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Nested deletion analysis of *Wheat streak mosaic virus* HC-Pro: Mapping of domains affecting polyprotein processing and eriophyid mite transmission

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Abstract

A series of in-frame and nested deletion mutations which progressively removed 5′-proximal sequences of the *Wheat streak mosaic virus* (WSMV) HC-Pro coding region (1152 nucleotides) was constructed and evaluated for pathogenicity to wheat. WSMV HC-Pro mutants with 5′-proximal deletions of 12 to 720 nucleotides systemically infected wheat. Boundary sequences flanking the deletions were stable and unaltered by passage through plants for all deletion mutants except HCD12 (lacking HC-Pro codons 3–6) that exhibited strong bias for G to A substitution at nucleotide 1190 in HC-Pro codon 2 (aspartic acid to asparagine). HC-Pro mutants with 5′-proximal deletions of up to 720 nucleotides retained autoproteolytic activity in vitro. In contrast, 5′-proximal deletion of 852 nucleotides of the HC-Pro coding region (HCD852) abolished both infectivity and in vitro proteolytic activity, confirming that the proteolytic domain of WSMV HC-Pro resides within the carboxy-terminal third of the protein and includes the cysteine proteinase motif (GYCY) conserved among four genera of the family Potyviridae. Inoculation of wheat with HC-Pro deletion mutants also bearing the GUS reporter gene revealed that HCD852 was unable to establish primary infection foci in inoculated leaves, indicating that processing of the P3 amino-terminus was essential. Deletion of as few as 24 nucleotides of HC-Pro (codons 3–10) eliminated transmission by the eriophyid mite vector *Aceria tosichella* Keifer. Collectively, these results demonstrated similar organization of proteinase and vector transmission functional domains among divergent HC-Pro homologues encoded by potyviruses and tritimoviruses.
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Keywords: Tritimovirus; Potyviridae; Vector transmission; Proteinase; Deletion mutants

Introduction

HC-Pro encoded by viruses of the genus *Potyvirus* performs multiple functions during infection of plants. The HC-Pro cysteine proteinase domain acts in *cis* to cleave the HC-Pro/P3 junction of the polyprotein (Carrington and Herndon, 1992; Carrington et al., 1989; Oh and Carrington, 1989). Non-persistent aphid transmission of potyviruses requires interaction of HC-Pro with both virions and vector food canal surfaces (Ammar et al., 1994; Atreya and Pirone, 1993; Atreya et al., 1992; Berger and Pirone, 1986; Govier and Kassanis, 1974; Thornbury et al., 1985; Wang et al., 1996). Potyvirus HC-Pro mediates suppression of post-transcriptional gene silencing (PTGS) (Anandalakshmi et al., 1998; Brigneti et al., 1998; Carrington et al., 2001; Kasschau and Carrington, 1998; Kasschau et al., 2003; Llave et al., 2000; Mallory et al., 2002; Palatnik et al., 2003; Vance and Vaucheret, 2001) and synergistic interactions with unrelated viruses in mixed infections (Pruss et al., 1997; Shi et al., 1997). Point substitutions and/or small insertions in potyvirus HC-Pro also affect replication and long-distance movement within infected plants (Cronin et al., 1995; Kasschau and Carrington, 2001; Kasschau et al., 1997; Klein et al., 1994; Sáenz et al., 2002).

Wheat streak mosaic virus (WSMV) is the type species of the genus *Tritimovirus* in the family Potyviridae (Stenger et al., 1998). As for other monopartite members of the family, WSMV encodes a polyprotein that is processed by viral proteinases. The amino-terminal region of the WSMV polyprotein includes a serine proteinase that cleaves the P1/HC-Pro junction (Choi et

al., 2002). WSMV HC-Pro has conserved cysteine proteinase motifs (Stenger and French, 2004). Other viral proteins are cleaved from the polyprotein in *trans* by the WSMV NIa proteinase (Choi et al., 2000b). Each WSMV proteinase appears to function in a manner similar to that of homologues encoded by potyviruses (Carrington and Dougherty, 1987; Carrington and Herndon, 1992; Carrington et al., 1989; Oh and Carrington, 1989; Verchot et al., 1991, 1992).

WSMV HC-Pro shares only limited (~16%) amino acid sequence identity with potyvirus HC-Pro (Stenger and French, 2004), and alignment of sequences outside of the carboxy-terminal proteinase domain is mostly ambiguous (French and Stenger, 2005). Nonetheless, WSMV HC-Pro also is multi-functional as determinants of semi-persistent transmission by the wheat curl mite (*Aceria tosichella* Keifer) mapped to the 5′-proximal half of WSMV HC-Pro (Stenger et al., 2005b). Replacement of HC-Pro with the corresponding coding region of potyviruses or rymoviruses was tolerated by WSMV with respect to systemic infection of wheat but not of oat or corn (Stenger and French, 2004). Surprisingly, WSMV also tolerated complete deletion of HC-Pro as a null mutant systemically infected wheat, corn and oat (Stenger et al., 2005a). Thus, WSMV HC-Pro is not required for replication or movement in plants such that host range restriction of WSMV bearing potyvirus or rymovirus HC-Pro replacements (Stenger and French, 2004) likely was due to interference.

We constructed a series of nested partial deletions of WSMV HC-Pro in the context of an infectious clone. The deletion mutant constructs were evaluated for function in a series of assays. Pathogenicity of the deletion mutants was assessed using RNA transcripts as inocula. The proteolytic domain of WSMV HC-Pro was defined in vitro by analysis of translation products synthesized in wheat germ extracts. Establishment of infection foci in inoculated leaves was monitored by histo-chemical assays for GUS expression following inoculation with deletion mutants bearing the GUS reporter gene. Necessity of 5′-proximal HC-Pro sequences for transmission by the wheat curl mite was determined in vector transmission assays. These experiments resulted in assignment of functional domains for WSMV HC-Pro and facilitated comparison of HC-Pro function among divergent genera of the family *Potyviridae*.

Results

Viability of HC-Pro nested deletion constructs

Sixteen plasmids (pS81HCD12–pS81HCD852) containing the WSMV-Sidney 81 genome with in-frame, 5′-proximal nested deletions of HC-Pro ranging in size from 12 to 852 nucleotides (nts) were constructed (Fig. 1A) and used as templates for in vitro transcription (HCD12–HCD852). With the exception of the largest deletion (HCD852), transcripts from each of the nested deletion constructs produced systemic infection of wheat (Table 1) with typical chlorotic streak symptoms. Although transcript inoculation resulted in a lower percentage of infected plants for some of the deletion mutants (Table 1), infection efficiency of viable deletion mutants

(HCD12–HCD720) and the positive control bearing the complete HC-Pro coding region (S81-SA) were similar upon sap transmission of progeny passaged to wheat seedlings (data not shown).

Infection by each deletion mutant was verified by reverse transcription-polymerase chain reaction (RT-PCR) of total RNA extracts from wheat using primers flanking the HC-Pro coding region (Fig. 1B). Size of the product amplified for each deletion mutant corresponded to that expected based on the length of sequences deleted. No product was amplified from total RNA extracted from uninoculated plants or from plants inoculated with HCD852 transcripts. To confirm that lack of infectivity of HCD852 transcripts was due to deletion of HC-Pro sequences and not spontaneous mutation elsewhere during propagation in *E. coli*, the HC-Pro insert of pS81HCD852 was replaced with the full-length HC-Pro insert of pS81-SA. As this replacement restored infectivity (Table 1), lack of infectivity for HCD852 transcripts mapped to HC-Pro. To confirm that spontaneous mutation during propagation in *E. coli* had not occurred in the residual portion of HC-Pro retained in pS81HCD852, the appropriate region of the plasmid was sequenced and found to be unaltered (data not shown).

To permit detection of primary infection foci on inoculated leaves, the GUS reporter gene was inserted into WSMV genomes bearing deletions in HC-Pro ranging in size from 192 to 852 nts. The GUS reporter gene was inserted in-frame between the P1 and HC-Pro coding regions as a *Sal*I fragment (Fig. 1A). A full-length WSMV construct (pWSMV-GUS-S1RN) bearing the GUS reporter gene at the same position (Choi et al., 2002) served as a positive control. Transcripts bearing the GUS gene were inoculated onto wheat seedlings; histochemical assays for GUS expression performed at 4 days post-inoculation (dpi) are presented in Fig. 2. Transcripts of all infectious deletion mutants bearing the GUS gene produced localized regions of GUS expression on inoculated leaves similar to that of GUS-S1RN. No GUS activity was detected on leaves of uninoculated wheat seedlings or on leaves of wheat seedlings inoculated with transcripts of the 852 nt deletion construct bearing GUS (HCD852GUS).

Spontaneous mutation of 12 nt deletion construct in plants

Sequencing (data not shown) of RT-PCR products amplified from plants infected with each deletion mutant confirmed that, in all cases except the 12 nt deletion, there was no alteration of sequences bordering the deletions. Sequences of two RT-PCR products amplified from HCD12-infected plants contained the same G to A substitution at WSMV-Sidney 81 nt 1190 (Fig. 3A) such that the second native codon of HC-Pro was altered from aspartic acid (GAC) to asparagine (AAC). Apparently, stability of the upstream boundary sequence was dependent upon the specific sequence removed at the 3′-proximal end of the deletion. The 12 nt deletion removed HC-Pro codons 3 to 6 (encoding QAVN). The G to A substitution at nt 1190 resulted in an asparagine codon positioned immediately downstream of the non-native valine codon introduced to generate the *Sal*I site (Fig. 3A). Of the sixteen nested deletion mutants, only HCD12
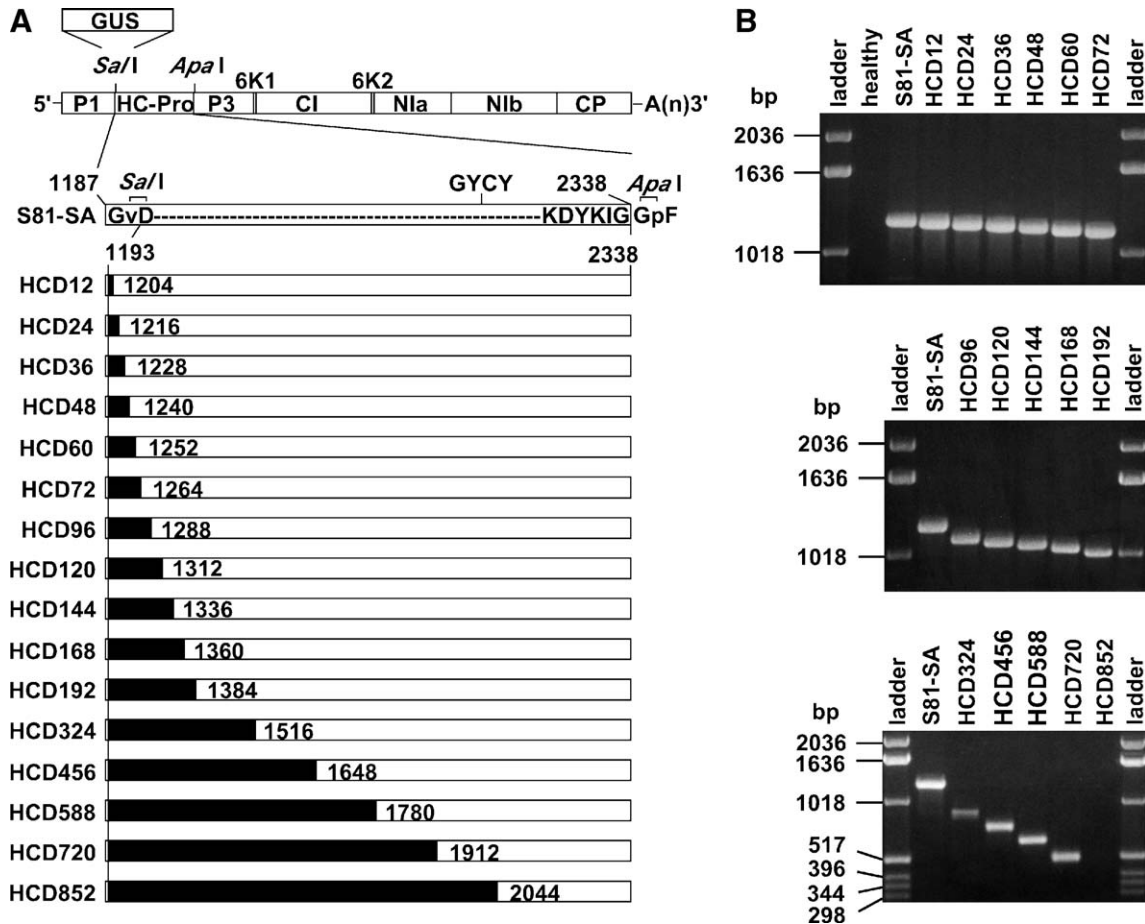
Fig. 1. Nested deletions of the HC-Pro coding region in *Wheat streak mosaic virus*-Sidney 81 (WSMV-Sidney 81). (A) A full-length genome of WSMV-Sidney 81 bearing engineered *Sal*I and *Apa*I sites (S81-SA) was modified by replacement of the HC-Pro coding region (WSMV-Sidney 81 nucleotides 1187–2338) with a series of in-frame, 5′-proximal nested deletions (HCD12–HCD852). HC-Pro sequences removed from each nested deletion construct are indicated in black. Each nested deletion had the same 5′-proximal boundary positioned between WSMV-Sidney 81 nts 1192 and 1193; coordinate of the 3′-proximal deleted nucleotide is indicated to the right of each deletion. Residues at the amino- and carboxy-termini of HC-Pro and the amino-terminus of P3 are indicated by a one-letter code for S81-SA. Non-native residues encoded by three base insertions used to generate the *Sal*I and *Apa*I sites are indicated in lower case. For WSMV-Sidney 81 genomes bearing deletions of 192 to 852 nucleotides in HC-Pro (HCD192-HCD852), a second series of constructs was generated in which the GUS reporter gene was inserted in-frame at the *Sal*I site. (B) RT-PCR assay for WSMV-Sidney 81 HC-Pro nested deletion mutants in wheat plants. Wheat seedlings were inoculated with transcripts bearing the complete genome of WSMV-Sidney 81 (S81-SA) or HC-Pro nested deletion mutants (HCD12–HCD852). RT-PCR products were amplified from total RNA samples extracted from uninoculated upper leaves of wheat plants 16–18 days post-inoculation using primers that flank the HC-Pro coding region. Presented are images of ethidium-bromide-stained RT-PCR products following electrophoresis in 1.2% agarose. Size of each product corresponded to the length of HC-Pro sequences retained in each deletion mutant construct. RNA extracted from uninoculated (Healthy) wheat plants served as template for a negative control reaction. Size in base pairs (bp) for DNA standards (ladder) is indicated at left. Note that no RT-PCR product (expected size 341 bp) was amplified from plants inoculated with transcripts bearing the 852 nucleotide deletion in the HC-Pro coding region (HCD852).

had these same two amino acid residues encoded by the two 3′-proximal codons of the deletion. In this context, translation (GVNKVIP...) of the HC-Pro amino-terminus for the 12 nt deletion mutant bearing the spontaneous substitution at nt 1190 was equivalent to HC-Pro with amino acid residues 2 to 4 (DQA) deleted (Fig. 3A).

The G to A substitution at nt 1190 fortuitously altered the *Sal*I cleavage site (GTCGAC to GTCAAC) in RT-PCR products of HCD12 progeny genomes. Loss of the *Sal*I site provided a means of identifying RT-PCR products bearing substitutions within the two codons immediately upstream of the deletion. A ~1.9 kbp RT-PCR product (WSMV-Sidney 81 nts 421 to 2373) was amplified from systemically infected, transcript-inoculated plants using primers HCR and P1V1 (Stenger et al., 2005a).

*Sal*I cleavage of the 1.9 kbp RT-PCR product from unaltered progeny genomes derived from HCD12 or S81-SA was expected to generate two fragments (1.2 and 0.7 kbp). RT-PCR products derived from genomes bearing the G to A substitution at nt 1190 would not be cleaved by *Sal*I (Fig. 3B). To ensure that *Sal*I digestions had gone to completion, each endonuclease reaction also included a 4.1 kbp supercoiled plasmid with a single *Sal*I site as an internal control.

Complete linearization of the internal control plasmid was observed for all *Sal*I digestions (Fig. 3C). Therefore, abundance of the 1.9 kbp product relative to the 1.2 and 0.7 kbp products represented abundance of unaltered progeny genomes relative to those bearing mutations in the two codons upstream of the deletion (Fig. 3C). The 1.9 kpb product from a plant infected

Table 1
Infectivity[a] of RNA transcribed from cloned DNA templates of *Wheat streak mosaic virus*-Sidney 81 with 5′-proximal nested deletions in the HC-Pro coding region

Transcript inoculum	Experiment 1	Experiment 2	Full-length HC-Pro replacement[b]
S81-SA[c]	10/10	10/10	ND
HCD12	10/10	9/10	ND
HCD24	9/10	8/10	ND
HCD36	9/10	9/10	ND
HCD48	10/10	9/10	ND
HCD60	8/10	8/10	ND
HCD72	9/10	10/10	9/10
None	0/10	0/10	ND
S81-SA[c]	10/10	10/10	9/10
HCD96	6/10	7/10	ND
HCD120	9/10	8/10	ND
HCD144	8/10	6/10	ND
HCD168	8/10	7/10	ND
HCD192	7/10	8/10	7/9
None	0/10	0/10	0/10
S81-SA[c]	8/10	10/10	8/9
HCD324	2/10	2/10	8/10
HCD456	5/10	8/10	9/10
HCD588	5/10	8/10	8/10
HCD720	4/10	5/10	8/10
HCD852	0/10	0/10	8/10
None	0/10	0/10	0/10

[a] Number of wheat plants systemically infected/number of wheat plants inoculated.
[b] Infectivity of transcripts after replacement of mutant HC-Pro with full-length HC-Pro. ND = not determined.
[c] Construct with full-length HC-Pro.

with S81-SA transcripts was completely digested with *Sal*I, as expected if the two codons were stable in this context. However, for six of eight RT-PCR products amplified from separate plants inoculated with HCD12 transcripts, a substantial portion of each product was not cleaved by *Sal*I, with half (four of eight) of these samples containing only trace amounts of the expected cleavage products. These results confirmed that mutation of the HCD12 *Sal*I site is a common event. Although it remains possible that mutations other than the G to A substitution at nt 1190 could have resulted in loss of the *Sal*I site, this transition was the only mutation detected in the RT-PCR products sequenced and also in clones of RT-PCR products from plants infected with progeny virus of HCD12 following vector transmission (described below). Thus, the G to A substitution at nt 1190 was considered to account for most, if not all, cases where the *Sal*I site was lost.

Proteinase domain mapping

Plasmids containing truncated viral genomes (WSMV-Sidney 81 nts 1–3786 with or without deletions in HC-Pro) were used as templates to program coupled transcription–translation reactions in vitro (Fig. 4A). Proteins expected upon complete or partial autoproteolysis of translation products by the WSMV P1 and HC-Pro proteinases are diagrammed in Fig. 4A. Mature P1 (41 kDa) and HC-Pro (44 kDa) proteins were present among translation products of the truncated genome
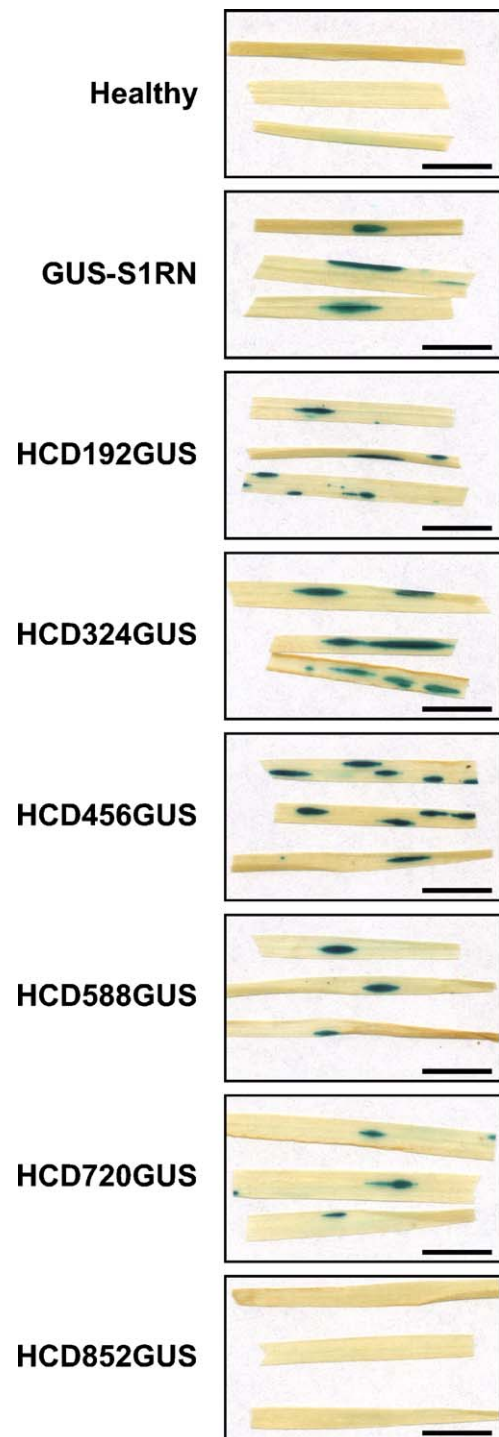

Fig. 2. Infection foci produced on inoculated leaves by *Wheat streak mosaic virus*-Sidney 81 (WSMV-Sidney 81) HC-Pro nested deletion mutants. Presented are images of histochemical assays for GUS expression (dark blue regions) in wheat leaf segments (~3–4 cm) harvested from plants 4 days post-inoculation with RNA transcribed from WSMV-Sidney 81 constructs bearing the GUS reporter gene and containing the full-length HC-Pro coding region (GUS-S1RN) or 5′-proximal nested deletions in the HC-Pro coding region ranging in size from 192 nucleotides (HCD192GUS) to 852 nucleotides (HCD852GUS). GUS assays of wheat leaf segments from uninoculated (Healthy) wheat plants served as a negative control. Note that infection foci were produced for all deletion mutant constructs except HCD852GUS. Black scale bars correspond to 1 cm.

(pSASB9) subcloned from pS81-SA (Fig. 4B). For truncated genomes also having deletions in HC-Pro, the size of the mature P1 protein remained unaltered. Also as expected, translation of the HC-Pro nested deletion mutants did not include a protein corresponding in size to full-length mature HC-Pro. Instead, smaller proteins of decreasing size (corresponding to size of the residual HC-Pro coding region) were observed among translation products of templates bearing deletions in HC-Pro ranging in size from 192 to 720 nts. A corresponding decrease in size of partially cleaved proteins also was observed for translation products of the deletion mutant templates. In contrast, no protein corresponding in size (~12 kDa) to the residual portion of HC-Pro remaining in HCD852 was observed. Although small polypeptides may, in some cases, be difficult to detect if the amino acid sequence contains few labeled methionine residues, the carboxy-terminal region of WSMV-Sidney 81 HC-Pro is methionine-rich. Furthermore, HCD852 translation products included a protein that corresponded in size (~65 kDa) to that expected if the HC-Pro proteinase was unable to cleave residual HC-Pro from the carboxy-terminal 53 kDa region of the translation product. Collectively, these results indicated that WSMV HC-Pro proteinase activity was retained upon transcription–translation of genomes bearing 5′-proximal deletions of the HC-Pro coding region of up to 720 nts but was eliminated by a larger deletion which included the highly conserved GYCY cysteine proteinase active site motif

(Fig. 4B). We further note that site-directed mutation of the active site motif or within the HC-Pro cleavage site generated similar noninfectious phenotypes (data not shown).

Vector transmission domain mapping

Progeny virus of a previously (Stenger et al., 2005a) constructed WSMV genome completely lacking HC-Pro (S81ΔHC12-5) was evaluated for transmissibility by the wheat curl mite. Groups of ten wheat curl mites reared on source plants infected with S81ΔHC12-5 did not transmit the HC-Pro null mutant to test plants (Table 2). In contrast, progeny virus of two genomes (S81-SA and S81SAS-12) bearing the complete HC-Pro coding region was transmitted from source plants to test plants by groups of wheat curl mites. S81-SA and S81SAS-12 differ from one another in that the latter is an intermediate construct with a second *Sal*I inserted immediately downstream of the engineered *Apa*I site in the 5′-proximal portion of P3 (Stenger et al., 2005a). No virus transmission to test plants was observed for groups of wheat curl mites reared on uninfected source plants. Infection status of all source and test plants was determined by RT-PCR as described (Stenger et al., 2005b).
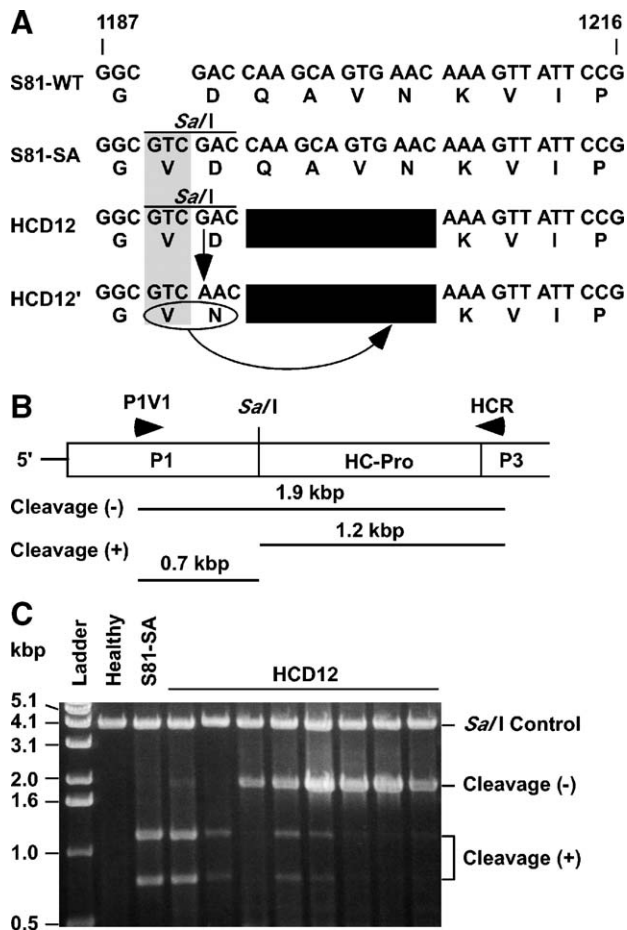

Fig. 3. Spontaneous mutation in the 5′-proximal deletion boundary sequence of HCD12 progeny recovered from systemically infected wheat. (A) Nucleotide sequence (presented as cloned DNA) of the 5′-proximal end of the HC-Pro coding region for *Wheat streak mosaic virus*-Sidney 81 (WSMV-Sidney 81) wild type (S81-WT), WSMV-Sidney 81 containing a three base insertion (gray shading) generating a *Sal*I site (S81-SA), a deletion derivative of S81-SA (HCD12) in which 12 nucleotides of the HC-Pro coding region have been deleted (black shading) and an altered progeny genome (HCD12′) derived from HCD12 in which WSMV-Sidney 81 nucleotide 1190 has undergone a G to A transition mutation (downward arrow) that alters the *Sal*I cleavage site. Encoded amino acid residues are indicated by a one-letter code. Nucleotide coordinates are those of S81-WT. Curved arrow indicates that two amino acid residues (within oval) encoded by HCD12′ immediately upstream of the deletion are the same two amino acids encoded by the 3′-proximal sequences of the deletion. (B) Schematic representation of a restriction fragment length polymorphism (RFLP) assay designed to distinguish between WSMV-Sidney 81 genomes with or without mutation at nt 1190. RT-PCR was accomplished using primers HCR and P1V1, yielding a 1.9 kbp product. RT-PCR products amplified from genomes having the mutation at nt 1190 will not be cleaved by *Sal*I [cleavage (−)], whereas RT-PCR products with an unaltered *Sal*I site will generate two cleavage products (0.7 and 1.2 kbp) upon digestion with *Sal*I [cleavage (+)]. (C) Application of the RFLP assay diagrammed in panel B. Presented are *Sal*I digests of RT-PCR products amplified from an uninfected wheat plant (Healthy) or wheat plants infected with transcripts of S81-SA or HCD12. To distinguish incomplete digestion of an homogeneous RT-PCR product bearing the *Sal*I site from complete digestion of a heterogeneous RT-PCR product exhibiting polymorphism within the *Sal*I site, an internal control substrate (a 4.1 kbp plasmid bearing a single *Sal*I site) was added to each endonuclease reaction. Following incubation with *Sal*I, the products were separated on a 1% agarose gel. In all cases, the internal *Sal*I control DNA was completely linearized (the 4.1 kpb product appearing in all samples), indicating that each *Sal*I digestion had gone to completion. *Sal*I digestion of the 1.9 kbp RT-PCR product from an S81-SA infected plant was completely digested with *Sal*I. In contrast, *Sal*I incubation of RT-PCR products amplified from eight plants inoculated with HCD12 transcripts gave variable results: two samples were completely (or nearly so) cleaved by *Sal*I, four samples were mostly insensitive to *Sal*I with cleavage products present in trace amounts, whereas the remaining two samples contained substantial amounts of both cleaved and uncleaved products.

A second series of wheat curl mite transmission assays (Table 3) was conducted using source plants infected with S81-SA or deletion mutants HCD12–HCD120 bearing small (12–120 nts) 5′-proximal nested deletions in HC-Pro. Groups of ten wheat curl mites efficiently transmitted S81-SA to test plants, as verified by RT-PCR. No infection was detected by RT-PCR of test plants colonized by groups of wheat curl mites transferred from uninfected source plants or from source plants infected with mutants having 5′-proximal deletions in HC-Pro of 24 to 120 nts. In contrast, HCD12 was vector transmissible as 73% of test plants assayed positive for infection by RT-PCR. Due to the small difference in size of RT-PCR products amplified from S81-SA and HCD12 (Fig. 1B), RT-PCR products from test plants infected with HCD12 were cloned and sequenced. Sequences of three independent clones were identical to that of

Table 2
Wheat curl mite transmission assays of *Wheat streak mosaic virus*-Sidney 81 containing (pS81-SA and pS81SAS12) or completely lacking (pS81ΔHC12-5) the HC-Pro coding region

Virus construct	Transmission to test plants [a]						
Source plant	A	B	C	D	E	F	Total
S81-SA	4/5	3/5	5/5	4/5	4/5	4/5	24/30
S81SAS12	5/5	3/5	4/5	4/5	5/5	4/5	25/30
S81ΔHC12-5	0/5	0/5	0/5	0/5	0/5	0/5	0/30
No virus	0/5	0/4	0/5	0/5	0/5	0/5	0/29

[a] Number of test plants infected/number of test plants inoculated.

HCD12 containing the G to A transition at nt 1190 (data not shown).

HCD12 genomes present in each infected source plant were evaluated for mutation within the *Sal*I site as described for Fig. 3. All six HCD12-infected source plants contained genotype mixtures as *Sal*I cleaved only a portion of the 1.9 kbp RT-PCR product. Among RT-PCR products amplified from 22 test plants infected with HCD12, only four were partially cleaved by *Sal*I; the remaining 18 were not cleaved by *Sal*I. In contrast, RT-PCR products amplified from both source and test plants infected with progeny virus of S81-SA were fully cleaved by *Sal*I. Fig. 5 shows the results of RT-PCR amplification and subsequent *Sal*I digestion (with inclusion of the internal control) for representative samples from source and test plants infected with S81-SA or HCD12.

Discussion

Proteinase domain maps to the carboxy-terminal third of WSMV HC-Pro

Infectivity and HC-Pro proteinase activity in vitro were retained by WSMV-Sidney 81 constructs with 5′-proximal deletions in HC-Pro up to 720 nts. Deletion of an additional
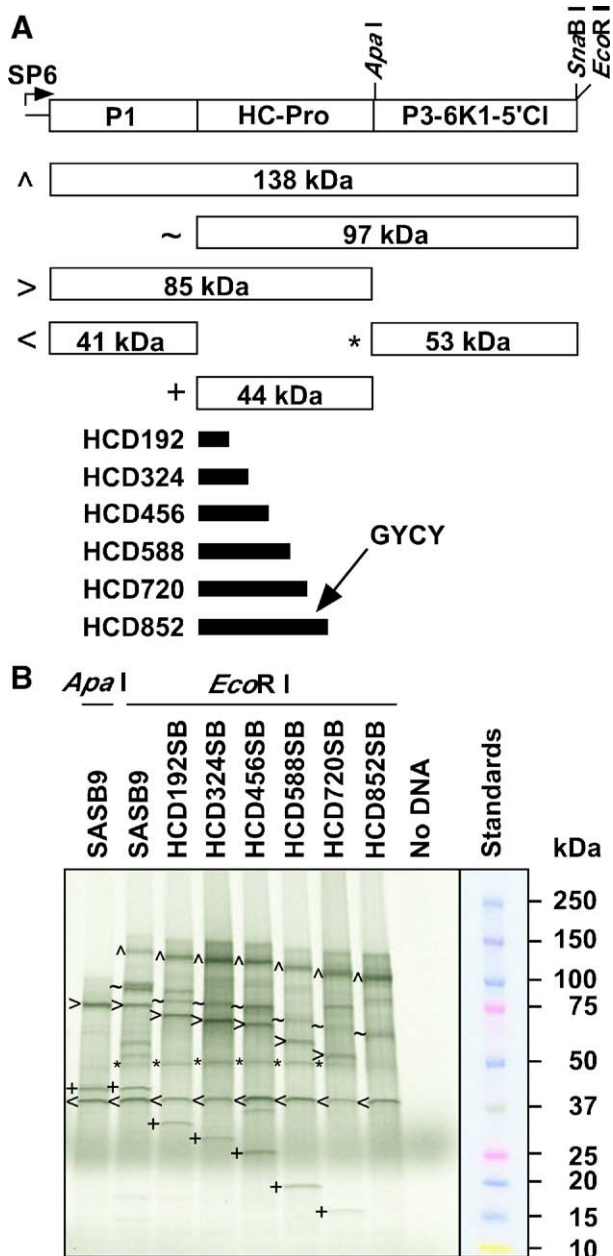

Fig. 4. In vitro proteinase activity of *Wheat streak mosaic virus*-Sidney 81 (WSMV-Sidney 81) HC-Pro nested deletion mutants. (A) Products expected upon coupled transcription–translation of truncated WSMV-Sidney 81 genomes (nucleotides 1–3786) with (HCD192SB-HCD852SB) or without (SASB9) 5′-proximal nested deletions in HC-Pro. Template DNAs were linearized with *Eco*RI (immediately downstream of the WSMV *Sna*BI site at nt 3786) or *Apa*I (immediately downstream of the HC-Pro/P3 junction). Expected translation products (^ ~ > < * +) upon partial or complete proteolysis by P1 and/or HC-Pro are shown with product size (kDa) indicated for the template containing full-length HC-Pro. The region of HC-Pro deleted from each template is indicated as a black bar. The location of the conserved cysteine proteinase active site motif (GYCY) is indicated with an arrow. (B) Autoradiograph of ^{35}S-labeled translation products following separation by electrophoresis in SDS–polyacryl-amide (4–15%) gradient gels. Lanes are identified by DNA template used to program coupled reactions and the restriction enzyme used to linearize templates prior to transcription. Sizes of stained protein standards are given in kilodaltons at the right. Symbols (^ ~ > < * +) to the left of each lane correspond to translation products expected upon complete or incomplete autoproteolysis by P1 and/or HC-Pro, as indicated in panel A. Note that translation products corresponding to mature HC-Pro are present (and of decreasing size) for templates bearing deletions in HC-Pro of up to 720 nucleotides but absent among translation products of HCD852 in which the GYCY motif has been deleted.

Table 3
Wheat curl mite transmission assays of *Wheat streak mosaic virus*-Sidney 81 (WSMV-Sidney 81) with 5′-proximal nested deletions in the HC-Pro coding region

Virus construct	Transmission to test plants [a]						
Source plant	A	B	C	D	E	F	Total
S81-SA [b]	5/5	5/5	5/5	9/10	10/10	M [c]	34/35
HCD12	3/5	3/5	5/5	5/5	1/5	5/5	22/30
HCD24	0/5	0/5	0/5	0/5	0/5	0/5	0/30
HCD48	0/5	0/5	0/5	0/5	0/5	0/5	0/30
HCD72	0/5	0/5	0/5	0/5	0/5	0/5	0/30
HCD96	0/5	0/5	0/5	0/5	0/5	0/5	0/30
HCD120	0/5	0/5	0/5	ND [d]	ND [d]	ND [d]	0/15
No virus	0/5	0/5	0/5	0/10	0/10	M [c]	0/35

[a] Number of test plants infected/number of test plants inoculated.
[b] WSMV-Sidney 81 with full-length HC-Pro.
[c] M = source plant mortality (drought) during acquisition access period.
[d] ND = not determined.

132 nts that included the conserved cysteine proteinase active site motif (GYCY) eliminated both proteinase activity in vitro and infectivity of transcripts inoculated onto wheat plants. These results demonstrated that the WSMV HC-Pro proteinase domain maps to the carboxy-terminal third of the protein and that processing of the HC-Pro/P3 junction was essential for infection of plants. GUS assays further indicated that the 852 nt deletion prevented establishment of primary infection foci. As the histochemical assay was sufficiently sensitive to detect GUS expression in individual cells (Choi et al., 2005), absence of GUS expression in inoculated leaves indicated that HCD852 transcripts were severely debilitated or, perhaps, completely defective for replication. Similar results have been reported for potyviruses in which elimination of HC-Pro proteinase activity concomitantly resulted in loss of infectivity and replication (Kasschau and Carrington, 1995). As the WSMV genome in which HC-Pro was completely deleted retained the ability to systemically infect plants (Stenger et al., 2005a), the lack of infectivity for HCD852 may be explained as a consequence of incorrect processing of the P3 amino-terminus.

Vector transmission domain includes sequences near the amino-terminus of WSMV HC-Pro

Previously, vector transmission assays of WSMV constructs bearing chimeric HC-Pro coding regions revealed that transmissibility by wheat curl mites mapped to the 5′-proximal half of HC-Pro (Stenger et al., 2005b). In the present series of experiments, the WSMV null mutant completely lacking HC-Pro was not vector transmissible, confirming that HC-Pro was required for transmission by the wheat curl mite and that loss of vector transmissibility upon replacement of HC-Pro with the corresponding region of other viruses cannot be explained as resulting from interference.

Deletion of as few as 24 nts near the 5′-proximal end of HC-Pro (codons 3–10) abolished wheat curl mite transmissibility. Of the deletion mutants tested, vector transmission only was observed for HCD12. Because progeny genomes derived from HCD12 transcripts frequently contained a substitution flanking

the deleted region, it was unclear whether HC-Pro amino acid residues 3–6 (the deletion as constructed) or residues 2–4 (those lacking upon translation of the spontaneous substitution mutant) were dispensable for vector transmission. Due to frequency and apparent selective advantage of the substitution mutation in infected plants, we could not ensure that HCD12 source plants contained only progeny genomes lacking the mutation at nt 1190. Indeed, all source plants containing progeny virus derived from HCD12 were infected with mixtures of both genotypes. Nonetheless, vector transmissibility of progeny virus derived from HCD12 coupled with loss of vector transmission for progeny viruses bearing 5′-proximal deletions of 24–120 nts collectively indicated that HC-Pro amino acid residues 5–10 (VNKVIP) contain an essential determinant of vector transmission. However, as wheat curl mite transmissibility required that chimeric HC-Pro coding regions contained sequences from WSMV both upstream and downstream of nt 1438 (Stenger et al., 2005b), the determinant identified here and located near the 5′-proximal end of the coding region does not constitute the sole and/or complete vector transmission determinant.
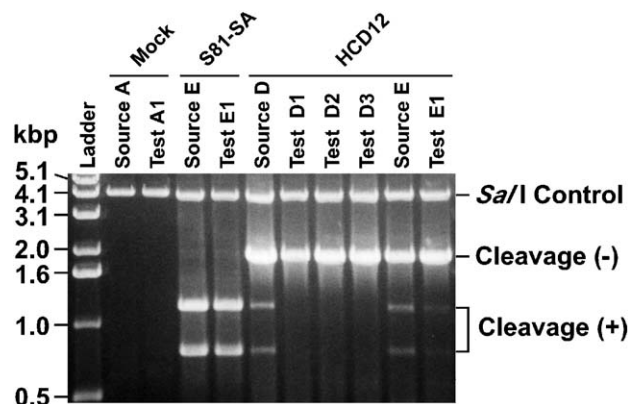

Fig. 5. Polymorphism at *Wheat streak mosaic virus*-Sidney 81 (WSMV-Sidney 81) nucleotide 1190 before and after transmission by the wheat curl mite. Presented is a restriction fragment length polymorphism assay for WSMV-Sidney 81 nt 1190 based on the presence (wild type G at nt 1190) or absence (mutation to A at nt 1190) of a *Sal*I site. Uninfected plants (mock) or plants infected with WSMV-Sidney 81, containing full-length HC-Pro (S81-SA) or bearing a 12 nt deletion in HC-Pro (HCD12), served as source plants. Wheat curl mites reared on source plants were transferred in groups of ten to test plants. RT-PCR of total RNA templates extracted from infected source and test plants resulted in amplification of a 1.9 kbp product (spanning WSMV-Sidney 81 nts 421–2373). No product was amplified from RNA samples extracted from uninfected source and test plants. *Sal*I digestion of RT-PCR products bearing the wild type G at nt 1190 was cleaved into two fragments of 1.2 and 0.7 kbp [Cleavage (+)]; RT-PCR products bearing the mutation at nt 1190 were not cleaved by *Sal*I [Cleavage (−)]. To ensure that *Sal*I reactions had gone to completion, each endonuclease reaction was supplemented with a 4.1 kbp plasmid known to have a single *Sal*I site (*Sal*I Control). In all cases, the internal control plasmid was completely linearized. For S81-SA, RT-PCR products were completely cleaved by *Sal*I, indicating that only the wild type G residue at nt 1190 was present in both source and test plants. In contrast, *Sal*I digestion of RT-PCR products amplified from HCD12-infected source plants resulted in a mixture of the 1.9, 1.2 and 0.7 kbp fragments, revealing polymorphism at nt 1190. Following wheat curl mite transmission of HCD12, test plant RT-PCR products were completely (test plants D1, D2, and D3) or mostly (test plant E1) insensitive to *Sal*I, indicating that wild type G residue at nt 1190 was either completely absent or, if present, occurred in lower abundance relative to the mutant A residue at nt 1190.

Organizations of HC-Pro functional domains of potyviruses and tritimoviruses are similar

Data presented here demonstrated that potyvirus and tritimovirus HC-Pro retain similarities with respect to organization of functional domains. As expected, the functional proteinase domain of WSMV HC-Pro mapped to the carboxy-terminal third of the protein and included the highly conserved cysteine proteinase active site motif GYCY. These data support the contention that HC-Pro homologues among divergent genera of the family Potyviridae perform similar roles with respect to polyprotein processing. Whether WSMV HC-Pro also acts as a suppressor of PTGS remains an open question that we are currently addressing.

Potyvirus aphid transmission was eliminated upon deletion or mutation of 5′-proximal sequences of HC-Pro (Atreya and Pirone, 1993; Atreya et al., 1992; Dolja et al., 1993). Similarly, deletion of sequences near the 5′-proximal end of HC-Pro abolished transmission of WSMV by the wheat curl mite. However, this does not demonstrate that tritimovirus and potyvirus HC-Pro homologues facilitate vector transmission by the same mechanism. Indeed, no interaction of WSMV HC-Pro and coat protein was observed in yeast two-hybrid and GST-fusion pull down assays (Choi et al., 2000a). Furthermore, potyvirus HC-Pro motifs essential for interaction with virions or vector surfaces (Blanc et al., 1998) are not present in WSMV HC-Pro, and the coat protein of WSMV-Sidney 81 lacks the DAG motif required for interaction of potyvirus coat protein with HC-Pro (Atreya et al., 1995; Blanc et al., 1997). Although the mechanism through which WSMV HC-Pro mediates transmission by the wheat curl mite remains to be elucidated, it is clear from the experiments presented here that the functional domains of HC-Pro needed for vector transmission of potyviruses and tritimoviruses are positioned similarly despite limited sequence conservation and significant differences in mode of virus transmission.

Materials and methods

Generation of nested deletion mutants

A full-length clone (pS81-SA) of WSMV-Sidney 81 bearing unique SalI and ApaI sites flanking the HC-Pro coding region has been described (Stenger and French, 2004). The SalI–ApaI fragment of pS81-SA containing HC-Pro was subcloned into pGEM5zf+ to produce pSHCA-17. Three series of in-frame, 5′-proximal nested deletions were generated by PCR using pSHCA-17 (1 ng) as template. To reduce the frequency of unintended mutations, PCR was accomplished using the Expand High Fidelity System (Roche). PCR primers employed were GEM5F2761 (located downstream of the pGEM5zf+ ApaI site) and 1 of 16 primers bearing the SalI site immediately upstream of ~20 nts that annealed to different locations in the HC-Pro coding region. PCR products were digested with SalI and ApaI, gel-purified, ligated to SalI–ApaI-digested pGEM5zf+ and transformed into E. coli DH5α. Inserts of clones with nested deletions were verified by sequencing, used to replace the SalI–

ApaI fragment of pS81-SA and subsequently transformed into E. coli JM109. A total of 16 clones of the Sidney 81 genome bearing 5′-proximal deletions in HC-Pro were generated (Fig. 1A). Each deletion derivative encoded the wild type 5′-proximal glycine codon (GGC) of WSMV-Sidney 81 HC-Pro followed by a non-native valine codon (GTC, introduced previously to generate the SalI site of pS81-SA), the native aspartic acid codon (GAC) in position 2 of WSMV-Sidney 81 HC-Pro, and thereafter by the first codon downstream of the deleted sequence. The first series of six nested deletions removed WSMV-Sidney 81 nts 1193–1264 in increments of 12 nts. The second series of five nested deletions included the 72 nts deleted in the first series and additional downstream sequences in increments of 24 nts through WSMV-Sidney 81 nt 1384. The third series of five nested deletions spanned the 192 nts deleted in the first two series (nts 1193–1384) and additional downstream sequences in increments of 132 nts through WSMV-Sidney 81 nt 2044.

Infectivity assays

RNA transcribed in vitro was mechanically inoculated to 7- to 10-day-old wheat seedlings (cv. Arapahoe) essentially as described (Choi et al., 1999). Non-inoculated wheat seedlings served as negative controls. Test plants were held in a growth chamber (16 h light per day; 22–25 °C) and monitored for symptom expression. Infection status of plants was determined by RT-PCR of total nucleic acid extracted (McNeil et al., 1996) from non-inoculated upper leaves 16–18 days post-inoculation (dpi). Primer HCR was used for RT, and primers HCR and HCF were used for PCR. Primers HCR and HCF (Stenger and French, 2004) anneal to WSMV-Sidney 81 sequences immediately upstream (HCF) or downstream (HCR) of the HC-Pro coding region, such that amplification of a 1193 bp product from progeny genomes derived from pS81-SA was expected.

The HC-Pro coding region of progeny genomes was sequenced to determine if deletion junctions present in plasmids used for in vitro transcription were retained by progeny genomes following systemic infection of plants. RT-PCR (conducted as described above) products were gel-purified, and sequences obtained from RT-PCR products were compared to those of S81-SA and the respective deletion mutant constructs used for in vitro transcription. For the non-infectious deletion mutant HCD852 (Fig. 1A), the HC-Pro coding region in the plasmid used for in vitro transcription was sequenced directly. To verify that the non-infectious phenotype of deletion mutant HCD852 was due to the intended mutation, rather than a spontaneous mutation elsewhere, the mutant fragment was replaced with the full-length SalI–ApaI fragment of pSHCA-17 and the resulting plasmid used to transcribe RNA inoculum.

Because deletion mutant HCD12 (Fig. 2A) displayed a strong bias in transcript-inoculated plants for a G to A substitution in HC-Pro codon 2 (GAC to AAC) and since this substitution altered the engineered SalI site (GTCGAC to GTCAAC), an assay was developed to determine frequency of this spontaneous mutation. RT-PCR was conducted on total RNA samples extracted from individual transcript-inoculated

plants using HCR as a primer in RT reactions and primers HCR and P1V1 (Stenger et al., 2005a) in PCR. The resulting RT-PCR products (1.9 kbp) were digested with SalI. Retention of the SalI site was expected to result in two fragments (1.2 and 0.7 kbp), whereas the G to A substitution was expected to produce an RT-PCR product not cleaved by SalI (Fig. 2B). To ensure that SalI digestions were complete rather than partial, each SalI digestion included ~0.2 μg of pSHCA-17 (4.1 kbp and containing a single SalI site) as an internal control.

GUS assays

A full-length infectious clone (pWSMV-GUS-S1RN) of WSMV-Sidney 81 bearing the GUS reporter gene sequence inserted at the engineered SalI site has been constructed (Choi et al., 2002). Active GUS protein is excised from the polyprotein at the P1/GUS junction by the P1 proteinase and at the GUS/HC-Pro junction by the NIa proteinase at an NIa proteinase cleavage site incorporated into the amino-end of the GUS protein. The GUS gene insert of pWSMV-GUS-S1RN was removed by digestion with SalI and inserted at the SalI site of WSMV-Sidney 81 genomes bearing nested deletions of HC-Pro ranging in size from 192 to 852 nts. The resulting constructs were transcribed and transcripts inoculated to wheat seedlings. Histochemical assays for GUS activity in inoculated leaves were performed 4 dpi as described (Choi et al., 2000b).

Proteinase assays

Coupled in vitro transcription (SP6 RNA polymerase)–translation (wheat germ system) was performed as described (Stenger et al., 2005a) using templates bearing 5′-proximal nested deletions in HC-Pro ranging in size from 192 to 852 nts. Fragments digested with BamHI (located upstream of the SP6 promoter/WSMV-Sidney 81 5′-end sequence junction) and SnaBI (WSMV-Sidney 81 nt 3786) were subcloned into BamHI–SmaI digested pUC8 to produce plasmids pHCD192SB, pHCD324SB, pHCD456SB, pHCD588SB, pHCD720SB and pHCD852SB (Fig. 4A). The corresponding fragment from pS81-SA also was subcloned into pUC8 (pSASB9) as a positive control. In vitro transcription–translation reactions were programmed with templates (~1 μg) digested with EcoRI (located immediately downstream of insert/pUC8 vector junction). An aliquot of template pSASB9 was digested with ApaI such that in vitro transcription–translation terminated immediately downstream of the HC-Pro/P3 junction. No template DNA was added to coupled transcription–translation reactions as a negative control. Translation products were labeled with ^{35}S-methionine and detected by autoradiography after electrophoresis in SDS-polyacrylamide (4–15%) gradient gels.

Vector transmission assays

Wheat curl mite transmission assays were performed as described (Stenger et al., 2005b). Individually caged source plants were prepared by mechanical inoculation of wheat seedlings using sap extracted from systemically infected, transcript-inoculated wheat plants. Infection status of source plants inoculated with the nested deletion mutants or constructs containing the complete HC-Pro coding region was verified by RT-PCR of the HC-Pro coding region using primers HCR and HCF. Infection status of source plants inoculated with progeny virus derived from a WSMV construct (pS81ΔHC12-5) in which the HC-Pro coding region was completely deleted was accomplished by RT-PCR amplification using primers HCR and P1V1 (Stenger et al., 2005a). Individually caged test plants were colonized by groups of ten wheat curl mites previously reared on source plants. Test plants were assayed for infection by RT-PCR, as described above, 4 weeks after transfer of wheat curl mites from source plants. For deletion mutant constructs transmissible by the wheat curl mite, verification of genotype in infected test plants was accomplished by sequencing plasmids bearing cloned inserts of RT-PCR products amplified from infected test plants.

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