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ABSTRACT

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The tritimovirus *Wheat streak mosaic virus* (WSMV) and the machlomovirus *Maize chlorotic mottle virus* (MCMV) each cause systemic chlorosis in infected maize plants. Infection of maize with both viruses produces corn lethal necrosis disease (CLND). Here, we report that complete deletion of the WSMV helper component-proteinase (HC-Pro) coding region had no effect on induction of CLND symptoms following coinoculation of maize with WSMV and MCMV. We further demonstrated that elevation of virus titers in double infections, relative to single infections, also was independent of WSMV HC-Pro. Thus, unlike potyvirus HC-Pro, WSMV HC-Pro was dispensable for disease synergism. Because disease synergism involving potyviruses requires HC-Pro-mediated suppression of posttranscriptional gene silencing (PTGS), we hypothesized that WSMV HC-Pro may not be a suppressor of PTGS. Indeed, WSMV HC-Pro did not suppress PTGS of a green fluorescent protein (GFP) transgene in an *Agrobacterium*-mediated coinfiltration assay in which potyvirus HC-Pro acted as a strong suppressor. Furthermore, coinfiltration with potyvirus HC-Pro, but not WSMV HC-Pro, resulted in elevated levels of the GFP target mRNA under conditions which trigger PTGS. Collectively, these results revealed significant differences in HC-Pro function among divergent genera of the family *Potyviridae* and suggest that the tritimovirus WSMV utilizes a gene other than HC-Pro to suppress PTGS and mediate synergistic interactions with unrelated viruses.

Coinfection of plants by two unrelated viruses may result in disease synergism with symptoms more severe than the additive effects of each virus individually. Corn lethal necrosis disease (CLND) is a field example of synergism caused by double infection of maize with the machlomovirus *Maize chlorotic mottle virus* (MCMV) and one of several maize-infecting potyviruses (13,25,41). Under laboratory conditions, the tritimovirus *Wheat streak mosaic virus* (WSMV) may be substituted for the potyvirus to experimentally generate CLND symptoms in double infections with MCMV (32). Separately, each virus induces systemic chlorosis but not necrosis. In contrast, infection of maize with both viruses causes extensive necrosis, stunting, and premature death of infected plants. Also, as with many synergistic interactions among unrelated plant viruses (2,7,13,15,16,30,34,43, 44,46), infection of maize with WSMV and MCMV may result in elevated virus titers compared with that observed in single infections (32).

Potyvirus helper component-proteinase (HC-Pro) mediates synergistic interactions among unrelated viruses in double infections (14,28,31,33,44) by posttranscriptional gene silencing (PTGS) suppression (1,6,8,20,22,45). Although WSMV HC-Pro shares cysteine proteinase functions in common with potyvirus HC-Pro (38) and is required for semipersistent transmission of WSMV by the wheat curl mite (38,39), a role for tritimovirus HC-Pro in disease synergism and suppression of PTGS has not been investigated. Recently, it was demonstrated (36) that WSMV tolerated complete deletion of the HC-Pro coding region (the P1/P3 junction was cleaved by P1 proteinase) without alteration of pathogenicity or virulence in maize. This unexpected observation prompted us to hypothesize that tritimovirus HC-Pro function may differ from that of potyvirus HC-Pro with respect to synergism and PTGS suppression. In this study, we evaluated disease phenotype and relative virus titer in maize plants co-infected with MCMV and progeny virus derived from WSMV constructs encoding or lacking HC-Pro. We further evaluated WSMV HC-Pro function using a well established transient assay for suppression of PTGS (29).

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MATERIALS AND METHODS

Virus isolates. The MCMV isolate used in these experiments was the same as that described by Goldberg and Brakke (13). MCMV was recovered from dried maize leaves (after nearly 20 years in storage at 4°C) by sap inoculation of maize inbred line 'SDp2'. MCMV infection of SDp2 plants caused mild systemic mottling symptoms and was verified by reverse transcription-polymerase chain reaction (RT-PCR) using primers for the coat protein (CP) gene (described below) based on the complete nucleotide sequence of MCMV (26; GenBank Accession no. NC_003627). The 673-bp RT-PCR product was cloned into pGEMT-easy (Promega, Madison, WI) and a consensus sequence (corresponding to MCMV nucleotides 3422 to 4094) based on four independent clones was compared with NC_003627. The consensus sequence was identical to that of the corresponding

region of NC_003627, except for two synonymous C to U transitions at nucleotides 3704 and 3929.

The WSMV genotypes evaluated in this study were based on an infectious cDNA clone (10) of the WSMV-Sidney 81 isolate (37; GenBank accession no. NC_001886). Three WSMV constructs (Fig. 1A) modified from the original infectious clone (pACYC-WSMV) were used as templates for in vitro transcription (10): pS81-SA (containing *Sal*I and *Apa*I sites flanking the HC-Pro coding region), pS81-SAS12 (containing an additional *Sal*I site immediately downstream of the *Apa*I site), and pS81ΔHC12-5 (completely lacking HC-Pro and derived from pS81-SAS12 by deletion of the *Sal*I fragment). Transcripts derived from all three WSMV constructs were previously shown to be infectious to wheat and stable upon passage by sap transmission to both wheat and SDp2 maize (35,36).

Establishment of single and double infections in maize inbred line SDp2. Symptomatic tissue (≈2 g) from leaves of SDp2 plants individually infected with MCMV or each WSMV genotype was ground in water (40 ml) with a mortar and pestle. To establish single infections, 10 ml of each inocula was diluted with 1 volume of water and applied mechanically to five SDp2 seedlings (two- to three-leaf stage). To establish double infections of MCMV and each WSMV genotype, mixtures of paired inocula (10 ml each) were mechanically inoculated onto five SDp2 seedlings each. Celite was added to all inocula as an abrasive. Uninoculated SDp2 seedlings served as negative controls. Following mechanical inoculation, seedlings were rinsed with water and placed in a growth chamber (16 h light/day, 24°C during the day and 21°C at night). Verification of single and double infections was accomplished by enzyme-linked immunosorbent assay (ELISA) and RT-PCR (described below). The entire experiment was repeated except that plants were maintained under different lighting conditions (12 h light per day) in the second experiment. In both experiments, plants representative of each virus inoculation treatment held beyond 30 days postinoculation (dpi) were moved to a greenhouse (because of growth chamber limits on plant height) and monitored for symptom development through 50 dpi.

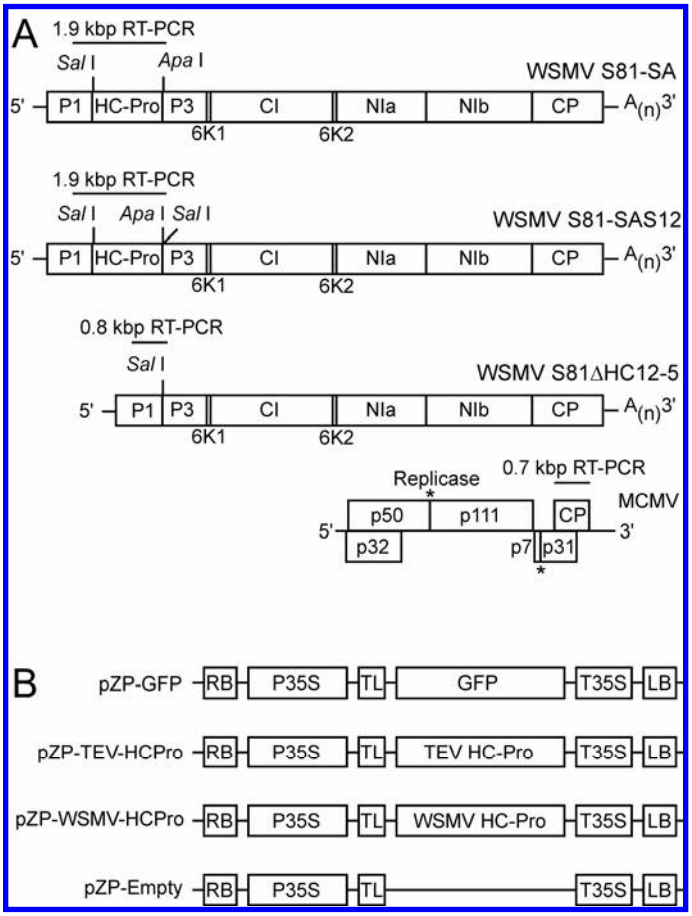

Fig. 1. Virus genome maps and construct diagrams. **A,** Genome maps of *Maize chlorotic mottle virus* (MCMV) and three *Wheat streak mosaic virus* (WSMV) genotypes (S81-SA, S81-SAS12, and S81ΔHC12-5) used in disease synergism assays. WSMV genotypes S81-SA and S81-SAS12 both encode helper component-proteinase (HC-Pro) and are identical except for a second *Sal*I site engineered into S81-SAS12. WSMV genotype S81ΔHC12-5 lacks the HC-Pro coding region and was constructed by deletion of the *Sal*I fragment of S81-SAS12. The location and size (in kilobase pairs) of reverse transcription-polymerase chain reaction products used to verify infection of plants are indicated above each genome map. **B,** Schematic representation of expression cassettes placed within the *Agrobacterium tumefaciens* binary shuttle vector pZP212 used in posttranscriptional gene silencing suppression assays. Abbreviations are as follows: right border (RB), tandem 35S promoter (P35S), *Tobacco etch virus* RNA leader (TL), green fluorescent protein (GFP), *Tobacco etch virus* HC-Pro (TEV HC-Pro), *Wheat streak mosaic virus* HC-Pro (WSMV HC-Pro), 35S terminator (T35S), and left border (LB).

RT-PCR. Total nucleic acid samples were extracted (24) from symptomatic, systemically infected SDp2 leaf tissue (≈0.5 g). Complimentary DNA was synthesized by reverse transcription using total nucleic acid samples as templates and a mixture of reverse primers MCMVR and HCR as described (36). MCMVR annealed to MCMV RNA nucleotides 4094 to 4075, whereas HCR annealed to WSMV-Sidney 81 sequences immediately downstream of the HC-Pro coding region (nucleotides 2373 to 2345). PCR amplification of MCMV or WSMV sequences was accomplished in separate reactions using cDNA as a template with primers MCMVR and MCMVF (MCMV nucleotides 3422 to 3441) or primers HCR and P1V1 (WSMV-Sidney 81 nucleotides 421 to 445). PCR conditions were as described previously (36) with the assays designed to be qualitative, not quantitative. Amplification of MCMV sequences was expected to generate an ≈0.7-kbp product; amplification of WSMV sequences was expected to generate an ≈1.9-kbp product for genotypes containing the HC-Pro coding region or an ≈0.8-kbp product from the WSMV genotype lacking HC-Pro (Fig. 1A). For each plant evaluated for relative virus titer by ELISA (described below), infection status was independently verified by RT-PCR.

Estimation of relative virus titer in single and double infections. Commercially available double antibody sandwich ELISA kits (Agdia, Elkhart, IN) were used to determine relative titer of MCMV or WSMV (39). Systemically infected, symptomatic leaf tissue (1 g) was sampled at two time points: 17 and 30 dpi in experiment 1 or 15 and 28 dpi in experiment 2. At each time point, tissue samples were collected from the second and third youngest leaves. This sampling strategy avoided necrotic areas that developed on older leaves of plants infected with both viruses.

Leaf samples were ground in 10 ml of ELISA extraction buffer. A 2-ml aliquot of each ground sample was subjected to brief centrifugation (30 s, 12,000 × *g*), and the supernatant was serially diluted (twofold increments) in extraction buffer. Serially diluted samples (corresponding to 50, 25, 12.5, 6.25, 3.12, and 1.56 mg of leaf tissue per ml) were applied to microtiter plates previously coated with antibodies to MCMV or WSMV. For each virus treatment at each time point, a total of four samples (two from each of two plants) were tested with each antiserum. Different plants were sampled at each time point. ELISA absorbance values were recorded when a positive-reacting sample first achieved a value of ≈2.2 optical density (OD) with MCMV antiserum or ≈1.7 OD with WSMV antiserum. Corresponding mean absorbance values of uninfected samples were ≈0.10 OD (MCMV antiserum) or ≈0.15 OD (WSMV antiserum).

Within each time point, mean ELISA absorbance values for each sample dilution were plotted (after subtraction of mean absorbance values obtained with uninfected plant samples) on a log-log scale (e.g., mean ELISA absorbance value as a function of

sample dilution). Relative titers of MCMV or WSMV were estimated for each virus treatment within a time point using the ELISA absorbance versus sample dilution plot of single infections with S81-SA or MCMV as standards. Thus, within each time point, virus titer of S81-SA or MCMV in single infections was assigned a value of 100% with titers of the remaining virus treatments for that time point estimated as a percentage relative to the appropriate standard. Each relative virus titer estimate was reported as the mean percentage of the appropriate standard based on mean ELISA absorbance values obtained for three or four serial dilutions that plotted within the range of the appropriate standard. Relative titers were only compared within a single time point as differences in absolute titer of samples used as standards at different time points were not assessed.

PTGS suppression assay. A transient in planta assay previously described by Qu et al. (29) was used to evaluate the potential of WSMV HC-Pro to suppress PTGS. *Tobacco etch virus* (TEV) HC-Pro served as a positive control in the PTGS suppression assay. The TEV HC-Pro coding region was amplified by PCR using pRTL2-0027 (provided by J. C. Carrington) as template; WSMV-Sidney 81 HC-Pro was amplified by PCR using pACYC-WSMV (10) as template. Both HC-Pro coding regions were amplified with virus-specific primers incorporating a start codon within a *Bsp*HI site (5′-proximal primers) and a stop codon followed by an *Xba*I site (WSMV) or *Kpn*I site (TEV) (3′-proximal primers). Primer sequences for TEV HC-Pro were 5′-TCATGAGCGACAAATCAATCTCTGAGGCATTCT-3′ and 5′-GGTACCTTATCCAACATTGTAAGTTTTCATTTC-3′; primer sequences for WSMV HC-Pro were 5′-TCATGAGCGGCGACCAAGCAGTGAACAAAG-3′ and 5′-TCTAGATTAGCCAATTTTGTAATCTTTCATTG-3′. The resulting PCR products were cloned into pGEMT-easy and verified by sequencing of the cloned inserts. Each HC-Pro coding region was excised from pGEMT-easy with *Bsp*HI and *Xba*I or *Kpn*I and used to replace the *Nco*I–*Xba*I or *Nco*I–*Kpn*I fragment (containing the *Turnip mosaic virus* [TuMV] P1/HC-Pro coding region) of pRTL2-TuMV. The resulting constructs contained the TEV or WSMV HC-Pro coding region as part of an expression cassette (35S promoter/TEV leader/HC-Pro/35S terminator) that was excised by digestion of flanking *Pst*I sites and positioned between the *Agrobacterium tumefaciens* T-DNA right and left borders (Fig. 1B) of the shuttle/binary plasmid pZP212 (provided by T. Clemente). The resulting pZP212 derivatives were electroporated into *A. tumefaciens* and selected by using the appropriate antibiotic regime. *A. tumefaciens* cultures were verified as harboring the appropriate pZP212 derivatives by PCR by using the HC-Pro primers described previously. Another pZP212 derivative containing only the 35S promoter/TEV leader/35S terminator was constructed and placed into *A. tumefaciens* (29) and served as an "empty vector" negative control (Fig. 1B).

A. tumefaciens cultures were grown and prepared for infiltration as described by Qu et al. (29). Attached leaves of transgenic *Nicotiana benthamiana* plants (line 16c provided by D. C. Baulcombe) constitutively expressing green fluorescent protein (GFP) were infiltrated with *A. tumefaciens* cultures or mock infiltrated with cell resuspension buffer as a negative control. *A. tumefaciens* containing a pZP212 derivative expressing GFP (29; Fig. 1B) was infiltrated into line 16c leaves to initiate PTGS of the endogenous GFP transgene. To evaluate PTGS suppression activity, *A. tumefaciens* bearing the GFP expression cassette was coinfiltrated in combination with *A. tumefaciens* cultures bearing TEV HC-Pro or WSMV HC-Pro expression cassettes or the empty vector control. Infiltrated plants were maintained in a growth chamber (12 h light/day, 24°C during the day and 21°C at night). Six days postinfiltration, GFP expression in the infiltrated leaves was assessed by fluorescence upon UV illumination. Images of UV-illuminated leaves were obtained with a digital camera by using both UV and yellow filters. For comparison, fluorescence of noninfiltrated leaves of nontransgenic *N. benthamiana* was photographed under the same conditions. GFP mRNA expression levels in infiltrated leaves were determined at 6 days postinfiltration by northern blotting of total RNA samples as described (29).

RESULTS

Symptom expression in single or double infections. The infection status of each inoculated SDp2 plant (five per treatment per replicate) was verified by qualitative RT-PCR assays during the course of both experiments. In all cases, RT-PCR indicated that plants inoculated with MCMV contained MCMV but not WSMV. Conversely, all plants inoculated with WSMV contained WSMV but not MCMV. All plants inoculated with mixtures of WSMV and MCMV contained both viruses. Because the PCR primers employed for WSMV detection flanked the HC-Pro coding region (Fig. 1A), the assay was able to distinguish infection by the HC-Pro deletion mutant S81ΔHC12-5 from the other two WSMV genotypes encoding HC-Pro (S81-SA and S81-SAS12) based on product size (0.8 kbp versus 1.9 kbp, respectively). No RT-PCR products were observed for uninfected control plants. Representative RT-PCR assays of plants sampled from experiment 1 at 17 dpi are shown in Figure 2.

Plants singly infected with MCMV developed mild chlorotic mottling symptoms beginning ≈7 to 10 dpi with similar symptoms appearing on new leaves as they expanded through 50 dpi in both experiments. No substantial differences in the timing or severity of symptoms were observed among plants individually inoculated with each of the three WSMV genotypes through 50 dpi in both experiments. In single infections, each WSMV genotype induced typical systemic mosaic symptoms that first appeared ≈9 to 13 dpi

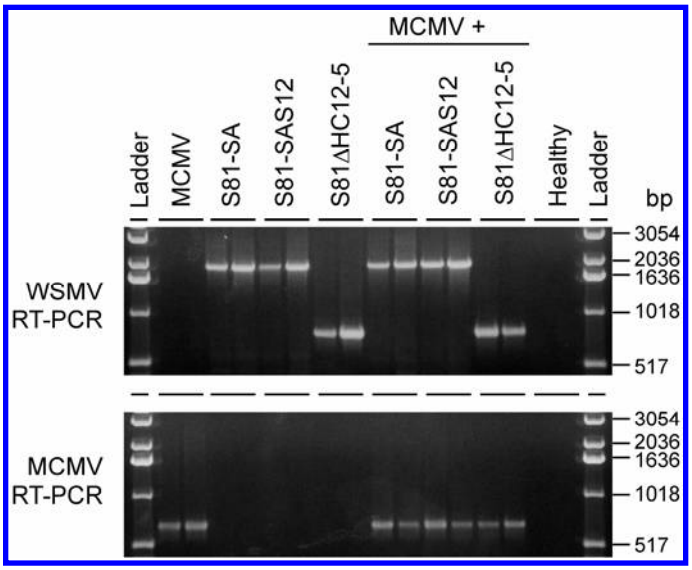

Fig. 2. Reverse transcription-polymerase chain reaction (RT-PCR) verification of single and double infections. Presented are images of 1% agarose gels following electrophoresis of RT-PCR products amplified from SDp2 maize plants singly or doubly infected with *Wheat streak mosaic virus* (WSMV) and/or *Maize chlorotic mottle virus* (MCMV). Lane labels correspond to RT-PCR amplification of samples (two each) extracted from uninfected plants (healthy) or plants infected with MCMV alone, with each of three WSMV genotypes (S81-SA, S81-SAS12, and S81ΔHC12-5) alone, or with MCMV and each of the three WSMV genotypes (MCMV+). Outer lanes (ladder) contain DNA standards with sizes indicated in kilobase pairs at right. RT-PCR was conducted using primers specific to WSMV (upper gel) or MCMV (lower gel). Amplification of MCMV generated a 0.7-kbp product; amplification of WSMV generated a 1.9-kpb product for genotypes encoding helper component-proteinase (HC-Pro) (S81-SA and S81-SAS12) or a 0.8-kbp product for S81ΔHC12-5 in which the HC-Pro coding region had been deleted. Note that the RT-PCR assay was designed to be qualitative and not quantitative.

Fig. 3. Expression of corn lethal necrosis disease (CLND) symptoms 30 days postinoculation. Presented are images of SDp2 maize plants singly or doubly infected with *Wheat streak mosaic virus* (WSMV) and/or *Maize chlorotic mottle virus* (MCMV). Plants singly infected with WSMV genotypes S81-SA, S81-SAS12 (both encoding helper component-proteinase [HC-Pro]), or S81ΔHC12-5 (HC-Pro deleted) expressed systemic chlorotic streak mosaic symptoms; plants singly infected with MCMV developed mild systemic chlorotic mottling. Plants doubly infected with MCMV and each of the three WSMV genotypes exhibited severe CLND symptoms characterized by extensive regions of systemic necrosis on fully developed leaves. Note that development of CLND symptoms was independent of WSMV HC-Pro. Plant shown at lower left is an uninoculated (healthy) control.

as isolated chlorotic streaks. At 15 to 17 dpi, plants singly infected with each WSMV genotype displayed more numerous, albeit still mostly separated, chlorotic streaks that by 28 to 30 dpi had coalesced. At 50 dpi, plants singly infected with each of the WSMV genotypes continued to exhibit chlorotic mosaic symptoms on newly developed leaves. Throughout both experiments, plants singly infected with MCMV or each of the three WSMV genotypes did not develop systemic necrosis.

Enhanced severity and accelerated development of symptoms were observed for all doubly infected SDp2 plants, regardless of which WSMV genotype was paired with MCMV. Systemic chlorotic streaks first appeared on doubly infected plants ≈7 to 9 dpi and were more numerous and of larger size compared with the initial systemic symptoms observed several days later for plants singly infected with WSMV. By 15 to 17 dpi, doubly infected plants were noticeably stunted, relative to singly infected plants, with patches of necrosis developing on older fully expanded leaves. By 28 to 30 dpi, doubly infected plants produced new growth with coalescent chlorotic streaks and extensive zones of necrotic tissue on older leaves. Figure 3 shows representative symptoms expressed by singly and doubly infected plants from experiment 1 at 30 dpi. By 50 dpi, singly infected plants reached the tassel growth stage with no evidence of systemic necrosis, whereas doubly infected plants failed to produce tassels, exhibited extensive systemic necrosis of leaves, and were moribund (Fig. 4).

Virus titers in single and double infections. Relative virus titers measured at two time points in each experiment are pre-

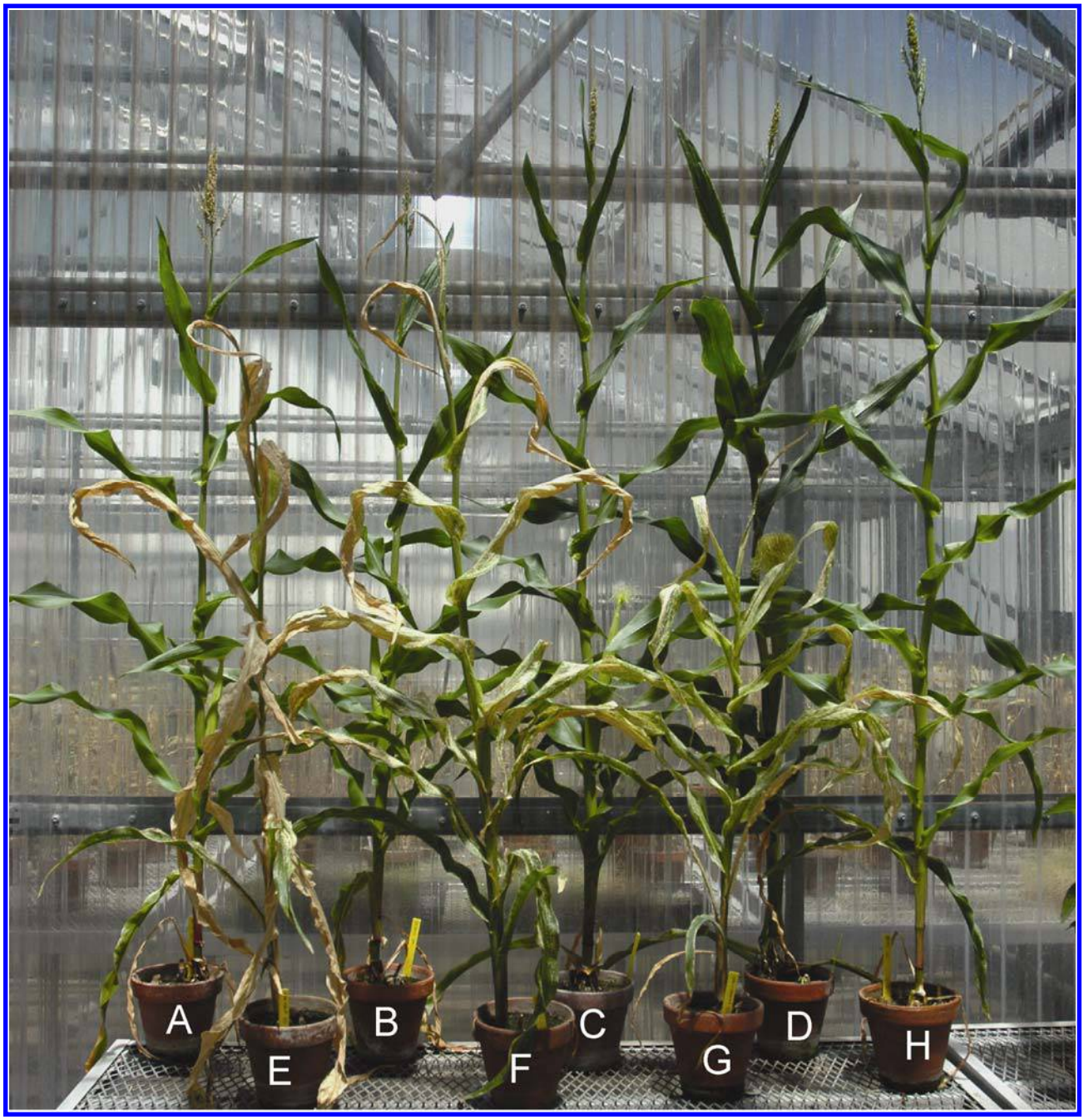

Fig. 4. Advanced symptoms of corn lethal necrosis disease (CLND) 50 days postinoculation. Presented are images of SDp2 maize plants singly or doubly infected with *Wheat streak mosaic virus* (WSMV) and/or *Maize chlorotic mottle virus* (MCMV). Plants **A to C** were singly infected with WSMV genotypes **A,** S81-SA or **B,** S81-SAS12 encoding helper component-proteinase (HC-Pro), or **C,** S81ΔHC12-5 lacking HC-Pro. Plant **D** is an uninfected SDp2 plant. Plants **E to G** were doubly infected with MCMV and WSMV genotypes **E,** S81-SA, **F,** S81-SAS12, or **G,** S81ΔHC12-5 and exhibited CLND symptoms. Plant **H** was infected with MCMV only. Note that development of CLND symptoms was independent of WSMV HC-Pro.

sented in Figure 5. In both experiments, there were minimal differences in titer among the three WSMV genotypes in single infections at 15 to 17 dpi. In contrast, titer of all three WSMV genotypes was increased approximately three- to sixfold in doubly infected plants at 15 to 17 dpi relative to S81-SA alone. However, at the second time point (28 to 30 dpi), relative titer of WSMV in doubly infected plants was not substantially greater than S81-SA alone and was, in most cases, less than that observed for S81-SA alone.

Coinfection with WSMV increased MCMV titer compared with single infections (Fig. 5). However, the mean increase in MCMV relative titer in doubly infected plants was less than twofold at the early time point (15 to 17 dpi) sampled in both experiments. At the later time point (28 to 30 dpi) sampled, mean relative titer of

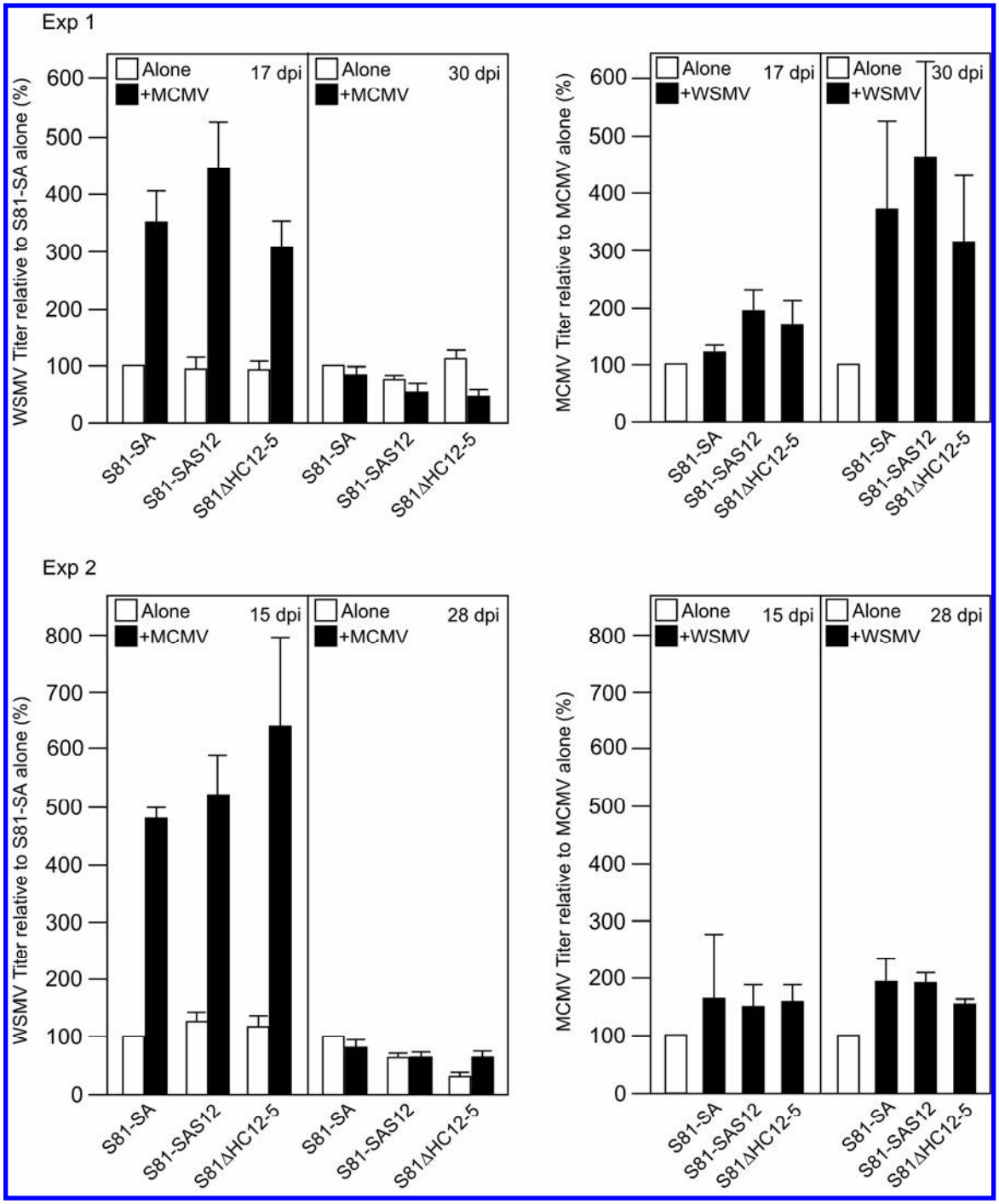

Fig. 5. Relative virus titers in SDp2 maize plants singly and doubly infected with *Wheat streak mosaic virus* (WSMV) and/or *Maize chlorotic mottle virus* (MCMV). Relative titers were estimated by enzyme-linked immunosorbent assay in two independent experiments (Exp 1 and Exp 2) at two time points (15 or 17 days postinoculation [dpi] and 28 or 30 dpi) using serial dilutions of plant sap as antigen and antiserum specific to either WSMV or MCMV. Within each time point, titer of WSMV S81-SA or MCMV was assigned a standard value of 100%. Relative virus titer of all other samples within the same time point and experiment was expressed as a percentage (mean ± SD) of the appropriate standard. Comparison of relative virus titers are valid within but not between time points. Relative virus titers for each experiment, time point, and virus are displayed above as bar graphs. Gray bars indicate single infections; black bars indicate double infections. WSMV genotypes S81-SA and S81-SAS12 encoded helper component-proteinase (HC-Pro); S81ΔHC12-5 is a WSMV deletion mutant lacking HC-Pro. MCMV titer was elevated in double infections with WSMV compared with MCMV alone at each time point in each experiment. In contrast, WSMV titer was elevated in double infections with MCMV only at the earliest time point sampled (15 and 17 dpi) in each experiment. Note that increased virus titers occurred in double infections independent of WSMV HC-Pro.

MCMV in doubly infected plants was three- to fourfold greater than for MCMV alone in one experiment, but less than twofold greater in the second experiment. Despite this variation, elevation of MCMV titer in double infections with WSMV was independent of HC-Pro.

WSMV HC-Pro lacks PTGS suppression activity. PTGS of the GFP transgene was induced upon infiltration of *N. benthamiana* line 16c leaves with *A. tumefaciens* bearing the GFP expression cassette but not by infiltration of cell resuspension buffer (Fig. 6). Coinfiltration of GFP transgenic plants with the *A. tumefaciens* culture containing the GFP expression cassette and a second *A. tumefaciens* culture expressing TEV HC-Pro resulted in high levels of GFP expression 6 days after infiltration, confirming TEV HC-Pro as a strong suppressor of PTGS. In contrast, an *A. tumefaciens* culture expressing WSMV HC-Pro did not exhibit PTGS suppression activity upon coinfiltration with the *A. tumefaciens* culture containing the GFP expression cassette (Fig. 6). Similarly, no PTGS suppression activity was detected when the GFP expressing *A. tumefaciens* culture was coinfiltrated with *A. tumefaciens* containing the empty vector control. In northern blots (Fig. 7), GFP mRNA levels were increased (as expected) by coinfiltration with *A. tumefaciens* expressing TEV HC-Pro but not by coinfiltration with *A. tumefaciens* expressing WSMV HC-Pro.

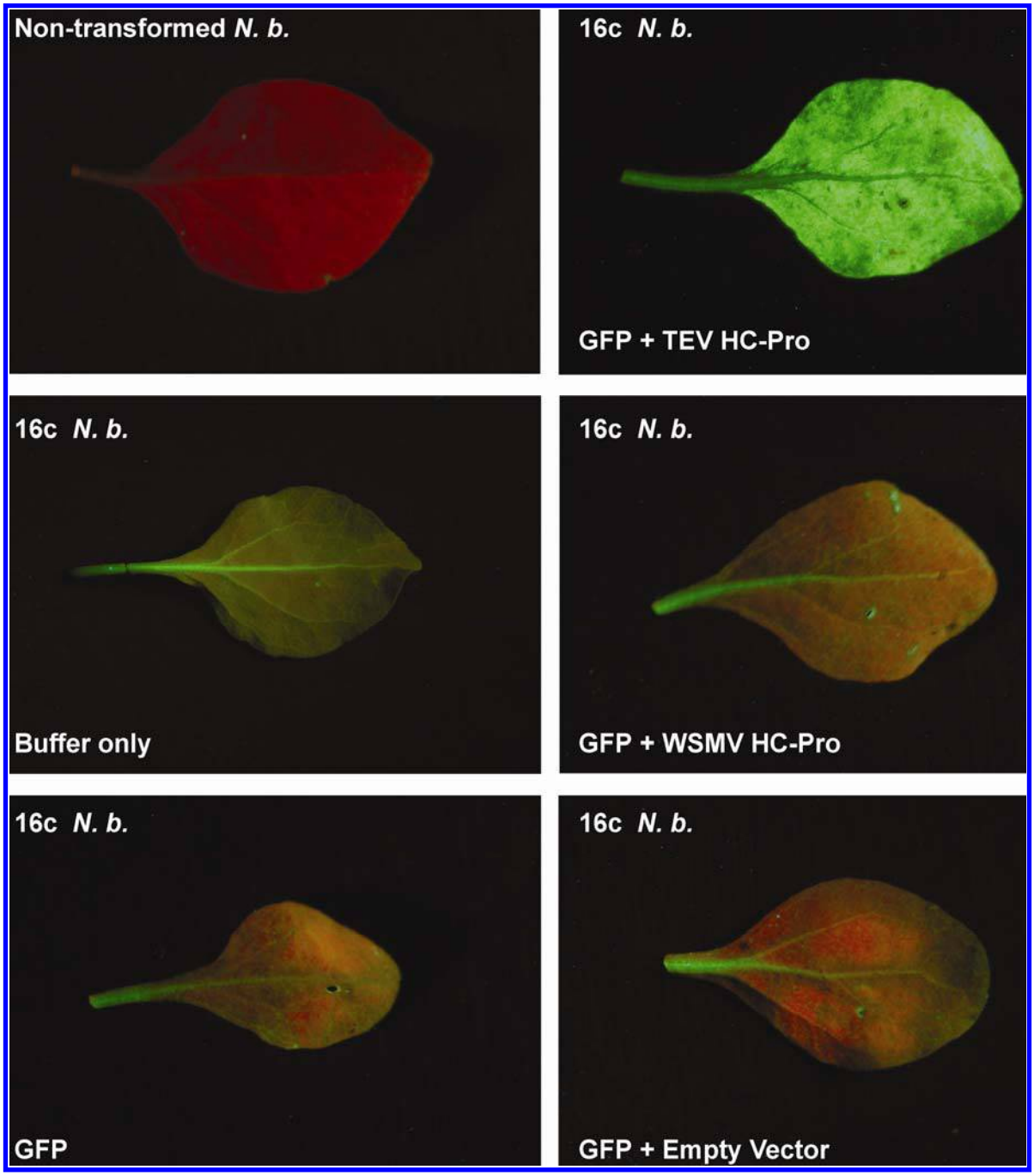

Fig. 6. Evaluation of *Wheat streak mosaic virus* (WSMV) helper component-proteinase (HC-Pro) in a posttranscriptional gene silencing suppression assay. Presented are images (photographed under UV illumination) of *Nicotiana benthamiana* leaves lacking green fluorescent protein (GFP) (nontransformed *N. b.*) or containing a constitutively expressed GFP transgene (16c *N. b.*) 6 days postinfiltration. Leaves were infiltrated with cell resuspension buffer (buffer only), or infiltrated with *Agrobacterium tumefaciens* cultures harboring binary Ti plasmids bearing an expression cassette for GFP alone or co-infiltrated with expression cassettes for GFP and *Tobacco etch virus* (TEV) HC-Pro, WSMV HC-Pro, or the Ti binary plasmid lacking an inserted gene (empty vector).

DISCUSSION

WSMV HC-Pro is dispensable for disease synergism. We successfully reproduced CLND in double infections of maize with WSMV and MCMV, whether or not the particular WSMV genotype encoded HC-Pro. The extensive necrosis, stunting, and premature death of doubly infected plants clearly satisfied the classic definition of disease synergism. We further confirmed the observation of Scheets (32) that WSMV virus titer was enhanced in doubly infected plants. However, in our experiments, WSMV titer was higher in doubly infected plants at 2 weeks but not 1 month after inoculation. We also found that titer of MCMV was greater in plants infected with both viruses compared with MCMV alone and, although the increase was of smaller magnitude than observed previously (32), the response was independent of WSMV HC-Pro. As Scheets (32) reproduced CLND symptoms in a different maize line (N28Ht) using a different isolate of MCMV (MCMV-Kansas) paired with WSMV-Sidney 81 and evaluated disease synergism under different environmental conditions, it is perhaps not surprising that some variation among the two studies was encountered. Nonetheless, our results clearly indicated that WSMV HC-Pro was not required for disease synergism.

Does WSMV encode a suppressor of PTGS? WSMV HC-Pro was unable to function as a suppressor of PTGS under conditions in which potyvirus HC-Pro was a strong suppressor of PTGS. This result can be interpreted in two ways: either WSMV HC-Pro does not suppress PTGS or the assay employed was not adequate to detect suppressor activity of WSMV HC-Pro. Although not all viral encoded suppressors of PTGS function in the *Agrobacterium*-mediated coinfiltration assay used here (23), many do, including an ever-increasing array of orthologous PTGS suppressors from unrelated viruses of both plants (9,17,19,23,29) and animals (21). Given that WSMV lacking HC-Pro remained competent to induce disease synergism with MCMV and since disease synergism in other systems is dependent on PTGS suppression (6,8,14,20,45), the simplest interpretation is that WSMV HC-Pro is not a suppressor of PTGS.

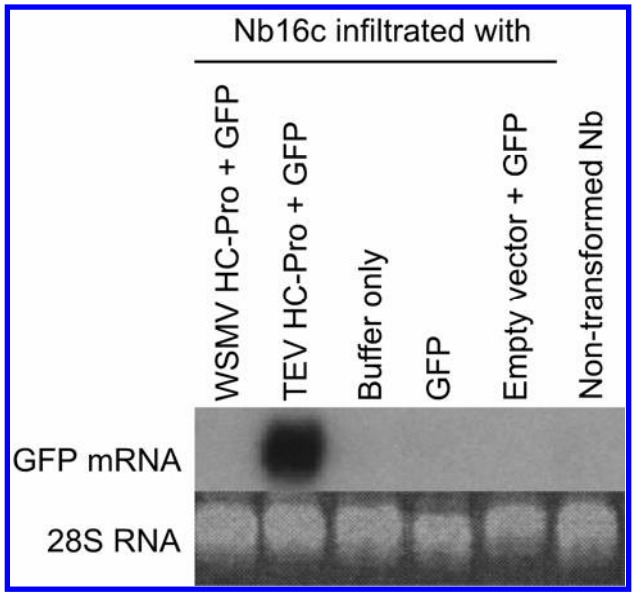

Fig. 7. Effect of *Wheat streak mosaic virus* (WSMV) helper component-proteinase (HC-Pro) on green fluorescent protein (GFP) mRNA levels in a post-transcriptional gene silencing (PTGS) suppression assay. Presented is a northern blot probed with ^{32}P-labeled GFP DNA (upper panel) of total RNA samples (5 µg) extracted from *Nicotiana benthamiana* (Nb) 16c leaves 6 days postinfiltration. Note that *Tobacco etch virus* (TEV) HC-Pro, but not WSMV HC-Pro, suppressed PTGS as evidenced by high levels of GFP mRNA. Ethidium bromide-stained 28S rRNA (lower panel) in total RNA samples served as loading controls.

The data reported here revealed significant differences in HC-Pro function among genera and lead to an obvious and testable hypothesis: Are PTGS suppression and disease synergism functions of a WSMV gene other than HC-Pro? Alternatively, but viewed by us as less probable, WSMV may not need a suppressor of PTGS for systemic infection of plants and induction of disease synergism. Given that *Cucumber vein yellowing virus* (a whitefly-transmitted species of the genus *Ipomovirus*) lacks an HC-Pro homologue and instead utilizes an imperfect repeat of P1 (designated as P1b) to accomplish PTGS suppression (42), the former hypothesis seems more likely. To address this, we are currently examining other WSMV genes for PTGS suppression and pathogenicity enhancement activity.

Tritimovirus and potyvirus HC-Pro are divergent in both sequence and function. The family *Potyviridae* contains a diverse collection of virus species assigned to six genera (4). Among these, at least four genera encode proteins designated as HC-Pro homologues: *Potyvirus*, *Tritimovirus*, *Ipomovirus* (e.g., some isolates of *Sweet potato mild mottle virus*), and *Rymovirus*. However, HC-Pro encoded by different genera share limited sequence identity, and alignment of HC-Pro amino acid sequences outside of the carboxy-terminal cysteine proteinase domain are mostly ambiguous and of low confidence (12).

Aside from recent experiments with WSMV (this report, 35,36,38–40), functional analyses of HC-Pro have been restricted to potyviruses. Nonetheless, there seems little doubt that HC-Pro of all four genera retain cysteine proteinase functions needed for polyprotein processing, as conserved motifs of the cysteine proteinase active site and cleavage site are readily identifiable in sequence comparisons. Furthermore, cysteine proteinase activity has been demonstrated directly for WSMV HC-Pro (38). Although vector transmission determinants map to the amino-terminal region of HC-Pro encoded by both potyviruses (3,5,11,18,27) and tritimoviruses (38,39), viruses of these two genera have distinctly different vector taxa and modes of transmission, making it unlikely that the exact same mechanism is employed. Whether other functions of HC-Pro demonstrated for potyviruses are conserved among genera remains an open question. Thus, systematic and comprehensive studies of HC-Pro encoded by genera of the family *Potyviridae* are needed to assess evolutionary relationships in a functional context.

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