Virus Research 339 (2024) 199277

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

The HC-Pro cistron of Triticum mosaic virus is dispensable for systemic infection in wheat but is required for symptom phenotype and efficient genome amplification

Satyanarayana Tatineni [a,b,*], Jeffrey Alexander [a], Frank Kovacs [c]

[a] United States Department of Agriculture-Agricultural Research Service, University of Nebraska-Lincoln, Lincoln, NE 68583, USA
[b] Department of Plant Pathology, University of Nebraska-Lincoln, Lincoln, NE 68503, USA
[c] Department of Chemistry, University of Nebraska-Kearney, Kearney, NE 68849, USA

ARTICLE INFO

Keywords:
Deletions
Functional genomics
Genome amplification
HC-Pro, Potyvirids
Symptom induction
Triticum mosaic virus
Wheat

ABSTRACT

Triticum mosaic virus (TriMV), the type species of the genus *Poacevirus* in the family *Potyviridae*, is an economically important wheat curl mite-transmitted wheat-infecting virus in the Great Plains region of the USA. In this study, the functional genomics of helper component-proteinase (HC-Pro) encoded by TriMV was examined using a reverse genetics approach. TriMV with complete deletion of HC-Pro cistron elicited systemic infection in wheat, indicating that HC-Pro cistron is dispensable for TriMV systemic infection. However, TriMV lacking HC-Pro caused delayed systemic infection with mild symptoms that resulted in little or no stunting of plants with a significant reduction in the accumulation of genomic RNA copies and coat protein (CP). Sequential deletion mutagenesis from the 5′ end of HC-Pro cistron in the TriMV genome revealed that deletions within amino acids 3 to 25, except for amino acids 3 and 4, elicited mild symptoms with reduced accumulation of genomic RNA and CP. Surprisingly, TriMV with deletion of amino acids 3 to 50 or 3 to 125 in HC-Pro elicited severe symptoms with a substantial increase in genomic RNA copies but a drastic reduction in CP accumulation. Additionally, TriMV with heterologous HC-Pro from other potyvirids produced symptom phenotype and genomic RNA accumulation similar to that of TriMV without HC-Pro, suggesting that HC-Pros of other potyvirids were not effective in complementing TriMV in wheat. Our data indicate that HC-Pro is expendable for replication of TriMV but is required for efficient viral genomic RNA amplification and symptom development. The availability of TriMV with various deletions in the HC-Pro cistron will facilitate the examination of the requirement of HC-Pro for wheat curl mite transmission.

1. Introduction

The family *Potyviridae* consists of 12 genera with more than 235 species and some of the potyvirids (members of the family *Potyviridae*) are of great economic importance affecting agriculturally important crops and trees worldwide (Inoue-Nagata et al., 2022; Tatineni and Hein, 2023). The genomes of potyvirids are monopartite except for the species of the genus *Bymovirus*, which contain bipartite genomes. Among the proteins encoded by the potyvirids, multifunctional helper component-proteinase (HC-Pro) is involved in several distinct processes of virus biology (Valli et al., 2018). The requirement of HC-Pro for aphid transmission of potyviruses is attributed to designating the name 'helper component' for this protein (Govier and Kassanis, 1974). HC-Pro is encoded in most of the potyvirids except in genera *Celavirus* and *Bymovirus* (Rose et al., 2019; Valli et al., 2018). The bipartite genome of *Bymovirus* members does not encode a designated HC-Pro, but RNA2 encodes a protein with amino acid domains with sequence similarities to those of potyvirus HC-Pro (You and Shirako, 2010). The members of the genus *Ipomovirus* with variable genome structures encode HC-Pro in sweet potato mild mottle virus (SPMMV) and tomato mild mottle virus genomes, but not in other known members of this genus (Dombrovsky et al., 2014).

Triticum mosaic virus (TriMV) is the type species of the genus *Poacevirus* in the family *Potyviridae* (Fellers et al., 2009; Tatineni et al., 2009). TriMV is a recently reported virus from the Great Plains region of the USA (Seifers et al., 2008). TriMV is transmitted by wheat curl mite

* Corresponding author.
E-mail address: Satyanarayana.tatineni@usda.gov (S. Tatineni).

https://doi.org/10.1016/j.virusres.2023.199277
Received 22 September 2023; Received in revised form 15 November 2023; Accepted 16 November 2023
Available online 30 November 2023

(*Aceria tosichella* Keifer) type 2 genotype but not by type 1 (Hein et al., 2012; McMechan et al., 2014; Seifers et al., 2009). TriMV is reported to predominantly co-infect wheat with wheat curl mite-transmitted wheat streak mosaic virus (WSMV) in the growers' fields (Burrows et al., 2009; Byamukama et al., 2013). TriMV and WSMV interact synergistically in co-infected wheat with an enhanced titer of both viruses causing exacerbated yield losses in growers' fields (Byamukama et al., 2012; Tatineni et al., 2019). The 10.3-kb single-stranded plus-sense RNA genome of TriMV is encapsidated in long flexuous filamentous particles of 15 × 550 to 909 nm (Seifers et al., 2008). The genome organization of TriMV is similar to those of *Potyvirus, Roymovirus, Rymovirus*, and *Tritimovirus* genera with a single large open reading frame that is translated into a large polyprotein. The polyprotein is cleaved into at least 10 mature proteins by three virus-encoded proteinases P1, HC-Pro, and NIa-Pro (Tatineni et al., 2009).

Viruses encode a limited number of proteins with multifunctionality for the successful completion of their life cycles (Firth, 2014). In the family *Potyviridae*, functional studies on multifunctional HC-Pro were extensively conducted only for the members of the genera *Potyvirus* and *Tritimovirus* (Valli et al., 2018). HC-Pro of potyvirids is a cysteine protease that contains a variable two-thirds of the N-terminal region involved in vector transmission and suppression of RNA silencing (Anandalakshmi et al., 1998; Atreya et al., 1992; Kasschau and Carrington, 1998; Stenger et al., 2005a; Valli et al., 2018). The potyviral HC-Pro facilitates virus transmission through aphids by forming a bridge between virions and insect mouthparts through the interaction of PTK and KITC motifs of HC-Pro with coat protein (CP) and insect mouthparts, respectively (Revers and Garcia, 2015). The HC-Pros of the members of *Potyvirus* and *Rymovirus* genera are involved in RNA silencing suppression through sequestration of small interfering RNAs (Anandalakshmi et al., 1998; Kasschau and Carrington, 1998; Qu and Morris, 2005; Young et al., 2012). The conserved C-terminal third of the protein contains a cysteine protease that is involved in polyprotein processing *in cis* (Kasschau and Carrington, 1995). The central domain of HC-Pro is involved in virus long-distance movement, virus amplification, and symptom development in host plants (Atreya et al., 1992; Kasschau et al., 1997; Stenger et al., 2005b).

The development of an infectious cDNA clone of TriMV facilitated the functional genomic analysis of NIa-Pro and CP as superinfection exclusion elicitors (Tatineni et al., 2015; Tatineni and French, 2016). The role of HC-Pro in TriMV biology is not known except that it is not required for the suppression of RNA silencing (Tatineni et al., 2012). In this study, the functional genomics of HC-Pro in TriMV biology was examined by deletion mutagenesis, followed by bioassay in wheat. The HC-Pro cistron is expendable for TriMV systemic infection of wheat and it was found that only amino acids 3 and 4 of HC-Pro can be deleted without affecting symptom phenotype and virus titer. Surprisingly, TriMV with deletion of amino acids 3 to 50 or 3 to 125 in HC-Pro elicited severe symptom phenotype compared to wild-type TriMV with increased accumulation of genomic RNAs but a drastic reduction in CP. Additionally, TriMV with heterologous HC-Pros from *Potyvirus* and *Tritimovirus* genera did not complement the functions of TriMV HC-Pro.

2. Materials and methods

2.1. Plant material and viruses

An infectious cDNA clone of TriMV isolate Nebraska (pTriMV-R; Tatineni et al., 2015) was the basis for all deletions introduced in HC-Pro cistron and for swapping with heterologous HC-Pros of turnip mosaic virus (TuMV), tobacco etch virus (TEV), and WSMV. Wheat cultivar (cv.) Tomahawk was used for inoculation of *in vitro* transcripts of TriMV (Tatineni et al., 2015) and its HC-Pro deletion mutants and for symptom phenotypic studies. The pasteurized soil mix consisting of 33 % each of clay loam soil and peat moss and 17 % each of sand and vermiculate was used to raise wheat seedlings in 20 cm diameter earthen pots.

2.2. Generation of TriMV HC-Pro deletion mutants

The complete deletion of HC-Pro cistron in the TriMV genome was created by precisely deleting the codons for amino acids 2 to 467 using mutagenic oligonucleotides comprising sequence on either side of deleting amino acid codons, followed by overlap extension PCR. The overlap extension PCR was performed with a plus-sense oligonucleotide Tr-260 [5′-GTGG*TCTAGA***ACCGCGG***ATTTAGGTGACACTATAG*AAAATTAAAGATCA-TATTACATAAAA-3′ containing *Xba*I (italics) and *Sac*II sites (bold), an SP6 RNA polymerase promoter (italics and underlined), followed by nts 1–26 of TriMV] and a minus-sense oligonucleotide Tr-94 (5′-ATTAACTAGT TCTCGAGAGGGTACTAATAT-3′, complementary to nts 4705 to 4676 of TriMV). The overlap extension PCR product was ligated into pTriMV-R (Tatineni et al., 2015) between *Xba*I (engineered in the plus-sense oligonucleotide) and *Xho*I (at nt 4689) restriction endonuclease sites to obtain pTriMV-ΔHC-Pro.

Deletion of the codons for amino acids 3 and 4, 3 to 8, 3 to 15, 3 to 25, 3 to 50, or 3 to 125 in the HC-Pro cistron of TriMV was introduced by using plus- and minus-sense oligonucleotides on either side of deletion, followed by overlap extension PCR with oligonucleotides Tr-260 and Tr-94. The overlap extension PCR products were ligated into pTriMV-R between *Xba*I and *Xho*I restriction endonuclease sites to obtain pTriMV-HC-ProΔ3–4aa, pTriMV-HC-ProΔ3–8aa, pTriMV-HC-ProΔ3–15 aa, pTriMV-HC-ProΔ3–25aa, pTriMV-HC-ProΔ3–50aa, and pTriMV-HC-ProΔ3–125aa. The presence of deleted amino acid codons in the HC-Pro cistron of cDNA clones was confirmed by nucleotide sequencing at Azenta Life Sciences (Burlington, MA, USA).

2.3. Engineering TriMV with heterologous HC-Pros

The sequence encoding HC-Pro amino acids 2 to 467 of TriMV was swapped with complete HC-Pro plus the first amino acid of P3 of TuMV (Lellis et al., 2002), TEV (Dolja et al., 1992), or WSMV (Choi et al., 1999) with overlap extension PCR, followed by ligation into pTriMV-R between *Xba*I and *Xho*I restriction endonuclease sites. The HC-Pro plus the first amino acid of P3 of TuMV, TEV, or WSMV was used to replace the TriMV HC-Pro amino acids 2 to 467 to facilitate efficient cleavage of TriMV P1 and heterologous HC-Pro from TriMV P3. The resulting TriMV with HC-Pro of TuMV, TEV, or WSMV were named TriMV-TuMV HC-Pro, TriMV-TEV HC-Pro and TriMV-WSMV HC-Pro, respectively. The presence of heterologous HC-Pros in pTriMV was confirmed by nucleotide sequencing at Azenta Life Sciences.

2.4. Generation of in vitro transcripts and inoculation of wheat seedlings

Plasmid DNAs were prepared from overnight grown 40-ml culture using a Bio-Rad plasmid midiprep kit (Bio-Rad, Hercules, CA). *In vitro* transcripts were generated in a 40 μl reaction volume from 1.0 μg of *Not*I-linearized plasmid DNAs as described (Tatineni et al., 2011). The freshly prepared *in vitro* transcripts were mixed with an equal volume of 2 % sodium pyrophosphate, pH 8.2 containing ~1.0 % baked Celite, and inoculated onto wheat seedlings at the single-leaf stage. Transcript-inoculated wheat seedlings were incubated in a greenhouse with 16 h natural/-artificial light at 22 to 30 °C. The upper uninoculated symptomatic leaves from *in vitro* transcripts inoculated plants were collected between 14 and 21 days postinoculation (dpi) and stored at −80 °C in 0.5 g aliquots for future symptom phenotypic studies.

2.5. Examination of symptom phenotype of TriMV and its HC-Pro deletion mutants

Crude sap of wheat leaves infected with *in vitro* transcripts at 1:20 dilution in 20 mM sodium phosphate buffer, pH 7.0, were inoculated to 3 to 4 pots (15 to 18 wheat seedlings per pot) per mutant at the single-leaf stage. Inoculated wheat plants were incubated in a greenhouse at 24 to 30 °C maximum and 18 to 22 °C minimum temperature with a 16 h

photoperiod of natural/supplemental light. The development of symptoms on upper uninoculated wheat leaves was observed at 7, 9, 14, and 21 dpi, and pictures of leaf symptoms were taken with a Nikon D750 camera. The symptom phenotype experiment was repeated 4 to 5 times, and the data were presented from one representative experiment.

2.6. Absolute quantification of TriMV genomic RNA copies

Symptomatic wheat leaves collected from 10 to 15 upper non-inoculated leaves, one leaf per plant from plants used for symptom phenotype, were ground into a fine powder in liquid nitrogen in a mortar and pestle. Total RNA was isolated from 100 mg powder in one ml TriPure reagent (Roche, Indianapolis, IN) essentially as described in Tatineni et al. (2010). Total RNA was suspended in 200 µl of distilled water and quantified in a NanoVue Plus Spectrophotometer (GE HealthCare, Piscataway, NJ). The first-strand cDNA was synthesized from 1.0 µg total RNA in a 10 µl volume, followed by absolute quantification of TriMV genomic RNA copies from 1 µl of cDNA in an Applied Biosystems 7300 Real-Time PCR system using primers and probe specific to TriMV as described in Tatineni et al. (2010). Statistical significance of genomic RNA copies accumulation of TriMV isolate Nebraska [TriMV-wild-type (TriMV-WT)] and its HC-Pro deletion mutants was determined with a one-way analysis of variance with Tukey's honestly significant difference.

2.7. RT-PCR

The presence of introduced deletions in HC-Pro cistron or heterologous HC-Pros in the TriMV genome was examined by reverse transcription (RT)-PCR method. Total nucleic acids were isolated from 100 mg of tissue collected from upper symptomatic leaves at 21 dpi as described in McNeil et al. (1996). RT-PCR was performed essentially as described in Tatineni et al. (2011), except using a plus-sense oligonucleotide Tr-289 (5′-TGAGATTGTCGAACAAGGGGACATGTAC-3′, corresponding to nts 1621–1648 of TriMV-WT) and a minus-sense oligonucleotide Tr-252 (5′-ATATACTTGGAATAGTCTTTCTGTAATA-3′, complementary to nts 3749–3722 of TriMV-WT). Five microliters of RT-PCR products were analyzed through 1.0% agarose gel electrophoresis in 1 x Tris-acetate buffer. RT-PCR products were gel-isolated using a PureLink Quick Gel Extraction kit (Invitrogen, Carlsbad, CA), followed by sequencing at Azenta Life Sciences.

2.8. Western blot assay

Accumulation of TriMV CP was examined by Western immuno-blot hybridization with CP-specific antibodies (Tatineni et al., 2013). Total proteins were isolated from 100 mg tissue powder that was also used for total RNA isolation, as described in Tatineni et al. (2011). Total proteins were separated on 4–20 % Tris-glycine-SDS polyacrylamide gels (Invitrogen). The gels were either stained with Coomassie brilliant blue R-250 for RuBisCo protein to determine the amount of protein loaded on gels or transferred onto polyvinylidene difluoride (PVDF) membranes. The PVDF membranes were probed with TriMV CP antibodies (1:10,000 dilution) as primary antibodies, followed by anti-rabbit horseradish peroxidase (HRP) conjugate (1:50,000 dilution) as secondary antibodies. Immunoreactive protein bands on PVDF membranes were developed with Immobilon Western blot substrate (Millipore, Billerica, MA) using the Molecular Imager ChemiDoc XRS+ with Image Lab software system (Bio-Rad). The intensities of TriMV CP major protein bands were quantified with the Molecular Imager ChemiDoc XRS+ system.

2.9. Phylogenetic analysis

Phylogenetic analysis of amino acid sequences of select HC-Pro sequences of the family *Potyviridae* was performed with the MEGA, version 11, analysis package (Tamura et al., 2021). The neighbor-joining method with the JTT matrix and pairwise gap deletion, with 1000 bootstrap replicates was used as the test of phylogeny.

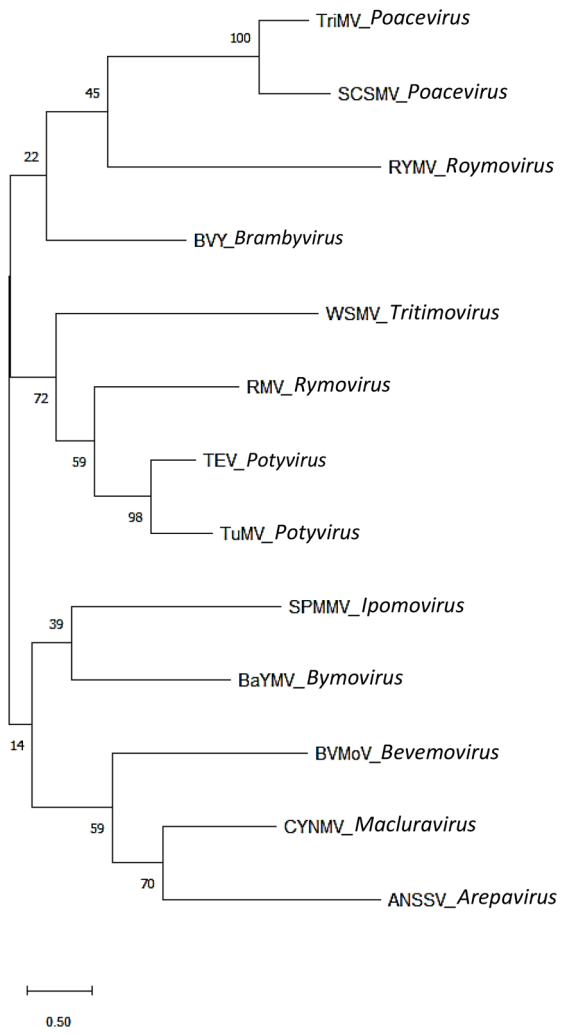

Fig. 1. Phylogenetic analysis of HC-Pro amino acid sequences of select viruses from the *Potyviridae* family. The unrooted bootstrap consensus phylogenetic tree was constructed with the MEGA v. 11 analysis package (Tamura et al., 2021) with the neighbor-joining method using the JTT matrix and pairwise gap deletion with 1000 bootstrap replicates. The numbers indicated at branch points are bootstrap support. The bar indicates the number of amino acid replacements per site. Potyvirids and their respective genera used in HC-Pro amino acid sequence analysis are indicated on the phylogenetic tree. ANSSV (areca palm necrotic spindle-spot virus); BVMoV (bellflower veinal mottle virus); BVY (blackberry virus Y); BaYMV (barley yellow mosaic virus); SPMMV (sweet potato mild mottle virus); CYNMV (Chinese yam necrotic mosaic virus), TriMV (Triticum mosaic virus), SCSMV (sugarcane streak mosaic virus); TuMV (turnip mosaic virus); TEV (tobacco etch virus); RYMV (rose yellow mosaic virus); RMV (ryegrass mosaic virus); and WSMV (wheat streak mosaic virus).

2.10. Predictive structural modeling

A three-dimensional structure of any part of TriMV HC-Pro or related HC-Pros of other potyvirids, specifically to the N-terminal portion is not available. Hence, we utilized predictive modeling for TriMV HC-Pro and its deletion mutants to find a possible structural reason for the functional effects that elicited severe symptom phenotype. We used Robetta, a protein structure prediction server (www.robetta.bakerlab.org) that uses RoseTTAFold, a deep learning-based modeling method with a good record of structure prediction (Baek et al., 2021). This type of predictive

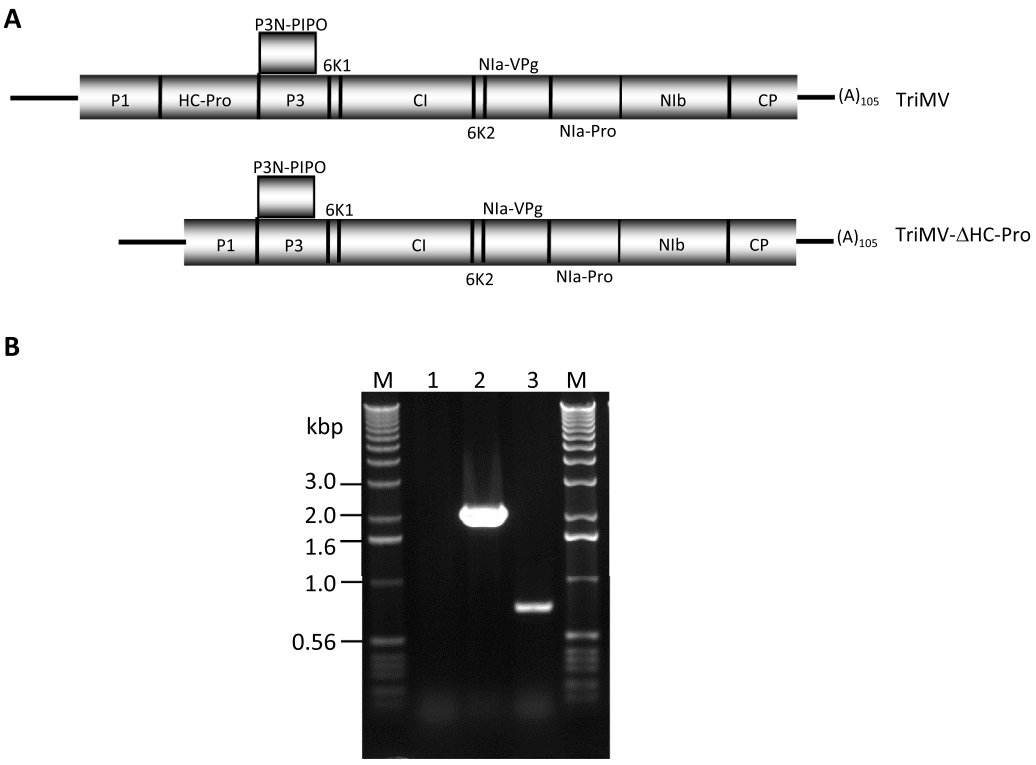

Fig. 2. Engineering HC-Pro deletion in an infectious cDNA clone of Triticum mosaic virus (TriMV). A. Genome organization of wild-type TriMV (TriMV-WT) and TriMV-ΔHC-Pro with cistrons encoded in a large open reading frame (ORF). The location of cleavage peptides in the large ORF is indicated with vertical lines. Amino acids 2 to 467 of the 467 amino acids of TriMV HC-Pro were deleted in TriMV-ΔHC-Pro. B. Agarose gel electrophoresis of RT-PCR products from wheat leaves infected with *in vitro* transcripts of TriMV-WT or TriMV-ΔHC-Pro at 21 days postinoculation. Reverse transcription, followed by PCR was performed with Tr-289 and Tr-252 (see materials and methods section). Total nucleic acids from mock-inoculated (lane 1), TriMV-WT-infected (lane 2), and TriMV-ΔHC-Pro-infected (lane 3) wheat were used for RT-PCR. Lanes M: 1.0 kbp DNA ladder.

modeling has greatly improved over the past several years through the use of artificial intelligence and has proven useful in gaining structural insight into protein function. We submitted the amino acid sequences for TriMV full-length HC-Pro and mutants with deletion of amino acids 3 to 25, 3 to 50, and 3 to 125 to the Robetta server using the RoseTTAFold method. We chose Robetta for predictive modeling because it consistently generated models with protease core structures that were highly superimposable with the crystal structure of the protease domain of the distantly related HC-Pro of TuMV (Guo et al., 2011). We used Chimera for structural visualization and superposition of the protein models (Pettersen et al., 2004).

3. Results

3.1. Phylogenetic analysis of TriMV HC-Pro

The HC-Pro cistron of TriMV encodes 467 amino acid residues, consisting of distinctive N-terminal and central domains and the C-terminal protease domain (Tatineni et al., 2009). Unlike members of the *Potyvirus* genus, TriMV HC-Pro does not have the KITC and PTK motifs necessary for aphid transmission, nor the FRNK motif involved in RNA silencing suppression (Valli et al., 2018). To compare HC-Pro sequences from different genera of the *Potyviridae* family, select potyvirids were used for sequence comparison and phylogenetic analyses. Pairwise sequence comparison showed that TriMV HC-Pro has only 10 to 22 % sequence identity at the amino acid level with HC-Pros of other genera. However, it has a 45.0 % amino acid sequence identity with sugarcane streak mosaic virus, another species of the genus *Poacevirus*, along with TriMV. Phylogenetic analyses revealed that TriMV formed a separate group along with the members of the *Brambyvirus* and *Roymovirus* genera in the *Potyviridae* family (Fig. 1). Furthermore, the C-terminal protease domain of TriMV HC-Pro has slightly higher sequence identity with the corresponding regions of other potyvirids compared to the N-terminal and central domains (data not shown). Overall, these findings suggest that TriMV HC-Pro has a low sequence identity with HC-Pros of potyvirids belonging to other genera in the family *Potyviridae*.

3.2. HC-Pro is expendable for systemic infection of TriMV in wheat

The requirement of HC-Pro for TriMV systemic infection of wheat was examined by deleting all 467 amino acids except for the first two amino acids to facilitate efficient cleavage of the preceding P1 protein (Fig. 2A). Thus, the first two amino acids serine and aspartic acid of HC-Pro will become part of the P3 protein in TriMV-ΔHC-Pro. *In vitro* generated RNA transcripts of TriMV-WT or TriMV-ΔHC-Pro were inoculated onto wheat seedlings cv. Tomahawk at the single-leaf stage and examined for the development of systemic symptoms in upper uninoculated leaves. Results showed that *in vitro* transcripts of TriMV-WT infected 58 to 86 % of wheat seedlings by 21 dpi, while TriMV-ΔHC-Pro facilitated systemic infection in only 20 to 23 % of wheat. Systemic infection of wheat plants by TriMV-ΔHC-Pro was further confirmed by RT-PCR with oligonucleotides Tr-289 and Tr-252 using total RNA isolated from upper uninoculated symptomatic leaves. Agarose gel electrophoresis of RT-PCR products revealed a ~2100 bp product in TriMV-WT-infected wheat, while TriMV-ΔHC-Pro-infected wheat had a ~750 bp product (Fig. 2B). These results indicate that the HC-Pro cistron is dispensable for TriMV systemic infection of wheat.

3.3. TriMV lacking HC-Pro elicited mild symptoms in wheat

We next conducted a study to explore the impact of HC-Pro on the

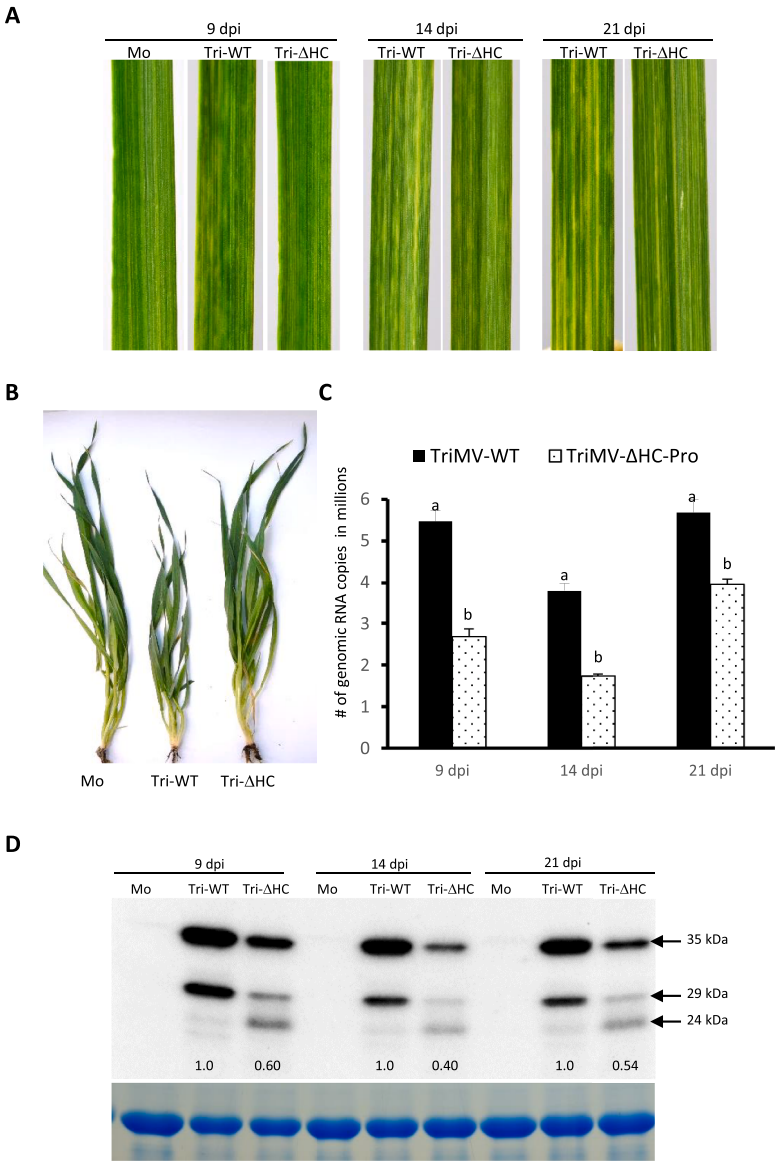

Fig. 3. Triticum mosaic virus (TriMV) HC-Pro is required for symptom development and efficient accumulation of genomic RNA and coat protein in wheat. A. Symptom phenotypes of TriMV-WT and TriMV-ΔHC-Pro on wheat cv. Tomahawk leaves at 9, 14, and 21 days postinoculation (dpi). Note TriMV with deletion of HC-Pro elicited mild chlorotic streaks and mosaic symptoms compared to large moderate chlorotic streaks, mosaic, and mottling symptoms by TriMV-WT. B. Effect of TriMV and TriMV-ΔHC-Pro on wheat growth at 21 dpi. Note TriMV with deletion of HC-Pro caused little or no stunting of wheat at 21 dpi. C. Reverse transcription real-time PCR assay for absolute quantification of genomic RNA copies of TriMV-WT and TriMV-ΔHC-Pro in wheat at 9, 14, and 21 dpi. The histograms represent the number of genomic RNA copies in 100 ng total RNA with standard error. The significant difference in genomic RNA accumulation between TriMV and TriMV-ΔHC-Pro at $P < 0.05$ at each time point is indicated with different letters. D. Western immunoblot analysis of total proteins from wheat leaves infected with TriMV-WT or TriMV-ΔHC-Pro at 9, 14, and 21 dpi. Arrows indicate a major protein of 35 kDa (CP) and two minor proteins of 29 and 24 kDa reacted with CP antibodies. Numbers below the Western blot indicate fold change in CP accumulation in TriMV-ΔHC-Pro-infected wheat compared to TriMV-WT. A Coomassie brilliant blue-stained SDS-PAGE gel below the Western blot shows the large subunit of wheat RuBisCo protein as a loading control for total protein per well. Tri-WT: TriMV-WT; Tri-ΔHC: TriMV-ΔHC-Pro; Mo: Mock.

manifestation of TriMV symptoms in wheat. The crude sap from wheat leaves infected with *in vitro* transcripts of TriMV-WT or TriMV-ΔHC-Pro was used as inoculum for wheat seedlings at the single-leaf stage. The onset of symptoms by TriMV-ΔHC-Pro was delayed by approximately 2 days compared to TriMV-WT. TriMV-WT and TriMV-ΔHC-Pro caused mild chlorotic streaks at 5 to 6 dpi and 7 to 8 dpi, respectively. At 9 dpi, TriMV-ΔHC-Pro elicited a few mild chlorotic streaks per leaf compared to several moderate chlorotic streaks by TriMV-WT (Fig. 3A). At 14 and 21 dpi, TriMV-ΔHC-Pro induced mild chlorotic streaks, mosaic, and mottling symptoms compared to large moderate chlorotic streaks and mosaic symptoms by TriMV-WT (Fig. 3A). No leaf yellowing symptoms were observed in wheat infected by TriMV-WT or TriMV-ΔHC-Pro at 21 dpi. At 21 dpi, wheat plants infected with TriMV-ΔHC-Pro induced little or no stunting of plants with a height of 41.9 ± 0.5 cm compared to a height of 41.2 ± 0.5 mock-inoculated plants (Table 1; Fig. 3B). However, wheat plants that were infected with TriMV-WT showed moderate stunting symptoms, with a height of 33.2 ± 0.7 cm (Table 1; Fig. 3B). These findings suggest that HC-Pro is essential for the development of foliar and stunting symptoms by TriMV in wheat.

3.4. HC-Pro is required for efficient genome amplification of TriMV

We next investigated the effect of HC-Pro deletion from the TriMV genome on the accumulation of genomic RNA in wheat. The absolute

Table 1
Effect of TriMV-WT, TriMV HC-Pro deletion mutants and TriMV with heterologous HC-Pros on wheat plant height at 21 days postinoculation.

Virus	Average plant height in cm±SE
Mock	41.2 ± 0.5 a
TriMV-WT	33.2 ± 0.7 b
TriMV-ΔHC-Pro	41.9 ± 0.5 a
TriMV-HC-ProΔ3–4aa	34.4 ± 0.3 b
TriMV-HC-ProΔ3–8aa	37.9 ± 0.7 c
TriMV-HC-ProΔ3–15aa	39.4 ± 0.8 a, c
TriMV-HC-ProΔ3–25aa	40.6 ± 0.4 a, c
TriMV-HC-ProΔ3–50aa	22.3 ± 0.8 d
TriMV-HC-ProΔ3–125aa	17.2 ± 0.7 e
TriMV-TuMV HC-Pro	41.3 ± 0.9 a
TriMV-TEV HC-Pro	39.4 ± 0.6 a, c
TriMV-WSMV HC-Pro	40.5 ± 0.6 a, c

Wheat seedlings inoculated with crude sap from *in vitro* transcripts-infected wheat leaves at the single-leaf stage.
Plant height was measured from 10 plants at 21 days postinoculation.
Plant height with different letters indicates significant differences ($P < 0.05$) between mutants.

quantification of TriMV genomic RNA copies was determined by RT-qPCR from wheat leaves systemically infected with TriMV-WT or TriMV-ΔHC-Pro at 9, 14, and 21 dpi. At 9 dpi, TriMV-WT accumulated at 5.47×10^6 genomic RNA copies compared to 2.67×10^6 copies of TriMV-ΔHC-Pro (Fig. 3C). At 14 and 21 dpi, TriMV-WT accumulated 3.79×10^6 and 5.68×10^6 genomic RNA copies compared to 1.73×10^6 and 3.94×10^6 copies in TriMV-ΔHC-Pro infected wheat, respectively (Fig. 3C). These findings suggest that the deletion of HC-Pro cistron from the TriMV genome significantly impacted the accumulation of TriMV genomic RNA copies in wheat.

Accumulation of CP in wheat infected by TriMV-WT or TriMV-ΔHC-Pro was examined by Western immunoblot analysis. Our findings showed that compared to TriMV-WT, deletion of HC-Pro cistron in TriMV led to a substantially less accumulation of CP at 0.60-, 0.40-, and 0.54-fold of TriMV-WT at 9, 14, and 21 dpi, respectively, in wheat (Fig. 3D). This indicates that while HC-Pro is not an absolute requirement for the systemic infection of TriMV in wheat, it plays a crucial role in the efficient amplification of TriMV genomic RNA and CP.

3.5. *Mapping HC-Pro amino acids required for TriMV symptom development and efficient genome amplification*

The above data suggested that TriMV lacking HC-Pro elicited mild symptoms with significantly reduced accumulation of genomic RNA copies and CP. We next examined HC-Pro amino acids required for TriMV symptom development and genome amplification by introducing a series of sequential deletion of codons for amino acids 3 and 4, 3 to 8, 3 to 15, 3 to 25, 3 to 50, and 3 to 125 (Fig. 3A). *In vitro* transcripts of TriMV with these deletions infected 60 to 86 % of wheat seedlings, similar to TriMV-WT. The role of these HC-Pro deletions in symptom development was examined by inoculating wheat seedlings with crude sap of *in vitro* transcripts-infected wheat leaves. At 14 and 21 dpi, TriMV with deletion of amino acids 3 and 4 in HC-Pro caused moderate chlorotic streaks, mosaic, and mottling symptoms similar to those caused by TriMV-WT (Fig. 4B). Moreover, wheat plants that were infected by TriMV lacking amino acids 3 and 4 in HC-Pro were stunted, with a height of 34.4 ± 0.3 cm, which was comparable to 33.2 ± 0.7 cm of TriMV-WT-infected plants (Table 1; Fig. 4C). TriMV with deletion of amino acids 3 to 8, 3 to 15, or 3 to 25 elicited mild chlorotic streaks, mosaic, and mottling symptoms with mild stunting symptoms compared to TriMV-WT-infected wheat plants (Table 1; Fig. 4B and C). Wheat plants infected with TriMV harboring a deletion of amino acids 3 to 8, 3 to 15, or 3 to 25 in HC-Pro induced slight stunting with a height of 37.9 ± 0.7 cm, 39.4 ± 0.8 cm, and 40.6 ± 0.4 cm, respectively, compared to the height of 41.2 ± 0.5 cm of mock-inoculated wheat plants (Table 1; Fig. 4C). TriMV with deletion of amino acids 3 and 4 but not beyond these amino acids elicited symptoms similar to TriMV-WT, suggesting that only amino acids 3 and 4 in HC-Pro are dispensable for symptom induction. The presence of introduced deletions in HC-Pro cistron was verified by RT-PCR (Fig. 4D), followed by nucleotide sequencing.

We next examined TriMV genomic RNA accumulation in different deletions at 14 and 21 dpi. At 14 dpi, deletion of amino acids 3 and 4 in HC-Pro accumulated similarly to TriMV-WT, but deletion of amino acids 3 to 8 or 3 to 15 accumulated similar to TriMV-ΔHC-Pro but significantly reduced levels compared to TriMV-WT (Fig. 5A). At 21 dpi, deletion of amino acids 3 and 4 in HC-Pro led to a slightly lower accumulation of genomic RNAs compared to TriMV-WT, but more compared to TriMV-ΔHC-Pro (Fig. 5A). On the other hand, removing amino acids 3 to 8 or 3 to 15 in HC-Pro resulted in reduced accumulation of genomic RNAs compared to TriMV-WT (Fig. 5A). At both 14 and 21 dpi, deletion of amino acids 3 to 25 in TriMV significantly affected the genomic RNA accumulation compared to TriMV-WT (Fig. 5A).

Based on the data, it appears that the deletion of amino acids 5 to 15 of HC-Pro in the TriMV genome has an impact on the early genome amplification process at 14 dpi. However, amino acids 16 to 25 are crucial for TriMV genome amplification at both 14 and 21 dpi.

Accumulation of CP from wheat leaves infected with HC-Pro deletion mutants of TriMV was examined at 14 and 21 dpi. Wheat leaves infected with TriMV harboring a deletion of amino acids 3 and 4 accumulated the CP similar to that of TriMV-WT (Fig. 5B, compare lanes 4 and 2). However, TriMV with a deletion of amino acids 3 to 8, 3 to 15, or 3 to 25 in HC-Pro accumulated CP substantially less at 0.21-, 0.16-, and 0.19-fold at 14 dpi, and at 0.22-, 0.30-, and 0.24-fold, respectively, of TriMV-WT (Fig. 5B).

3.6. *TriMV with deletion of amino acids 3 to 50 or 3 to 125 in HC-Pro elicited severe symptoms in wheat*

The above studies revealed that HC-Pro amino acids located between 5 and 25 are required for TriMV symptom development and virus titer. We next extended deletions to amino acids 3 to 50 and 3 to 125 in HC-Pro to further examine the requirement of amino acids located between 26 and 125 for symptom phenotype and genomic RNA accumulation (Fig. 4A). Unexpectedly, TriMV with deletion of amino acids 3 to 50 or 3 to 125 in HC-Pro elicited severe chlorotic streaks, mosaic, mottling symptoms with severe stunting of plants at 14 and 21 dpi (Fig. 4B and C). At 21 dpi, wheat plants infected with TriMV with deletion of amino acids 3 to 50 or 3 to 125 were significantly stunted to a height of 22.3 ± 0.8 cm and 17.2 ± 0.7 cm, respectively, compared to the height of 33.2 ± 0.7 cm of TriMV-WT-infected and 41.2 ± 0.5 cm of mock-inoculated plants (Table 1; Fig. 4C). These two mutants caused a significantly increased accumulation of TriMV genomic RNA copies at both 14 and 21 dpi compared to TriMV-WT (Fig. 5A). TriMV with deletion of amino acids 3 to 50 or 3 to 125 accumulated genomic RNA copies at 14.3×10^6 and 12.3×10^6 at 14 dpi, and 14.7×10^6 and 9.4×10^6 at 21 dpi, respectively, compared to 3.8×10^6 and 5.7×10^6 of TriMV-WT (Fig. 5A). These data indicate that deletion of amino acids 3 to 125 in HC-Pro-resulted in a severe symptom phenotype of TriMV with a substantial increase in genomic RNA amplification. Examination of CP accumulation from wheat infected with TriMV harboring a deletion of amino acids 3 to 50 or 3 to 125 revealed that CP accumulated at substantially reduced levels at 0.26- to 0.28-fold at 14 dpi and 0.13- to 0.16-fold at 21 dpi compared to TriMV-WT (Fig. 5B). Increased accumulation of genomic RNA copies without a corresponding increase in CP accumulation is an unusual phenomenon in severe symptom-inducing HC-Pro deletions in TriMV. However, the reason behind this distinctive phenotype of HC-Pro deletion mutants is not known.

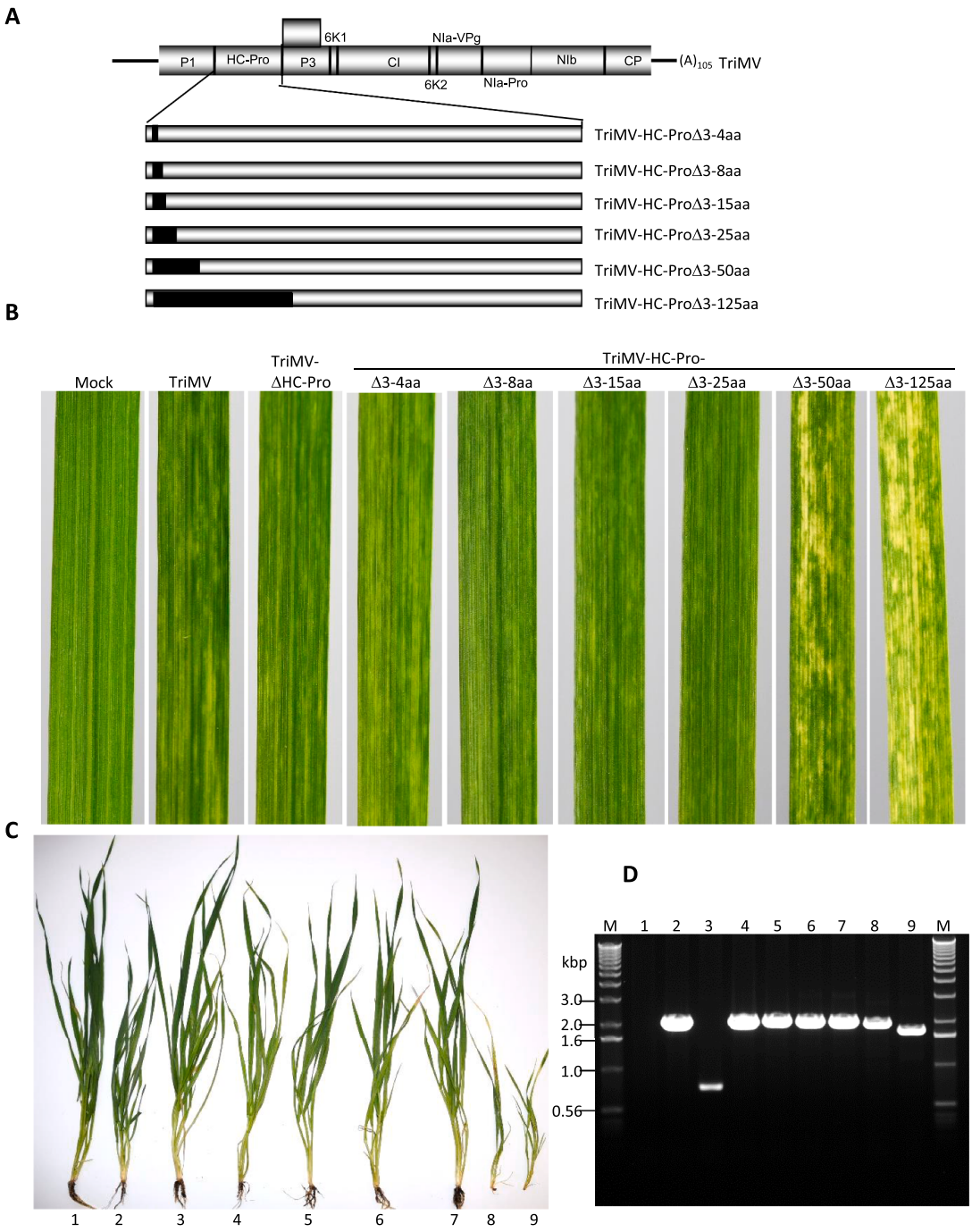

Fig. 4. Mapping the HC-Pro cistron of Triticum mosaic virus (TriMV) required for symptom development. A. Schematic diagram of the genomic organization of TriMV with engineered deletions (in black rectangles) in the HC-Pro cistron. B. Symptom phenotypes of wild-type TriMV (TriMV-WT) and TriMV HC-Pro deletion mutants at 14 days postinoculation (dpi). Note that HC-Pro with deletion of amino acids 3 and 4 elicited symptoms similar to TriMV-WT, and deletion of amino acids 3 to 8, 3 to 15, or 3 to 25 elicited mild symptoms similar to TriMV-ΔHC-Pro. TriMV with deletion of amino acids 3 to 50 or 125 induced severe symptoms compared to TriMV-WT. C. Effect of TriMV-WT and HC-Pro deletion mutants on wheat growth at 21 dpi. TriMV with deletion of amino acids 3 and 4 induced stunting of wheat similar to TriMV-WT; deletion of amino acids 3 to 8, 3 to 15, and 3 to 25 induced little or no stunting of plants similar to that of TriMV-ΔHC-Pro; and deletion of amino acids 3 to 50 or 125 caused severe stunting of wheat. D. Agarose gel electrophoresis of reverse transcription-PCR products from wheat infected with TriMV-WT or HC-Pro deletion mutants at 21 dpi. The numbers in C and D represent mock-inoculated wheat (1), TriMV-WT (2), TriMV-ΔHC-Pro (3), TriMV with deletion of amino acids 3 and 4 (4), 3 to 8 (5), 3 to 15 (6), 3 to 25 (7), 3 to 50 (8), and 3 to 125 (9) in HC-Pro. Lanes M in D represents a 1.0 kbp DNA ladder.

3.7. Heterologous HC-Pros did not complement the HC-Pro functions of TriMV

We examined whether heterologous HC-Pros from *Potyvirus* and *Tritimovirus* species will complement TriMV symptom development and efficient genomic RNA amplification. The HC-Pro cistron of TriMV was precisely replaced with those of TuMV, TEV, or WSMV (Fig. 6A). *In vitro* transcripts of TriMV with heterologous HC-Pro sequences infected 20 to 26 % of wheat seedlings similar to that of TriMV-ΔHC-Pro. Infection of wheat by TriMV with heterologous HC-Pros was further confirmed by RT-PCR, followed by nucleotide sequencing (Fig. 6D).

Wheat infected with TriMV harboring heterologous HC-Pros elicited

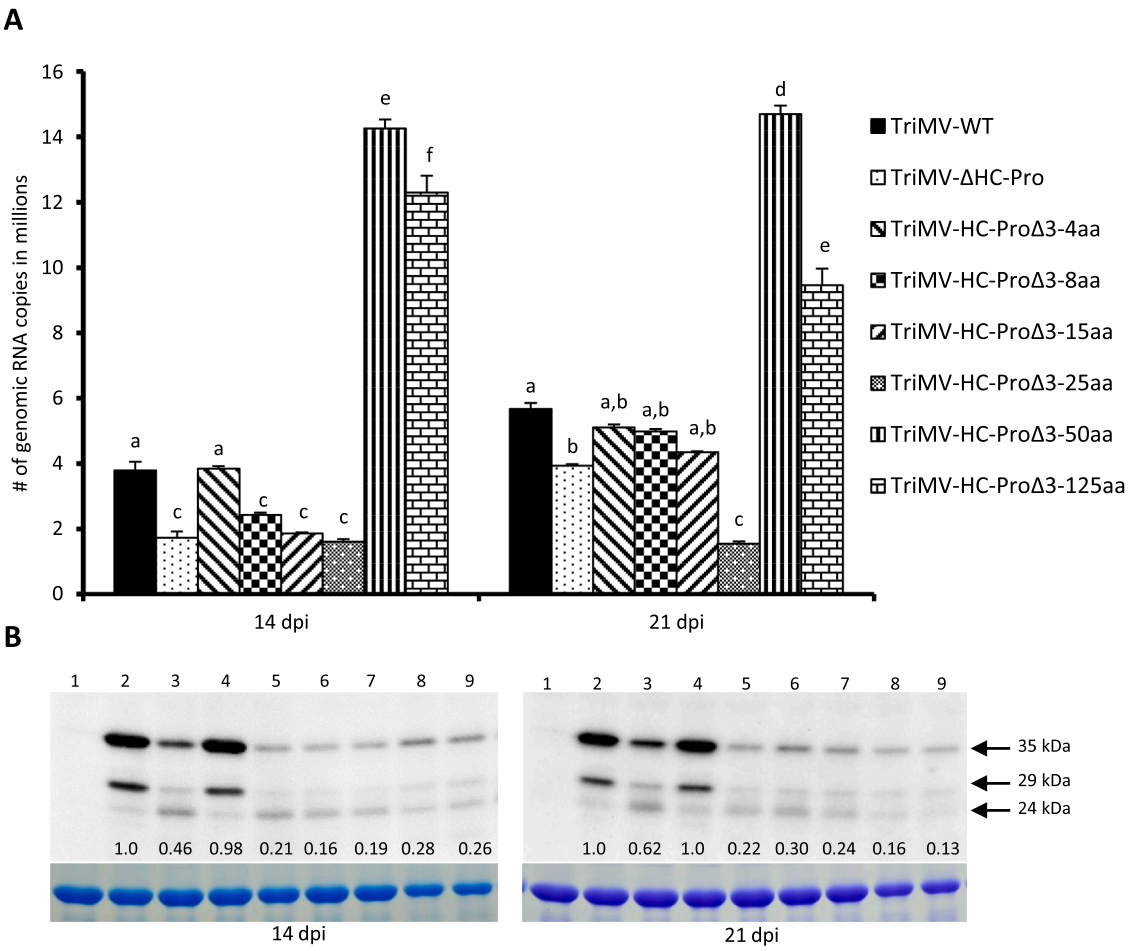

Fig. 5. Effect of deletions in the HC-Pro cistron of Triticum mosaic virus (TriMV) on genomic RNA amplification and CP accumulation in wheat. A. Absolute quantification of TriMV genomic RNA copies by reverse transcription real-time PCR in total RNA isolated from wheat leaves infected with TriMV-WT or HC-Pro deletion mutants at 14 and 21 days postinoculation (dpi). The number of TriMV genomic RNA copies in 100 ng of total RNA at 14 and 21 dpi is indicated in histograms with standard error. Different letters above the histograms indicate significant differences in genomic RNA accumulation among different mutants at $P < 0.05$, while the same letters indicate no significant difference. B. Western blot analysis of total proteins from wheat leaves infected with TriMV or HC-Pro deletion mutants with CP antibodies at 14 and 21 dpi. Note deletion of HC-Pro amino acids 3 to 50 or 3 to 125 caused a significant increase in TriMV genomic RNA accumulation but not CP , while deletion of amino acids 3 to 8, 3 to 15, and 3 to 25 caused a reduction in both genomic RNA and CP accumulation. Numbers below the Western blot indicate fold change in CP accumulation of HC-Pro deletion mutants compared to TriMV-WT. The amount of total protein loaded in each well for Western blot was shown in a Coomassie brilliant blue-stained SDS-PAGE gel showing the large subunit of wheat RuBisCo protein. The numbers in B represent mock-inoculated wheat (1), TriMV-WT (2), TriMV-ΔHC-Pro (3), TriMV with deletion of amino acids 3 and 4 (4), 3 to 8 (5), 3 to 15 (6), 3 to 25 (7), 3 to 50 (8), and 3 to 125 (9) in HC-Pro.

symptoms similar to that of TriMV-ΔHC-Pro with mild chlorotic streaks, mosaic, and mottling at 9, 14, and 21 dpi compared to moderate chlorotic streaks, mosaic, and mottling symptoms of TriMV-WT (Fig. 6B). Wheat plants infected by TriMV with heterologous HC-Pros induced minimal stunting of plants with a height of 39.4 ± 0.6 to 41.3 ± 0.9 cm compared to 41.2 ± 0.5 cm of mock-inoculated and 33.2 ± 0.7 cm of TriMV-WT-infected wheat plants (Table 1; Fig. 6C).

The role of heterologous HC-Pros in TriMV genome amplification was examined by extracting total RNA at 14 and 21 dpi, followed by absolute quantification of TriMV genomic RNA. At 14 and 21 dpi, TriMV with HC-Pro of TuMV, TEV, or WSMV accumulated similar to that of TriMV-ΔHC-Pro, but at significantly reduced levels compared to TriMV-WT (Fig. 7A). At 14 and 21 dpi, TriMV with heterologous HC-Pros accumulated CP at 0.18- to 0.4-fold of TriMV-WT (Fig. 7B). These data indicate that HC-Pros from TuMV, TEV, or WSMV did not complement the HC-Pro functions of TriMV for symptom development and efficient accumulation of genomic RNA and CP.

4. Discussion

In this study, the functional role of HC-Pro in TriMV biology was examined by deletion mutagenesis in an infectious cDNA clone (Tatineni et al., 2015). We found that HC-Pro is dispensable for systemic infection of TriMV but is required for symptom development and virus titer. Deletion mutagenesis analyses revealed that only amino acids 3 and 4 but not 5 to 25 of HC-Pro are expendable for TriMV symptom phenotype and virus accumulation. Unexpectedly, deletion of amino acids 3 to 50 or 3 to 125 in HC-Pro significantly increased TriMV symptom severity and genomic RNA with a drastic reduction in CP accumulation.

In vitro transcripts of TriMV with complete deletion of HC-Pro systemically infected wheat seedlings led to the conclusion that HC-Pro is expendable for TriMV infection of wheat. Previous research has shown that WSMV with a complete deletion of HC-Pro cistron was also able to infect wheat systemically (Stenger et al., 2005b). Although *in vitro* transcripts of TriMV-ΔHC-Pro infected wheat inefficiently, the crude sap from transcripts-infected wheat resulted in 95 to 100 % infection,

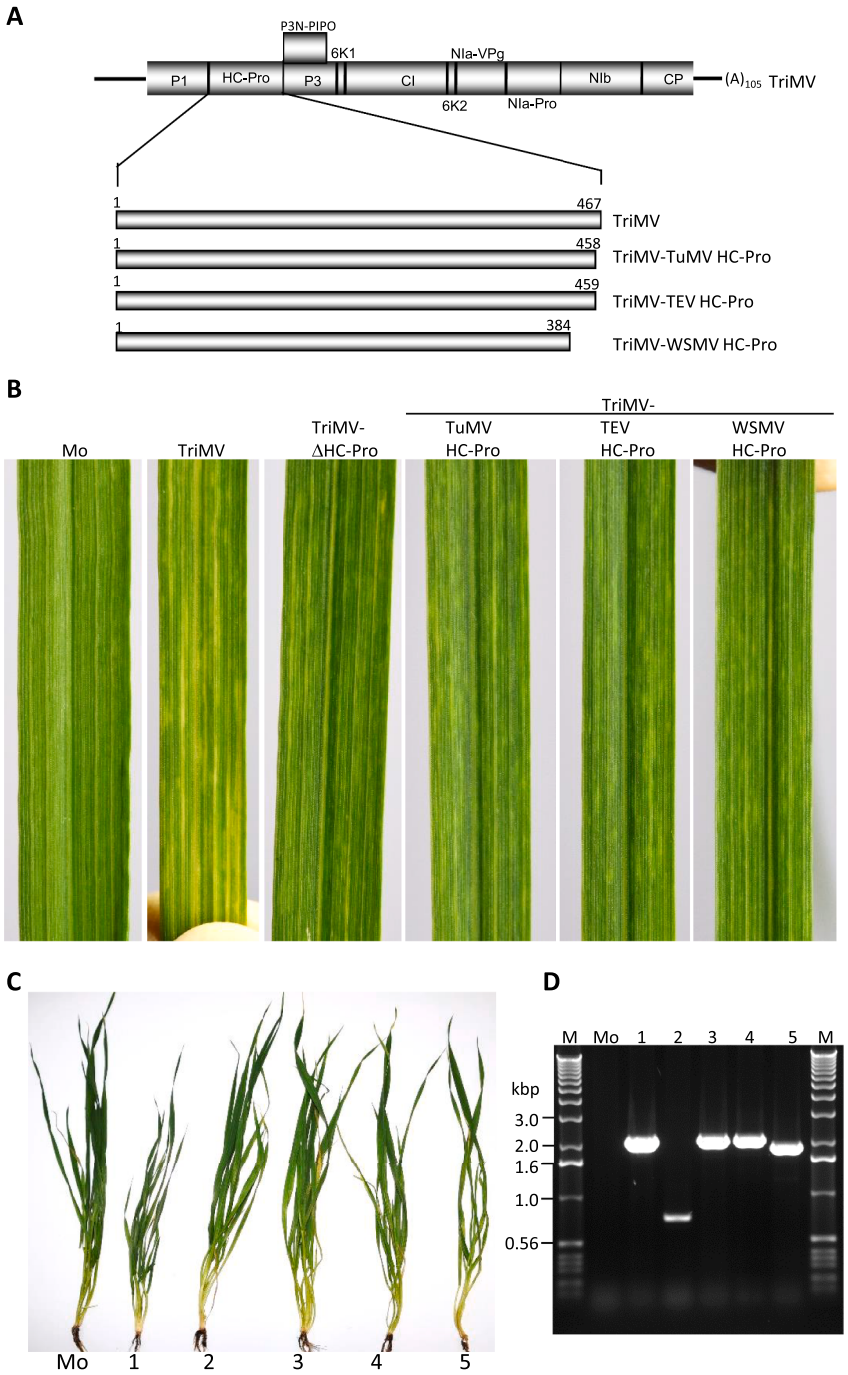

Fig. 6. Heterologous HC-Pros in Triticum mosaic virus (TriMV) did not complement severe symptom induction and efficient genomic RNA amplification in wheat. A. Schematic diagram of TriMV showing the replacement of TriMV HC-Pro with that of turnip mosaic virus (TuMV; 458 amino acids), tobacco etch virus (TEV; 459 amino acids), or wheat streak mosaic virus (WSMV; 384 amino acids). B. Symptom phenotype of TriMV with heterologous HC-Pro in wheat at 14 days post-inoculation (dpi). Note that TriMV with HC-Pro from TuMV, TEV, or WSMV elicited symptoms similar to those of TriMV-ΔHC-Pro. C. Effect of TriMV with heterologous HC-Pros on wheat growth at 21 dpi. Wheat infected by TriMV with HC-Pro from TuMV, TEV, or WSMV induced mild or no stunting of wheat similar to TriMV-ΔHC-Pro. D. Reverse transcription PCR analysis of total nucleic acids from wheat infected with TriMV harboring HC-Pro cistron of TuMV, TEV, or WSMV for the stability of inserted cistron. PCR was performed with Tr-289 and Tr-253 as described in the materials and methods section. The numbers in C and D represent mock-inoculated wheat (Mo); TriMV-WT (1); TriMV-ΔHC-Pro (2) TriMV-TuMV HC-Pro (3); TriMV-TEV HC-Pro (4); and TriMV-WSMV HC-Pro (5). Lanes M in D represents a 1.0 kbp DNA ladder.

similar to WSMV without HC-Pro (Stenger et al., 2005b). These findings indicate that HC-Pro is not essential for TriMV replication and movement, which are both critical functions of the virus. In contrast, the HC-Pro of the members of the genus *Potyvirus* is indispensable for systemic infection (Valli et al., 2018). Previous research on WSMV has indicated that the HC-Pro cistron is a wheat curl mite transmission determinant (Stenger et al., 2005a; Young et al., 2007). It is also possible that TriMV HC-Pro might be involved in wheat curl mite transmission. However, examining the requirement of HC-Pro for wheat curl mite transmission of TriMV is beyond the scope of the current investigation.

Potyviral HC-Pro was reported to be involved in the development of symptoms and genome amplification (Kasschau et al., 1997; Shiboleth

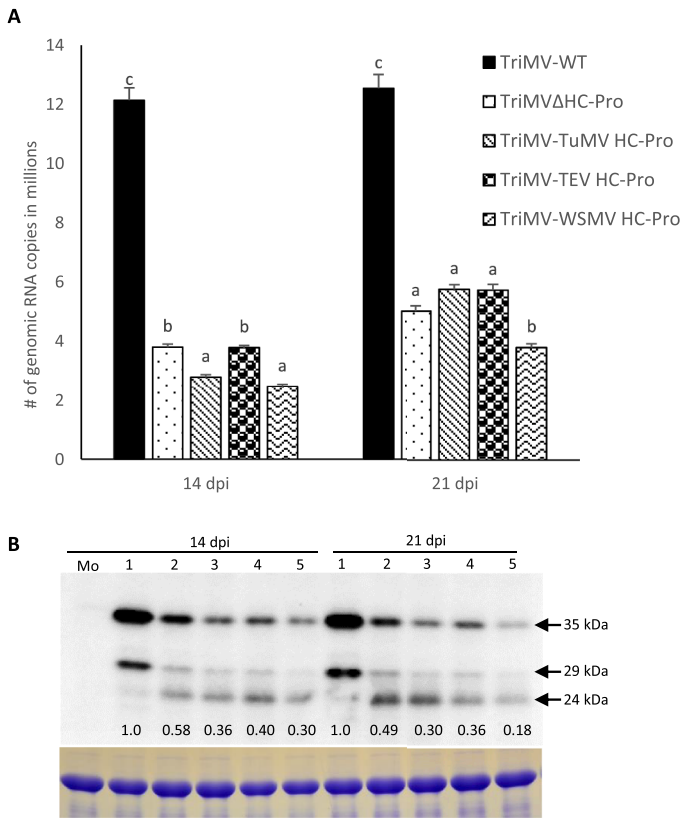

Fig. 7. TriMV with heterologous HC-Pro affected accumulation of genomic RNA copies and coat protein (CP). A. Absolute quantification of TriMV genomic RNA copies from wheat infected with TriMV harboring heterologous HC-Pro from turnip mosaic virus (TuMV), tobacco etch virus (TEV), or wheat streak mosaic virus (WSMV) at 14 and 21 days postinoculation (dpi). Reverse transcription real-time PCR was used for the quantification of TriMV genomic RNA copies per 100 ng of total RNA was shown in histograms with standard error. Different letters above the histograms represent significant differences in genomic RNA accumulation among different mutants at $P < 0.05$ at each time point, while the same letters indicate no significant difference. B. Western immunoblot analysis of total proteins from wheat leaves infected with TriMV harboring heterologous HC-Pro from TuMV (lane 3), TEV (lane 4), or WSMV (lane 5) at 14 and 21 dpi. Lane Mo: Mock-inoculated wheat; Lane 1: TriMV-WT; Lane 2: TriMV-ΔHC-Pro. TriMV CP antibodies in Western blots reacted with a major CP of 35 kDa and two minor proteins of 29 and 24 kDa. Note that accumulation of TriMV CP was drastically reduced in wheat leaves infected with TriMV harboring heterologous HC-Pro. Numbers below the Western blot indicate fold change in CP accumulation of TriMV with heterologous HC-Pros over TriMV-WT. A Coomassie-stained SDS-PAGE gel below the Western immunoblot showed a large fragment of wheat RuBisCo protein as a loading control for total protein loaded per well.

et al., 2007; Valli et al., 2018). In wheat, the absence of HC-Pro in TriMV resulted in delayed mild symptoms, as opposed to moderate chlorotic streaks, mosaic, and mottling symptoms observed in plants infected with TriMV-WT. In addition, TriMV-ΔHC-Pro did not cause any noticeable stunting symptoms in wheat, unlike TriMV-WT which resulted in moderate stunting. These findings indicate that HC-Pro is a key factor in symptom development by TriMV in wheat. In contrast, WSMV with HC-Pro deletion displayed similar symptoms as wild-type WSMV in wheat (Stenger et al., 2005a). Furthermore, HC-Pro also plays a role in efficient TriMV genomic RNA amplification. Our data showed that the absence of HC-Pro led to a significant reduction in TriMV genomic RNA accumulation compared to TriMV-WT. Additionally, the accumulation of CP was also substantially reduced at 9, 14, and 21 dpi compared to TriMV-WT. These results suggest that HC-Pro is essential for efficient replication of TriMV in wheat, and the reduced accumulation of genomic RNA and CP in the absence of HC-Pro may have contributed to mild symptoms observed in wheat.

Accumulation of genomic RNA copies and CP of TriMV with deletion of amino acids 3 and 4 in HC-Pro was similar to those of TriMV-WT but not in mutants comprising deletions between amino acids 4 and 25. These data suggest only amino acids 3 and 4 in HC-Pro are dispensable for TriMV symptom induction and genomic RNA amplification. Unexpectedly, deletion of amino acids 3 to 50 or 3 to 125 elicited more severe chlorotic streaks, mosaic, and leaf yellowing symptoms with severely stunted plants compared to moderate symptoms with no leaf yellowing by TriMV-WT. The severe symptom phenotype of TriMV with deletion of amino acids 3 to 50 or 3 to 125 in HC-Pro was accompanied by a dramatic increase in genomic RNA accumulation compared to wild-type virus, but the CP was reduced similarly to TriMV-ΔHC-Pro. Why did the enhanced symptom phenotype with increased accumulation of genomic RNAs did not reflect similar levels of increase in CP accumulation? Does this suggest that increased accumulation of genomic RNA but not CP of TriMV with deletion of amino acids 3 to 50 or 3 to 125 is responsible for enhanced symptom phenotype? Deleting amino acids 3 to 50 or 3 to 125 in HC-Pro might have affected CP translation, stability, or both, which might have resulted in differential accumulation of genomic RNA and CP. Alternatively, TriMV with deletion of amino acids 3 to 50 or 3 to 125 may have contributed to increased levels of HC-Pro accumulation, which could have led to more severe symptoms. However, we were unable to perform experiments to confirm this hypothesis due to the unavailability of HC-Pro antibodies. It is also possible that these deletion mutants might have interacted more efficiently with host susceptibility genes than TriMV-WT. Previously, deletions in the CP of WSMV resulted in an enhanced symptom phenotype with increased accumulation of both genomic RNA copies and CP (Tatineni et al., 2014, 2017).

Additionally, deletion of amino acids 3 to 50 or 3 to 125 could have resulted in structural changes in HC-Pro, leading to more severe symptom phenotypes due to alterations in the high-ordered protein structures. To investigate this further, we used the Robetta protein structure predicted server (Baek et al., 2021) to generate the predictive structural models for HC-Pro amino acid sequences for full-length and for deletion of amino acids 3 to 25 (mild mutant), and 3 to 50 and 3 to 125 (severe mutants). Our analysis revealed that when all of these structures are oriented with the C-terminal protease domain in the same orientation, the N-terminal domains overlap and point in the same direction (Fig. 8). The severe symptom-inducing HC-Pro mutants with deletion of amino acids 3 to 50 or 3 to 125 lacked α-helical extensions and allowed for closer packing of the N-terminal domains compared to the full-length and mild mutant (deletion of amino acids 3 to 25) models (Fig. 8, compare C and D with A and B). This altered domain packing may have contributed to the increased virulence of these deletion mutants. It is important to note that these findings are similar to observations made of HC-Pro from lettuce mosaic virus, where the deletion of amino acids 1 to 100 appears to cause N-terminal domain packing interactions not observed in full-length HC-Pro (Plisson et al., 2003).

We tested the compatibility of HC-Pros from different potyvirids in the TriMV genome for symptom development and RNA amplification. Our findings show that TriMV with heterologous HC-Pros resulted in mild symptoms with no or little stunting of plants, similar to TriMV-ΔHC-Pro. The presence of heterologous HC-Pros did not support TriMV genomic RNA accumulation, as it accumulated at significantly reduced levels compared to TriMV-WT. This suggests that heterologous HC-Pros with 10 to 22 % amino acid sequence identity did not function well in the TriMV genome. The phenotypes of TriMV with heterologous HC-Pros were similar to TriMV-ΔHC-Pro, which may have been caused by incompatible protein-protein interactions with wheat or other TriMV-encoded proteins. Replacement of HC-Pro of tobacco vein mottle virus and plum pox virus with corresponding cistron of zucchini yellow mosaic virus and TEV, respectively, resulted in viable chimeric viruses but produced attenuated symptoms, suggesting only partial functional

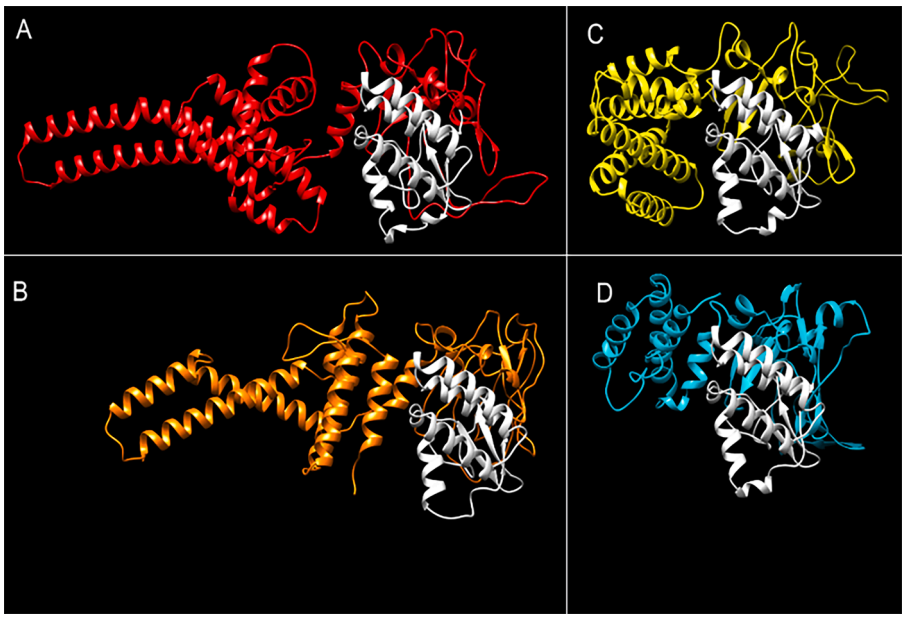

Fig. 8. Predicted structural models of Triticum mosaic virus (TriMV) HC-Pro and its deletion mutants using the Robetta protein structure prediction server. Predicted ribbon structures of TriMV full-length HC-Pro (A), mild symptoms inducing TriMV HC-Pro mutant with deletion of amino acids 3 to 25 (B), and severe symptom inducing mutants with deletion of amino acids 3 to 50 (C), or 3 to 125 (D). The protease core structure (white ribbon) of TriMV HC-Pro is shown in the same orientation for the full-length and its three deletion mutants.

compatibility of HC-Pro (Atreya and Pirone, 1993; Sáenz et al., 2002). In contrast, the reciprocal exchange of HC-Pros with 49 % amino acid identity between soybean mosaic virus and clover yellow vein virus resulted in functionally compatible chimeric viruses (Wang et al., 2020). WSMV with heterologous HC-Pros from different potyvirids resulted in a viable virus because WSMV HC-Pro is dispensable for systemic infection but elicited varied symptom phenotypes (Stenger et al., 2004).

In conclusion, the HC-Pro cistron is expendable for TriMV systemic infection in wheat but is crucial for eliciting severe symptoms and achieving efficient genomic RNA and CP accumulation. Except for amino acids 3 and 4 in HC-Pro, the progressive deletions up to amino acid 25 in TriMV elicited increasingly mild symptoms. However, deletion of HC-Pro amino acids 3 to 50 or 3 to 125 in TriMV induced severe symptoms with a drastic increase and decrease in genomic RNA and CP accumulation, respectively. This is an unusual anomaly where severe symptoms are induced by enhanced genomic RNA but not the CP. Further research is needed to understand why TriMV lacking amino acids 3 to 50 or 3 to 125 causes severe symptom phenotypes in wheat with differential accumulation of genomic RNA and CP. The availability of a range of HC-Pro deletion mutants would facilitate determining the requirement of HC-Pro for wheat curl mite transmission of TriMV.

Author Statement

All authors reviewed and approved to submit the revised manuscript.

Funding

This study was in part supported by a grant from the National Science Foundation (#1758912) and USDA-ARS (#3042–21000–035–000D).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

All data generated in this study are included in this published article.

Acknowledgments

We thank Valerian Dolja, Oregon State University, for providing pCB-TuMV-GFP and pTEV-7DA-HAT clones. We also would like to thank Jonathan Horrell for his technical assistance, and Sourav Pal for developing TriMV with heterologous HC-Pro sequences. USDA is an equal opportunity provider and employer. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

References

Anandalakshmi, R., Pruss, G.J., Ge, X., Marathe, R., Mallory, A.C., Smith, T.H., Vance, V. B., 1998. A viral suppressor of gene silencing in plants. Proc. Natl. Acad. Sci. U.S.A. 95, 13079–13084.

Atreya, C.D., Atreya, P.L., Thornbury, D.W., Pirone, T.P., 1992. Site-directed mutations in the potyvirus HC-Pro gene affect helper component activity, virus accumulation, and symptom expression in infected tobacco plants. Virology 191, 106–111.

Atreya, C.D., Pirone, T.P., 1993. 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. Proc. Natl. Acad. Sci. U.S.A. 90, 11919–11923.

Baek, M., DiMaio, F., Anishchenko, I., Dauparas, J., Ovchinnikov, S., Lee, G.R., Wang, J., Cong, Q., Kinch, L.N., Schaeffer, R.D., Millán, C., Park, H., Adams, C., Glassman, C. R., DeGiovanni, A., Pereira, J.H., Rodrigues, A.V., van Dijk, A.A., Ebrecht, A.C., Opperman, D.J., Sagmeister, T., Buhlheller, C., Pavkov-Keller, T., Rathinaswamy, M. K., Dalwadi, U., Yip, C.K., Burke, J.E., Garcia, K.C., Grishin, N.V., Adams, P.D., Read, R.J., Baker, D., 2021. Accurate prediction of protein structures and interactions using a three-track neural network. Science 373, 871–876.

Burrows, M., Franc, G., Rush, C., Blunt, T., Ito, D., Kinzer, K., Olson, J., O'Mara, J., Price, J., Tande, C., Ziems, A., Stack, J., 2009. Occurrence of viruses in wheat in the Great Plains region, 2008. Plant Health Progress. Online publication. https://doi.org/10.1094/PHP-2009-0706-01-RS.

Byamukama, E., Tatineni, S., Hein, G.L., Graybosch, R.A., Baenziger, P.S., French, R., Wegulo, S.N., 2012. Effects of single and double infections of winter wheat by Triticum mosaic virus and Wheat streak mosaic virus on yield determinants. Plant Dis. 96, 859–864.

Byamukama, E., Seifers, D.L., Hein, G.L., De Wolf, E., Tisserat, N.A., Langham, M.A.C., Osborne, L.E., Timmerman, A., Wegulo, S.N., 2013. Occurrence and distribution of Triticum mosaic virus in the central Great Plains. Plant Dis. 97, 21–29.
Choi, I.R., French, R., Hein, G.L., Stenger, D.C., 1999. Fully biologically active *in vitro* transcripts of the eriophyid mite-transmitted wheat streak mosaic tritimovirus. Phytopathology 89, 1182–1185.
Dolja, V.V., McBride, H.J., Carrington, J.C., 1992. Tagging of plant potyvirus replication and movement by insertion of -glucuronidase into the viral polyprotein. Proc. Natl. Acad. Sci. U.S.A. 89, 10208–10212.
Dombrovsky, A., Reingold, V., Antignus, Y., 2014. Ipomovirus-an atypical genus in the family Potyviridae transmitted by whiteflies. Pest Manag. Sci. 70, 1553–1567.
Fellers, J.P., Seifers, D., Ryba-White, M., Joe Martin, T., 2009. The complete genome sequence of Triticum mosaic virus, a new wheat-infecting virus of the High Plains. Arch. Virol. 154, 1511–1515.
Firth, A.E., 2014. Mapping overlapping functional elements embedded within the protein-coding regions of RNA viruses. Nucleic Acids Res. 42, 12425–12439.
Govier, D.A., Kassanis, B., 1974. Evidence that a component other than the virus particle is needed for aphid transmission of potato virus Y. Virology 57, 285–286.
Guo, B., Lin, J., Ye, K., 2011. Structure of the autocatalytic cysteine protease domain of potyvirus helper-component proteinase. J. Biol. Chem. 286, 21937–21943.
Hein, G.L., French, R., Siriwetwiwat, B., Amrine, J.W., 2012. Genetic characterization of North American populations of wheat curl mite and dry bulb mite. J. Econ. Entomol. 105, 1801–1808.
Inoue-Nagata, A.K., Jordan, R., Kreuze, J., Li, F., López-Moya, J.J., Mäkinen, K., Ohshima, K., Wylie, S.J., ICTV Report Consortium., 2022. ICTV virus taxonomy profile. Potyviridae. J. Gen. Virol. 103, 17.
Kasschau, K.D., Carrington, J.C., 1995. Requirement for HC-Pro processing during genome amplification of tobacco etch potyvirus. Virology 209, 268–273.
Kasschau, K.D., Cronin, S., Carrington, J.C., 1997. Genome amplification and long-distance movement functions associated with the central domain of tobacco etch potyvirus helper component–proteinase. Virology 228, 251–262.
Kasschau, K.D., Carrington, J.C., 1998. A counter defensive strategy of plant viruses: suppression of posttranscriptional gene silencing. Cell 95, 461–470.
Lellis, A.D., Kasschau, K.D., Whitham, S.A., Carrington, J.C., 2002. Loss-of-susceptibility mutants of Arabidopsis thaliana reveal an essential role for eIF (iso) 4E during potyvirus infection. Curr. Biol. 12, 1046–1051.
McMechan, A.J., Tatineni, S., French, R., Hein, G.L., 2014. Differential transmission of Triticum mosaic virus by wheat curl mite populations collected in the Great Plains. Plant Dis 98, 806–810.
McNeil, J.E., French, R., Hein, G.L., Baenziger, P.S., Eskridge, K.M., 1996. Characterization of genetic variability among natural populations of wheat streak mosaic virus. Phytopathology 86, 1222–1227.
Pettersen, E.F., Goddard, T.D., Huang, C.C., Couch, G.S., Greenblatt, D.M., Meng, E.C., Ferrin, T.E., 2004. UCSF Chimera-a visualization system for exploratory research and analysis. J. Comput. Chem. 25, 1605–1612.
Plisson, C., Drucker, M., Blanc, S., German-Retana, S., Le Gall, O., Thomas, D., Bron, P., 2003. Structural characterization of HC-Pro, a plant virus multifunctional protein. J. Biol. Chem. 278, 23753–23761.
Qu, F., Morris, T.J., 2005. Suppressors of RNA silencing encoded by plant viruses and their role in viral infections. FEBS Lett. 579, 5958–5964.
Revers, F., García, J.A., 2015. Molecular biology of potyviruses. Adv. Virus Res. 92, 101–199.
Rose, H., Döring, I., Vetten, H.J., Menzel, W., Richert-Pöggeler, K.R., Maiss, E., 2019. Complete genome sequence and construction of an infectious full-length cDNA clone of celery latent virus - an unusual member of a putative new genus within the Potyviridae. J. Gen. Virol. 100, 308–320.
Sáenz, P., Salvador, B., Simón-Mateo, C., Kasschau, K.D., Carrington, J.C., García, J.A., 2002. Host-specific involvement of the HC protein in the long-distance movement of potyviruses. J. Virol. 76, 1922–1931.
Seifers, D.L., Martin, T.J., Harvey, T.L., Fellers, J.P., Stack, J.P., Ryba-White, M., Haber, S., Krokhin, O., Spicer, V., Lovat, N., Yamchuk, A., 2008. Triticum mosaic virus: a new virus isolated from wheat in Kansas. Plant Dis. 92, 808–817.
Seifers, D.L., Martin, T.J., Harvey, T.L., Fellers, J.P., Michaud, J.P., 2009. Identification of the wheat curl mite as the vector of Triticum mosaic virus. Plant Dis. 93, 25–29.
Shiboleth, Y.M., Haronsky, E., Leibman, D., Arazi, T., Wassenegger, M., Whitham, S.A., Gaba, V., Gal-On, A., 2007. The conserved FRNK box in HC-Pro, a plant viral suppressor of gene silencing, is required for small RNA binding and mediates symptom development. J. Virol. 81, 13135–13148.
Stenger, D.C., French, R., 2004. Functional replacement of wheat streak mosaic virus HC-Pro with the corresponding cistron from a diverse array of viruses in the family *Potyviridae*. Virology 323, 257–267.
Stenger, D.C., Hein, G.L., Gildow, F.E., Horken, K.M., French, R., 2005a. Plant virus HC-Pro is a determinant of eriophyid mite transmission. J. Virol. 79, 9054–9061.
Stenger, D.C., French, R., Gildow, F.E., 2005b. Complete deletion of wheat streak mosaic virus HC-Pro: a null mutant is viable for systemic infection. J. Virol. 79, 12077–12080.
Tatineni, S., French, R., 2016. The coat protein and NIa protease of two *Potyviridae* family members independently confer superinfection exclusion. J. Virol. 90, 10886–10905.
Tatineni, S., Hein, G.L., 2023. Plant viruses of agricultural importance: current and future perspectives of virus disease management strategies. Phytopathology 113, 117–141.
Tatineni, S., Ziems, A.D., Wegulo, S.N., French, R., 2009. Triticum mosaic virus: a distinct member of the family *Potyviridae* with an unusually long leader sequence. Phytopathology 99, 943–950.
Tatineni, S., Graybosch, R.A., Hein, G.L., Wegulo, S.N., French, R., 2010. Wheat cultivar-specific disease synergism and alteration of virus accumulation during co-infection with wheat streak mosaic virus and Triticum mosaic virus. Phytopathology 100, 230–238.
Tatineni, S., McMechan, A.J., Hein, G.L., French, R., 2011. Efficient and stable expression of GFP through Wheat streak mosaic virus-based vectors in cereal hosts using a range of cleavage sites: formation of dense fluorescent aggregates for sensitive virus tracking. Virology 410, 268–281.
Tatineni, S., Qu, F., Li, R., Morris, T.J., French, R., 2012. Triticum mosaic poacevirus enlists P1 rather than HC-Pro to suppress RNA silencing-mediated host defense. Virology 433, 104–115.
Tatineni, S., Sarath, G., Seifers, D., French, R., 2013. Immunodetection of Triticum mosaic virus by DAS-and DAC-ELISA using antibodies produced against coat protein expressed in Escherichia coli: potential for high-throughput diagnostic methods. J. Virol. Methods 189, 196–203.
Tatineni, S., Kovacs, F., French, R., 2014. Wheat streak mosaic virus infects systemically despite extensive coat protein deletions: identification of virion assembly and cell-to-cell movement determinants. J. Virol. 88, 1366–1380.
Tatineni, S., McMechan, A.J., Bartels, M., Hein, G.L., Graybosch, R.A., 2015. *In vitro* transcripts of wild-type and fluorescent protein-tagged Triticum mosaic virus (family *Potyviridae*) are biologically active in wheat. Phytopathology 105, 1496–1505.
Tatineni, S., Elowsky, C., Graybosch, R.A., 2017. Wheat streak mosaic virus coat protein deletion mutants elicit more severe symptoms than wild-type virus in multiple cereal hosts. Mol. Plant-Microbe Interact. 30, 974–983.
Tatineni, S., Alexander, J., Gupta, A.K., French, R., 2019. Asymmetry in synergistic interaction between wheat streak mosaic virus and Triticum mosaic virus in wheat. Mol. Plant-Microbe Interact. 32, 336–350.
Tamura, K., Stecher, G., Kumar, S., 2021. MEGA 11: molecular evolutionary genetics analysis version 11. Mol. Biol. Evol. https://doi.org/10.1093/molbev/msab120.
Valli, A.A., Gallo, A., Rodamilans, B., López-Moya, J.J., García, J.A., 2018. The HC-Pro from the *Potyviridae* family: an enviable multitasking helper component that every virus would like to have. Mol. Plant Pathol. 19, 744–763.
You, Y., Shirako, Y., 2010. *Bymovirus* reverse genetics: requirements for RNA2-encoded proteins in systemic infection. Mol. Plant Pathol. 11, 383–394.
Young, B.A., Hein, G.L., French, R., Stenger, D.C., 2007. Substitution of conserved cysteine residues in wheat streak mosaic virus HC-Pro abolishes virus transmission by the wheat curl mite. Arch. Virol. 152, 2107–2111.
Young, B.A., Stenger, D.C., Qu, F., Morris, T.J., Tatineni, S., French, R., 2012. Tritimovirus P1 functions as a suppressor of RNA silencing and an enhancer of disease symptoms. Virus Res. 163, 672–677.
Wang, Y., Xu, W., Abe, J., Nakahara, K.S., Hajimorad, M.R., 2020. Precise exchange of the helper-component proteinase cistron between soybean mosaic virus and clover yellow vein virus: impact on virus viability and host range specificity. Phytopathology 110, 206–214.