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B. A. Young
USDA-ARS, University of Nebraska-Lincoln

Gary L. Hein
University of Nebraska-Lincoln, ghein1@unl.edu

Roy C. French
USDA-ARS, University of Nebraska-Lincoln, rfrench2@unl.edu

Drake C. Stenger
USDA-ARS, San Joaquin Valley Agricultural Sciences Center, dstenger@fresno.ars.usda.gov

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Brief Report

Substitution of conserved cysteine residues in wheat streak mosaic virus HC-Pro abolishes virus transmission by the wheat curl mite

B. A. Young[1], **G. L. Hein**[2], **R. French**[1], **D. C. Stenger**[1]

[1] United States Department of Agriculture – Agricultural Research Service and Department of Plant Pathology, University of Nebraska, Lincoln, NE, USA
[2] Department of Entomology, University of Nebraska Panhandle Research Center, Scottsbluff, NE, USA

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Summary

Substitutions in the amino-proximal region of wheat streak mosaic virus (WSMV) HC-Pro were evaluated for effects on transmission by the wheat curl mite (*Aceria tosichella* Keifer). Alanine substitution at cysteine residues 16, 46 and 49 abolished vector transmission. Although alanine substitution at Cys_{20} had no effect, substitution with arginine reduced vector transmission efficiency. Random substitutions at other positions (Lys_7 to Asn, Asn_{19} to Ile, and Arg_{45} to Lys) did not affect vector transmission. These results suggest that a zinc-finger-like motif (His_{13}-X2-Cys_{16}-X29-Cys_{46}-X2-Cys_{49}) in WSMV HC-Pro is essential for vector transmission.

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Wheat streak mosaic virus (WSMV) is the type species of the genus *Tritimovirus* in the family *Potyviridae* [15]. Whereas viruses of the genus *Potyvirus* are transmitted non-persistently by aphids, WSMV is transmitted semi-persistently by the wheat curl mite (*Aceria tosichella* Keifer). Both tritimoviruses [14, 17] and potyviruses [7, 19] encode HC-Pro homologues required for vector transmission. Although HC-Pro sequence conservation among the two genera is limited [6], functional domains of WSMV HC-Pro [16, 18] involved in vector transmission (amino-proximal) and polyprotein maturation (carboxy-proximal) are positioned similarly to that of potyvirus HC-Pro.

Alignment of WSMV HC-Pro with potyvirus HC-Pro is mostly ambiguous except for the proteinase domain in the carboxy-proximal third of the protein [6]. Nonetheless, the amino-proximal region of both potyvirus and tritimovirus HC-Pro are cysteine-rich. HC-Pro of both genera self-interact [5, 8, 11, 20]; mutations introduced into the cysteine-rich region of potyvirus HC-Pro have been shown to abolish self interaction [20] and aphid transmission [1, 2, 9]. Whether amino acid substitutions in the corresponding region of WSMV HC-Pro affect virus transmission by the wheat curl mite is unknown. Here, we address this issue by evaluating infectivity and vector transmission phe-

Correspondence: Drake C. Stenger, USDA-ARS, San Joaquin Valley Agricultural Sciences Center, 9611 South Riverbend Avenue, Parlier, CA 93648, USA
e-mail: dstenger@fresno.ars.usda.gov

notypes of WSMV bearing amino acid substitutions in the amino-proximal region of HC-Pro.

An infectious cDNA clone of the WSMV Sidney 81 isolate was constructed previously [4] and subsequently modified [12] to contain unique *Sal*I and *Apa*I sites (pS81-SA) flanking the HC-Pro coding region. This *Sal*I–*Apa*I fragment was subcloned into pALTER (Promega, Madison, WI) and served as a template for site-directed mutagenesis. Codons for each of four cysteine residues (Cys_{16}, Cys_{20}, Cys_{46}, and Cys_{49}) in the 5′-proximal coding region of WSMV HC-Pro were mutated individually to alanine codons. Site-directed mutagenesis also was used to generate double mutants in which pairs of cysteine codons ($Cys_{16} + Cys_{20}$ and $Cys_{46} + Cys_{49}$) were altered to alanine codons in the same construct. Primers (sequences in WSMV minus strand) used for site-directed mutagenesis (underline denotes nucleotide substitutions) were 5′-TTG CGCAGTTGTTGTTGGCCACATCGTGATATTT CGG-3′ (Cys_{16} to Ala), 5′-CAGGTAACTTGCTGC GTTGTTGTTGCACA-3′ (Cys_{20} to Ala), 5′-GTTC TACATTGGTCTGCCCTCAGATTATG-3′ (Cys_{46} to Ala), 5′-CCACTCTGTTCTTGCTTGGTCGCA CCTC-3′ (Cys_{49} to Ala), 5′-TCCAGGTAACTTGC TGCGTTGTTGTTTGCCACATCGTGATATTTC-3′ (Cys_{16} to Ala and Cys_{20} to Ala), and 5′-TTCC ACTCTGTTCTTGCTTGGTCTGCCCTCAGATT ATGTA-3′ (Cys_{46} to Ala and Cys_{49} to Ala). The four single and two double mutations were verified by nucleotide sequencing. Each mutant *Sal*I–*Apa*I fragment was excised from the pALTER derivatives and subsequently used to replace the *Sal*I–*Apa*I fragment of pS81-SA. RNA was transcribed from each mutant construct and inoculated to wheat seedlings (cv. Arapahoe) essentially as described [4]. Transcripts from all six mutant constructs were infectious to wheat and generated wild-type systemic symptoms (Table 1). The infection status of transcript-inoculated plants was verified by reverse transcription-polymerase chain reaction (RT-PCR) of the HC-Pro region as described [12]. The stability of mutations introduced into WSMV HC-Pro was assessed by sequencing of plasmids bearing cloned inserts of RT-PCR products amplified from systemically infected test plants. No evidence for reversion was seen; all cysteine-to-alanine substitutions were retained by WSMV in systemically infected plants (data not shown).

Table 1. Infectivity of wheat streak mosaic virus with cysteine-to-alanine substitutions in HC-Pro

WSMV genotype[a]	Experiment 1[b]		Experiment 2[b]	
	Symptoms	RT-PCR	Symptoms	RT-PCR
Cys_{16} to Ala	10/10	10/10	9/10	4/4
Cys_{20} to Ala	10/10	10/10	10/10	4/4
Cys_{16} to Ala + Cys_{20} to Ala	7/10	7/10	10/10	4/4
Cys_{45} to Ala	7/10	9/10	8/10	4/4
Cys_{49} to Ala	9/10	10/10	9/10	4/4
Cys_{45} to Ala + Cys_{49} to Ala	8/10	9/10	9/10	4/4
No virus control	0/10	0/4	0/10	0/4

[a] Subscript denotes HC-Pro amino acid coordinate. Stability of mutations verified by sequencing of RT-PCR products cloned from systemically infected wheat plants.
[b] Number of plants infected/number of inoculated plants evaluated. Infection status of plants determined by visual inspection for symptoms and by RT-PCR.

Previously, a series of 35 randomly generated point mutations in WSMV HC-Pro were evaluated for systemic infectivity, the ability to generate primary infection foci, and autoproteolytic activity [18]. Among the random mutations analyzed, four nonsynonymous point substitutions (Lys_7 to Asn, Asn_{19} to Ile, Cys_{20} to Arg, and Arg_{45} to Lys) that occurred within the amino-proximal region of WSMV HC-Pro had no effect on pathogenicity or proteinase activity. These four randomly generated mutations, along with the cysteine-to-alanine site-directed mutations described above, were evaluated for phenotypic effects on virus transmission by the wheat curl mite.

Wheat curl mite transmission assays were conducted essentially as described [17]. Wheat seedlings to be used as source plants for vector transmission assays were inoculated with transcripts derived from each of the ten mutant constructs. Wheat seedlings inoculated with transcripts of pS81-SA or non-inoculated wheat seedlings served as positive or negative (mock) control source plants, respectively. Total RNA was extracted [10] from leaf samples 12–14 days post inoculation and used as template to determine infection status of source

Table 2. Vector transmission of wheat streak mosaic virus (WSMV) mutants bearing amino acid substitutions in HC-Pro

WSMV Genotype	Transmission to test plants[a]						
	Acquisition source plant						
	Experiment 1			Experiment 2			All[c]
	A	B	C	D	E	F	
Cys_{16} to Ala	0/4	0/4	0/4	0/4	0/4	ND[b]	0/20 (0%)
Cys_{20} to Ala	3/4	3/4	4/4	3/4	3/4	4/4	20/24 (83%)*
Cys_{16} to Ala & Cys_{20} to Ala	0/4	0/4	0/4	0/4	0/4	ND[b]	0/20 (0%)
Cys_{46} to Ala	0/4	0/4	0/4	0/4	0/4	ND[b]	0/20 (0%)
Cys_{49} to Ala	0/4	0/4	0/4	0/4	0/4	0/4	0/24 (0%)
Cys_{46} to Ala & Cys_{49} to Ala	0/4	0/4	0/4	0/4	0/4	0/4	0/24 (0%)
Lys_7 to Asn	4/4	4/4	3/4	3/4	3/4	2/4	19/24 (79%)*
Asn_{19} to Ile	4/4	4/4	4/4	2/4	2/4	4/4	20/24 (83%)*
Cys_{20} to Arg	0/4	3/4	1/4	0/4	0/4	0/4	4/24 (17%)
Arg_{45} to Lys	4/4	4/4	4/4	1/4	3/4	4/4	20/24 (83%)*
Wild type (S81-SA)	3/4	4/4	3/4	4/4	4/4	3/4	21/24 (87%)*
No virus (mock)	0/4	0/4	0/4	0/4	0/4	0/4	0/24 (0%)

[a] Number of test plants infected/number test plants inoculated.
[b] *ND* Not determined.
[c] Asterisk denotes vector transmission efficiency not significantly different ($P > 0.05$) from wild type (S81-SA) based on ANOVA and Tukey's multiple comparison tests.

plants by RT-PCR as described [12]. The presence of each mutation was verified by sequencing of one RT-PCR product per mutant per experimental replicate (data not shown). Source plants (three per mutant per experimental replicate, except as noted in Table 2) were each colonized with ten wheat curl mites transferred from an aviruliferous colony. After a three-week acquisition access period, wheat curl mite progeny were transferred in groups of ten from each source plant to healthy wheat seedlings serving as test plants (four per source plant). Following a four-week inoculation access period, test plants were harvested and leaves stored at $-80\,^{\circ}C$. To prevent movement of wheat curl mites among plants, each source plant and each test plant was individually caged. The infection status of each test plant was determined by RT-PCR as described above.

Results of the wheat curl mite transmission assays are presented in Table 2. Of the four single cysteine-to-alanine substitutions, the only mutant that retained vector transmission competence was Cys_{20} to Ala. The remaining three single substitution mutants and the two WSMV genomes bearing double cysteine-to-alanine mutations were not transmitted by the wheat curl mite. Three of the four randomly generated substitution mutants (Lys_7 to Asn, Asn_{19} to Ile, and Arg_{45} to Lys) were transmitted by the wheat curl mite at levels (79–83%) similar to that of the positive control S81-SA (87%). The mutant bearing the Cys_{20}-to-Arg substitution retained vector transmission competence, albeit at a reduced level (17%) compared to S81-SA or the alanine substitution mutant at Cys_{20} (83%). All test plants colonized with wheat curl mites transferred from uninoculated (mock) source plants tested negative for WSMV infection by RT-PCR. Direct sequencing of RT-PCR products (two per mutant construct) amplified from WSMV-infected test plants revealed no evidence for reversion (data not shown) following transmission by the wheat curl mite.

We propose that the amino-proximal region of WSMV HC-Pro contains a non-canonical zinc finger motif [3] (His_{13}-X2-Cys_{16}-X29-Cys_{46}-X2-C_{49}), in which the three conserved cysteine residues ($Cys_{16,46,49}$), along with a conserved histidine resi-

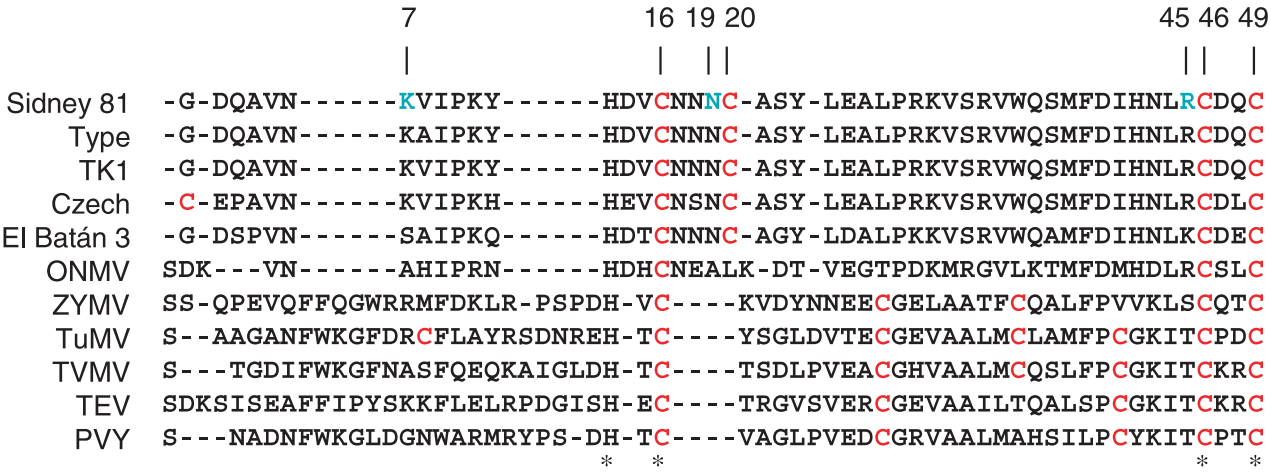

Fig. 1. Multiple alignment of the amino-proximal region of HC-Pro for five wheat streak mosaic virus (WSMV) strains (Sidney 81, Type, TK1, Czech, and El Batán 3), oat necrotic mottle virus (ONMV), and members of five species of the genus *Potyvirus* (zucchini yellow mosaic virus [ZYMV], turnip mosaic virus [TuMV], tobacco vein mottling virus [TVMV], tobacco etch virus [TEV], and potato virus Y [PVY]). Coordinates of amino acid substitutions introduced into Sidney 81 HC-Pro are indicated at the top. Cysteine residues are shown in red, and includes four Sidney 81 residues mutated to alanine (and also arginine for Cys_{20}). Green denotes other Sidney 81 HC-Pro amino acid residues subject to substitution mutations. Asterisks (*) at the bottom denote a conserved histidine residue and three conserved cysteine residues constituting a zinc-finger-like motif in WSMV HC-Pro (His_{13}-X2-Cys_{16}-X29-Cys_{46}-X2-Cys_{49})

due (His_{13}) in a favorable position relative to a cysteine residue (His-X2-C), constitute the key residues that interact with a zinc ion (Fig. 1). This same zinc-finger-like motif occurs in HC-Pro of oat necrotic mottle virus (ONMV), the tritimovirus species most closely related to WSMV [13], and is similar to that conserved among divergent potyviruses (Fig. 1). That mutations in the zinc-finger-like motif of HC-Pro for both potyviruses and WSMV abolished vector transmission suggests that, despite very different modes of transmission by distinct vector taxa, at least some aspect(s) of the mechanism by which HC-Pro mediates vector transmission is similar among genera of the family *Potyviridae*. Although a fourth cysteine residue (Cys_{20}) could be mutated to alanine without affecting transmission by the wheat curl mite, substitution of this residue with arginine reduced vector transmission efficiency. If the hypothesis (wheat curl mite transmission of WSMV requires zinc bound to the zinc-finger-like motif of HC-Pro) is correct, then these observations suggest that Cys_{20} does not directly interact with the metal ion, but instead is likely part of a loop structure which tolerates some substitutions. Interestingly, ONMV HC-Pro has leucine at this position rather than cysteine (Fig. 1), further suggesting that the zinc-finger-like motif in tritimovirus HC-Pro does not require Cys_{20}. To test the hypothesis stated above, non-transmissible WSMV HC-Pro mutants should be evaluated for zinc ion binding.

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