION

Two naturally occurring potyviruses, PVC and ZYMV (5, 6), have a Glu residue in place of Lys at positions in their polyproteins comparable to TVMV-307E (4), and their HC-Pro proteins cannot mediate virus transmission by aphids. Thus it seems that a highly basic residue such as Lys or Arg at this position in HC-Pro plays a key role in potyvirus helper component activity, as does the Gly of the DAG motif in the N terminus of the potyviral coat protein (16, 17) in determining virion transmissibility. In the case of TVMV, replacement of the Lys residue also results in a selective disadvantage as evidenced by the fact that the Lys-307 to Glu substitution eventually reverts to the wild-type Lys 5–6 weeks after infection.

It is evident from our data, obtained with TVMV, that there are other critical residues within the conserved N terminus that may affect the transmission activity of HC-Pro. One such example is residue 291, where the loss of helper component activity occurred with TVMV-291S. Since this mutation is well within the HC-Pro N terminus, it is conceivable that the mutation may affect whatever tertiary structure the Lys-307 may impart to TVMV helper component activity by way of Cys coordination as discussed before (4). In fact the Ser substitutions in the putative finger motif of the N terminus suggest that if His and Cys residues participate in HC-Pro structure, the most likely ones are His-280, Cys-282, Cys-310, and, by analogy with other viral protein fingers, Cys-313. These could form a finger, $HXCX_{27}CX_2C$, similar to the structures observed in other viral proteins such as the E6 protein of papilloma viruses (PV), $CX_2CX_{29}CX_2C$ or

$CX_2CX_{34}CX_2C$ and the Tax protein of human T-cell leukemia virus type I (HTLV-I), $CX_5CX_{13}CX_5C$ or the alternate structure $CX_6CX_{12}CX_2H$ (18, 19). Although there is no experimental evidence, such as is the case for the PV and HTLV-I proteins, that a metal ion coordinates this structure in potyviruses, it has been established that magnesium ion-containing buffers maintain the helper component activity in *in vitro* assays of aphid transmission (20). In the case of TEV, helper component activity was found to be lost when the deletions in the HC-Pro included this Cys motif (21). It has recently been found that in another potyvirus, ZYMV, a change from Thr to Ala in the C-terminal half of HC-Pro within the Phe-Thr-Lys conserved motif (5) also greatly reduces transmission activity of HC-Pro (B. Raccah, personal communication). Overall these studies suggest that a specific structure or folding of HC-Pro is necessary for its transmission activity.

The lack of infectivity of the TVMV transcripts containing the GUS insertion or deletions in the HC-Pro coding region is somewhat paradoxical. On one hand, this result does not seem surprising, in view of the importance of sequences in this region for TVMV virulence and viability, as discussed here and previously (4). On the other hand, transcripts containing the GUS insertion and resultant progeny virions containing deletions in the N terminus of the HC-Pro were infectious in the case of another solanaceous potyvirus, TEV (9, 21). One explanation for this discrepancy is that it is not the HC-Pro protein or its N terminus *per se* that is involved in infectivity of the RNA, but that the nucleotide sequence of the HC-Pro gene interacts with specific host component(s) that function in the infection process. It is reasonable to speculate that in a given potyvirus/host combination, a specific sequence within the HC-Pro gene recognizes and interacts with a host component(s). For instance, in the TVMV HC-Pro gene, the sequence corresponding to the HC-Pro N terminus might be the host-interacting domain, whereas in the case of TEV, it might be the sequence located downstream of the N terminus. This could explain why the deletions in the N terminus of TEV HC-Pro which occur as a result of GUS insertion survived (9, 21), whereas in the case of TVMV, when the critical HC-Pro N-terminal sequences are either disrupted by GUS insertion or removed by directed deletions, nonviability of the TVMV transcript results. One observation that is consistent, however, is that the site-directed mutations in the N terminus of TVMV HC-Pro described here and previously (4) and the spontaneous deletions in the N terminus of TEV HC-Pro (21) both result in a significant reduction in virus concentrations.

The TVMV-ZYMV chimera is, to our knowledge, the first such infectious potyvirus combination to be reported. The reason the chimeric transcripts infect *N. benthamiana* but not *N. tabacum*, while the progeny virions from *N. benthamiana* can infect *N. tabacum*, is not clear at this time. It could be that *N. benthamiana*, which is a host for both viruses, is more sensitive to the level of inoculum in the transcripts. Alternatively, a mutational adaptation may take place in *N. benthamiana* that allows infection of *N. tabacum*. In any case, the production of viable chimeric potyviruses should provide a means to understand the regulation of the host range of this important group of plant viruses, which individually have rather restricted host ranges but collectively infect plants in a wide range of families.

We are grateful to B. Raccah, J. Chappell, and E. Hiebert for providing plasmids containing the ZYMV HC-Pro gene, GUS gene, and watermelon mosaic virus 1 (WMV-1) amorphous inclusion protein antibodies respectively, and to P. L. Atreya, D. W. Thornbury, J. J. Lopez-Moya, M. Chu, S. L. Tracy, and S. Dutton for their help in various aspects of the project. Financial support was provided by grants from the U.S. Department of Agriculture National Research Initiative Competitive Grants Program (91-37303-6505), U.S.-Israel Binational Agricultural Research and Development Fund (US-2088-91RC), the R. J. Reynolds Corp., and U.S. Department of Agriculture Agricultural Research Service Cooperative Agreement 58-6430-1-126.

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