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Wheat streak mosaic virus coat protein is a determinant for vector transmission by the wheat curl mite

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ABSTRACT

Wheat streak mosaic virus (WSMV; genus *Tritimovirus*; family *Potyviridae*), is transmitted by the wheat curl mite (*Aceria tosichella* Keifer). The requirement of coat protein (CP) for WSMV transmission by the wheat curl mite was examined using a series of viable deletion and point mutations. Mite transmission of WSMV was completely abolished with deletions comprising CP amino acids 58–100. In contrast, the amino-proximal (amino acids 6–27 and 36–57) and carboxy-terminal (14 amino acids) regions of CP were expendable for mite transmission. Mutation of aspartic acid residues at amino acid positions 289 or 326 (D289A or D326A) at the carboxy-proximal region of CP significantly reduced mite transmission. Remarkably, every wheat plant infected by mutants D289A or D326A through mite transmission but not with *in vitro* transcripts contained a second-site mutation of R131C and N275H, respectively. Collectively, these data demonstrate for the first time that CP is a determinant for an eriophyid-transmitted plant virus.

1. Introduction

Vector transmission is necessary for dissemination of most of the plant viruses. Plant viruses have evolved with precise interactions between viral and vector proteins for vector-specific transmission, and these interactions determine the nature and efficiency of transmission (Ng and Falk, 2006; Whitefield et al., 2015). Among plant virus vectors, arthropods, nematodes, fungi, and protists play important roles in the spread and survival of plant viruses in agricultural ecosystems. Arthropods are numerically and economically the most significant vectors of plant viruses. Hemipterans, such as aphids and whiteflies are considered to be the most common vectors of plant viruses; however, the family Eriophyidae (class Arachnida; subclass Acari) includes eleven species of mites that serve as vectors of several plant viruses belong to the families *Potyviridae*, *Secoviridae*, *Alphaflexiviridae*, and *Betaflexiviridae*, and the genus *Emaravirus* (Stenger et al., 2016). Eriophyid mites are microscopic in nature with an average length of 200 μm with only two pairs of well-developed legs attached to the prodorsum just behind the head (Lindquist, 1996).

The family *Potyviridae* consists of the largest number of economically important viruses (Adams et al., 2011). This virus family is taxonomically divided into eight genera based on sequence homology and nature of vectors. The members of the family *Potyviridae* are transmitted by aphids (*Potyvirus* and *Macluravirus*), whiteflies (*Ipomovirus*), eriophyid mites (*Rymovirus*, *Tritimovirus*, and *Poacevirus*), plasmodiophorid (*Bymovirus*), and an unknown vector (*Brambyvirus*) (Whitefield et al., 2015). Among the family *Potyviridae*, viral determinants of vector transmission have been reported only for aphid-transmitted (genus *Potyvirus*) and wheat curl mite-transmitted (genus *Tritimovirus*) viruses. The CP and helper component-proteinase (HC-Pro) of members of the genus *Potyvirus* (Atreya et al., 1991; Atreya and Pirone, 1993) and HC-Pro of *Wheat streak mosaic virus* (WSMV) (Stenger et al., 2005b, 2006), the type species of the genus *Tritimovirus* (Stenger et al., 1998), have been reported as viral determinants of vector transmission. A conserved motif, asp-ala-gly (DAG) located near the amino-proximal region of the CP of most potyviruses, but absent in the CP of WSMV isolate Sidney 81, has been implicated in aphid transmission (Atreya et al., 1991, 1995; Harrison and Robinson, 1988).

WSMV is the most economically important virus of wheat (*Triticum aestivum* L.) in the Great Plains region of the USA (Brakke, 1987; French and Stenger, 2004), and it is transmitted by an eriophyid mite, the wheat curl mite (*Aceria tosichella* Keifer) (Slykhuis, 1955). The mode of WSMV transmission by wheat curl mites is not clearly defined. However, WSMV is thought to be persistently transmitted by wheat curl mite, as it must be acquired by juveniles and mites remain viruliferous through molting and as adults (Siriwetwiwat, 2006; Slykhuis, 1967). The 9,384-nucleotide (nt) single-stranded RNA genome of WSMV is encapsidated in flexuous filamentous virion particles of ~700 nm ×

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10–15 nm (Stenger et al., 1998). In contrast to multi-functional HC-Pro of potyviruses (Anandalakshmi et al., 1998; Carrington et al., 1989; Kasschau and Carrington, 1998; Kasschau et al., 1997), WSMV HC-Pro is dispensable for systemic infection of wheat (Stenger et al., 2005a). HC-Pro of WSMV was identified as a determinant of mite transmission with a crucial role for the cysteine-rich amino-terminal region (Stenger et al., 2005b, 2006; Young et al., 2007). Though potyviral CP has been reported as a determinant for aphid transmission (Revers and Garcia, 2015), there is no information on the role of WSMV CP in wheat curl mite transmission.

Recently, we reported that the CP cistron of WSMV tolerated extensive deletions and point mutations at the amino-proximal and carboxy-terminal regions (Tatineni and French, 2014; Tatineni et al., 2011a, 2014, 2017). WSMV mutants with deletions comprising CP amino acids 36–84 accumulated similarly or more efficiently in wheat compared to wild-type virus with no noticeable effect on virion formation (Tatineni et al., 2014, 2017). In contrast, WSMV with deletion of CP amino acids 6–27, 85–100, or carboxy-terminal 14 amino acids infected wheat systemically but accumulated at reduced levels as these amino acids are required for efficient cell-to-cell movement and/or virion assembly (Tatineni et al., 2014). Additionally, negatively charged aspartic acid residues at amino acid positions 289, 290, and 326 at the carboxy-proximal region of CP are dispensable for systemic infection of wheat but not for maize (*Zea mays* L.) inbred line SDp2 (Tatineni and French, 2014).

The role of CP in WSMV transmission by the wheat curl mite was examined using a series of viable deletion and point mutations. The results demonstrate amino acids 58–100 are required for WSMV transmission by wheat curl mites, with aspartic acid residues at amino acid positions 289 and 326 required for efficient mite transmission. Interestingly, wheat infected by mutants D289A or D326A harbored a second-site mutation of R131C and N275H, respectively, but only when transmitted by mites. Collectively, these data revealed that CP is a determinant of WSMV transmission by the wheat curl mite.

2. Results

2.1. WSMV CP amino acids 36–57 are dispensable for mite transmission

WSMV CP contains three motifs comprising amino acids SGSGS (flexible linker) at positions 36–40, 43–47, and 53–57, and deletion of these motifs, either individually or together (deletion of amino acids 36–57), resulted in systemically infected wheat with no appreciable effects on virus accumulation (Tatineni et al., 2017). Mutants WSMV-CPΔ36-40aa (SGSGS-1), WSMV-CPΔ43-47aa (SGSGS-2), WSMV-CPΔ53-57aa (SGSGS-3), and WSMV-CPΔ36-57aa (all three motifs) were used for wheat curl mite transmission to examine the requirement of SGSGS motifs for mite transmission (Fig. 1A).

Single-mite transmission of SGSGS deletion mutants was conducted from four source plants in two independent experiments, and results presented in Table 1. An analysis of variance showed that there was no interaction between the two independent mite transmission experiments ($F = 0.28$; $df = 11, 26$; $P = 0.9846$); therefore, data from these two experiments were combined for the analysis. Mite transmission of WSMV-CPΔ36-40aa ranged from 20% to 90% from four source plants with an average transmission of 55%, WSMV-CPΔ43-47aa ranged from 40% to 100% with 62% average transmission, and WSMV-CPΔ53-57aa ranged from 50% to 100% with average mite transmission rate of 77%. Mite transmission of WSMV-CPΔ36-57aa ranged from 40% to 70% with 50% average transmission (Table 1). Under the same experimental conditions, wild-type virus was transmitted from 30% to 60% with an average transmission rate of 50%.

There were no differences in mite presence between treatments ($F = 1.58$; $df = 12, 35$; $P = 0.1320$), indicating that mite transmission differences between treatments were not the result of differences in mite establishment and survival. Virus transmission differed among WSMV constructs ($F = 6.39$; $df = 12, 35$; $P = 0.0001$) with the greatest transmission occurring for WSMV-CPΔ53-57aa (77%), followed by WSMV-CPΔ43-47aa (62%), WSMV-CPΔ36-40aa (55%), WSMV-CPΔ36-57aa (50%), and WSMV-S81 (wild-type) (50%). WSMV-CPΔ53-57aa had significantly greater transmission than all other mutants except WSMV-CPΔ43-47aa. In contrast, no significant differences occurred between WSMV-S81 and WSMV-CPΔ43-47aa, WSMV-CPΔ36-40aa, and WSMV-CPΔ36-57aa. These data indicate that CP amino acid sequences comprising the SGSGS motifs are dispensable for wheat curl mite transmission of WSMV.

Total RNA extracted from the source and select mite-transmitted plants was subjected to RT-PCR using CP specific primers (Fig. 1B), followed by nucleotide sequencing. All mutants retained the introduced deletions, and no other second-site mutation was found in the CP cistron. Wheat curl mite transmission from buffer-inoculated (mock) source plants resulted in no infection of test plants indicating that there was no significant movement or contamination of source plants or mite movement between test plants following infestations.

2.2. WSMV CP amino acids 58–100 are required for mite transmission

WSMV mutants with deletion of amino acids 58–84, 49–83, 36–84, 85–100 or 36–100 in CP failed to be transmitted by single wheat curl mites transferred from four source plants (0 out of 40 test plants infected) (Fig. 1A; Table 1). Wild-type WSMV had an average mite transmission of 50%. These data revealed that WSMV mutants with deletions comprising amino acids 58–100 in CP failed to be transmitted by the wheat curl mite using a single mite per test plant. Virus source plants examined for the presence of WSMV with introduced deletions in the CP cistron by RT-PCR (Fig. 1B), followed by nucleotide sequencing revealed that all virus source plants retained the introduced deletions in the CP cistron.

Since the above mite transmission experiments were performed with a single mite per test plant, it is possible that failure to detect mite transmission of CP deletion mutants could be due to reduced efficiency of vector transmission. To exclude this possibility, vector transmission of WSMV mutants harboring deletion of amino acids 36–84, 49–83, or 85–100 in CP was performed using five mites per test plant across three source plants per mutant virus (Table 2). Vector transmission of wild-type WSMV ranged from 80% to 90%, with an average transmission rate of 83% (25 out of 30 test plants infected) (Table 2). In contrast, WSMV mutants bearing a deletion comprising amino acids 36–84, 49–83, or 85–100 in CP failed to be transmitted by mites (0 out of 30 test plants infected for each deletion mutant). Since CP amino acids 36-57 are expendable for mite transmission, the above data conclusively demonstrated that CP amino acids 58–100 are required for WSMV transmission by wheat curl mites.

2.3. The amino-proximal and carboxy-terminal regions of WSMV CP are dispensable for mite transmission

Wheat plants systemically infected with WSMV-CPΔN6-27aa and WSMV-CPΔC14aa (Tatineni et al., 2014) were used for mite transmission using 3–4 source plants per mutant (Table 1). Mite transmission of WSMV-CPΔN6-27aa ranged from 0% to 10% with an average transmission rate of 7% (2 out of 30 plants infected) (Table 1). WSMV-CPΔC14aa transmitted by wheat curl mites at 0–20% with an average transmission rate of 10% (4 out of 40 plants infected). These data revealed that vector transmission of WSMV-CPΔC14aa (10%) and WSMV-CPΔN6-27aa (7%) were significantly lower than wild-type WSMV-S81 (50%). However, reduced vector transmission of these mutants could be due to CP amino acids 6–27 and 14 amino acids at the carboxy-terminal region that are required for efficient virion assembly/cell-to-cell movement as reported by Tatineni et al. (2014). No differences in transmission occurred between WSMV-CPΔC14aa and WSMV-CPΔN6-27aa ($t = 0.16$; $df = 1, 35$; $P = 0.6954$). Mite transmission source and

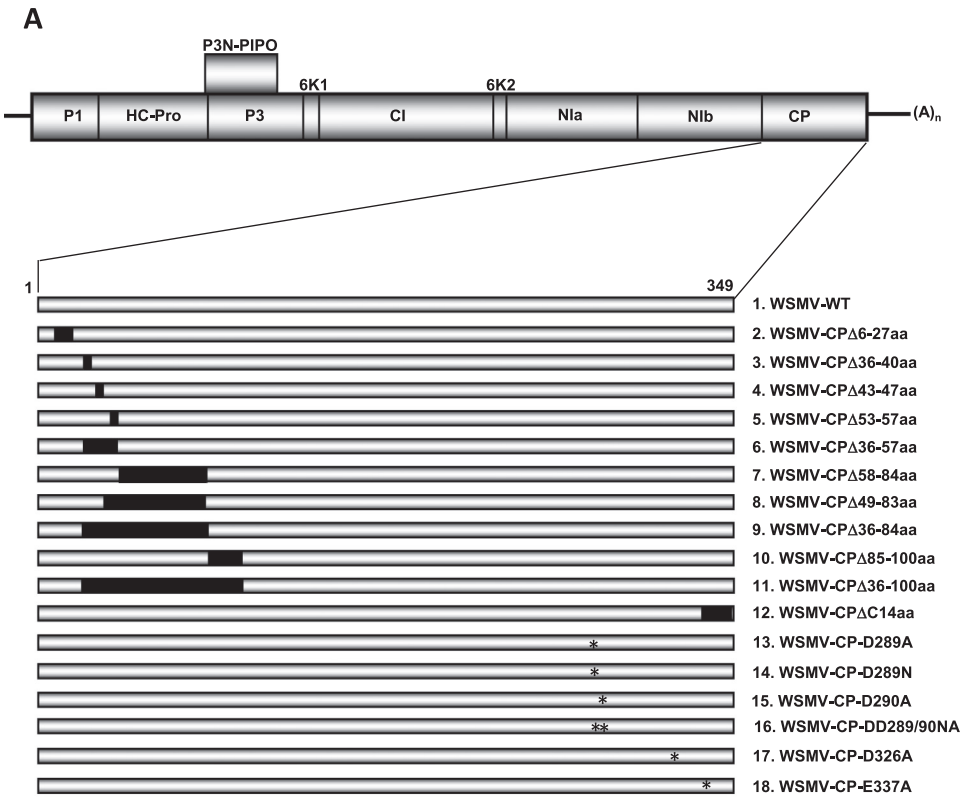

Fig. 1. (A). Schematic diagram of the genomic organization of *Wheat streak mosaic virus* (WSMV) with individual mature proteins indicated inside the large polyprotein. The location of P3N-PIPO is indicated. Vertical lines inside the large ORF indicate the position of cleavage peptides. Expanded view of coat protein (CP) with engineered deletions or point mutations is indicated below the genomic organization of WSMV. Engineered deletion and point mutations in CP are indicated with solid boxes and asterisks, respectively, and were described previously in Tatineni et al., (2014, 2017) and Tatineni and French (2014). (B). RT-PCR amplification of CP cistron from wheat curl mite-source plants that were infected with WSMV mutants harboring deletion or point mutations. The numbers above agarose gels correspond to WSMV CP mutants indicated by numbers in panel A. The sizes of 1.0 kbp DNA ladder bands (lane L) are indicated on the right. Lane M: Mock inoculated wheat; P: pSP6-WSMV-S81 as a positive control for PCR.

Table 1
Wheat curl mite transmission assays of *Wheat streak mosaic virus* (WSMV) coat protein (CP) deletion mutants[a].

Mutant	Virion assembly/cell-to-cell movement	Wheat curl mite transmission				
		Expt. 1[d]	Expt. 2[d]			
			Source plant 1	Source plant 2	Source plant 3	Average % transmission[f]
WSMV-S81 (WT)	+ + +/+ + +[b]	6/10	3/10	5/10	6/10	50% b[g]
WSMV-CPΔ36–40aa	+ + +/+ + +[c]	9/10	7/10	4/10	2/10	55% b
WSMV-CPΔ43–47aa	+ + +/+ + +[c]	10/10	5/10	4/10	6/10	62% ab
WSMV-CPΔ53–57aa	+ + +/+ + +[c]	9/10	5/10	10/10	7/10	77% a
WSMV-CPΔ36–57aa	+ + +/+ + +[c]	7/10	4/10	5/10	4/10	50% b
WSMV-CPΔ58–84aa	+ + +/+ + +[c]	0/10	0/10	0/10	0/10	0% c
WSMV-CPΔ49–83aa	+ + +/+ + +[c]	0/10	0/10	0/10	0/10	0% c
WSMV-CPΔ36–84aa	+ + +/+ + +[b]	0/10	0/10	0/10	0/10	0% c
WSMV-CPΔ85–100aa	+ +/+ +[b]	0/10	0/10	0/10	0/10	0% c
WSMV-CPΔ36–100aa	+ ±/+ ±[b]	0/10	0/10	0/10	0/10	0% c
WSMV-CPΔC14aa	+ + +/+[b]	1/10	1/10	0/10	2/10	10% c
WSMV-CPΔN6–27aa	+/+[b]	ND[e]	0/10	1/10	1/10	7% c
Mock	–	0/10	0/10	0/10	0/10	0% c

[a] Test plants infested with viruliferous mites were harvested at 25 days postinfestation except WSMV-CPΔ85–100, WSMV-CPΔ36–100, WSMV-CPΔC14aa, and WSMV-CPΔN6-27aa (harvested at 32 days postinfestation). Wheat curl mite transmission experiments were performed using a single viruliferous mite per test plant. All source and test plants were assayed for WSMV infection by DAC-ELISA.

[b] Virion assembly/cell-to-cell movement of WSMV CP deletion mutants was reported in (Tatineni et al., 2014).

[c] Virion assembly/cell-to-cell movement of WSMV CP deletion mutants was reported in (Tatineni et al., 2017).

[d] Number of plants infected/number plants infested.

[e] ND: not determined.

[f] Average transmission from Experiment 1 and 2.

[g] Different letters indicate significant differences ($P < 0.05$) in virus transmission between mutants.

Table 2
Wheat curl mite transmission of *Wheat streak mosaic virus* (WSMV) coat protein (CP) deletion mutants with five viruliferous mites per test plant[a].

Mutant	Mite transmission to test plants[b]			
	Source plant 1	Source plant 2	Source plant 3	Average % transmission
WSMV-Sidney	8/10	9/10	8/10	83%
WSMV-CPΔ36–84aa	0/10	0/10	0/10	0%
WSMV-CPΔ49–83aa	0/10	0/10	0/10	0%
WSMV-CPΔ85–100aa	0/10	0/10	0/10	0%
Mock	0/10	0/10	0/10	0%

[a] Test plants infested with viruliferous mites were harvested at 25 days postinfestation. All source and test plants were assayed for WSMV infection by DAC-ELISA.
[b] Number of plants infected/number plants infested.

progeny wheat plants were subjected to RT-PCR amplification of CP cistron (Fig. 1B), followed by nucleotide sequencing revealed that all mutants retained the introduced deletions with no unintended mutations in the CP cistron.

2.4. The carboxy-proximal aspartic acid residues are required for efficient mite transmission

The role of negatively charged aspartic acid residues located at amino acid positions 289, 290, and 326 in WSMV transmission by the wheat curl mite was examined by performing mite transmission assays with WSMV mutants bearing the following point mutations in the CP: D289A, D289N, D290A, DD289/290NA, or D326A (Tatineni and French, 2014). As a control, a glutamic acid residue at amino acid position 337 was mutated to alanine (E337A), and was included in vector transmission assays (Fig. 1A). Virus transmission varied by WSMV mutant ($F = 2.63$; df = 6, 19; $P = 0.0498$). Mite transmission of wild-type WSMV-S81 (55%, 11 out of 20 test plants infected) was not significantly different ($F = 0.42$; df = 1, 19; $P = 0.5231$) from transmissions of WSMV-CP-D290A (47%, 7 out of 15 test plants infected) and WSMV-CP-E337A (45%, 9 out of 20 test plants infected) (Table 3). These data indicate that mutation of aspartic acid and glutamic acid residues at amino acids positions 290 and 337, respectively, did not significantly affect WSMV transmission by wheat curl mites.

In contrast, virus transmission was significantly lower ($t = -2.21$; df = 19; $P = 0.0394$) for mutant DD289/290NA (20%, 4 out of 20 test plants infected) when compared with WSMV-S81 (55%). Mutants D326A, D289N, and D289A also were transmitted poorly by mites at 15% (3 out of 20 test plants infected), 13% (2 out of 15 test plants infected), and 15% (3 out of 20 test plants infected) (Table 3), respectively, suggesting that aspartic acid residues at positions 289 and 326 are required for efficient WSMV transmission by mites. Mite presence did not differ between treatments ($F = 0.53$; df = 6, 19; $P = 0.7783$) indicating that transmission differences were not due to variations in mite establishment and survival.

Total RNA extracted from wheat infected by aspartic acid mutants through mite transmission was used for RT-PCR amplification of CP cistron (Fig. 1B), followed by nucleotide sequencing for the presence of any unintended second-site mutations. Wheat infected through mite transmission of mutants D289N, D290A, DD289/90NA, or E337A retained the introduced mutation with no other second-site mutation in the CP cistron. In contrast, all three wheat plants infected by mutants D289A or D326A through mite transmission retained the introduced mutation, but additionally found a second-site mutation of R131C and N275H, respectively (Table 3). No mutations were observed in the CP cistron from wheat plants infected by the wild-type virus through wheat curl mites.

2.5. Second-site mutations found in CP cistron are specific to wheat curl mite transmission

The presence of a second-site mutation in wheat infected by mutants D289A or D326A via mite transmission prompted us to examine whether these second-site mutations are specific to mite transmission or occurred in wheat infected by *in vitro* transcripts. *In vitro* transcripts of mutants D289A or D326A were inoculated onto wheat seedlings at the single-leaf stage. The CP cistron was amplified from total RNA extracted from wheat infected with *in vitro* transcripts of mutants D289A or D326A at 14 days postinoculation (dpi), followed by cloning into pGEM-T. The presence of second-site mutations in the CP cistron was examined by sequencing 15 independent clones for each mutant and found that the progeny virus retained the introduced mutation in all sequenced clones, but no other second-site mutation was found. These data suggest that second-site mutations observed in mutants D289A- or D326A-infected wheat by mites might have occurred during the process of mite transmission.

2.6. Effect of second-site mutations on wheat curl mite transmission efficiency

The above experiments suggest that mutants D289A and D326A might have acquired second-site mutations during the process of wheat curl transmission. It is not known whether these second-site mutations may cause increased vector transmission of respective mutant viruses. Vector transmission of double mutants carrying an introduced mutation plus a second-site mutation, D289A + R131C and D326A + N296H, was performed along with parental mutants (D289A and D326A) and wild-type virus as controls in two independent mite transmission experiments.

An analysis of variance showed that there was no significant interaction between two independent mite transmission experiments ($F = 0.19$; df = 4, 30; $P = 0.9442$); therefore, data from these two

Table 3
Wheat curl mite transmission assays of *Wheat streak mosaic virus* (WSMV) coat protein (CP) aspartic acid mutants using a single viruliferous mite per test plant[a].

Mutant	Mite transmission[b]	Second-site mutation	# of plants with second site mutation
WSMV-S81 (WT)	5/5, 2/5, 3/5, 1/5: **11/20 (55%)** a[d]	None[c]	None
WSMV-CP-D289A	1/5, 1/5, 1/5, 0/5: **3/20 (15%)** b	R131C	3
WSMV-CP-D289N	1/5, 0/5, 1/5: **2/15 (13%)** b	none	None
WSMV-CP-D290A	3/5, 2/5, 2/5: **7/15 (47%)** ab	none	None
WSMV-CP-DD289/90NA	1/5, 1/5, 0/5, 2/5: **4/20 (20%)** b	none	None
WSMV-CP-D326A	0/5, 2/5, 1/5, 0/5: **3/20 (15%)** b	N275H	3
WSMV-CP-E337A	1/5, 2/5, 2/5, 4/5: **9/20 (45%)** ab	none	None

[a] Test plants infested with viruliferous mites were harvested at 25 days postinfestation. All source and test plants were assayed for WSMV infection by RT-PCR and/or DAC-ELISA.
[b] Number of plants infected/number plants infested per source plant. The total number of plants infected/number of plants infested (percentage of transmission) from all source plants was indicated in bold.
[c] Coat protein was sequenced from 2 to 4 wheat plants infected through transmissions by single mites.
[d] Different letters indicate significant difference between mutants at $P < 0.05$.

Table 4
Effect of aspartic acid mutation and its *in vivo* second-site mutation in the coat protein (CP) on mite transmission efficiency of *Wheat streak mosaic virus* (WSMV)[a].

Mutant	Experiment 1		Experiment 2		% mite transmission[c]
	Mite transmission[b]	% mite transmission	Mite transmission[b]	% mite transmission	
WSMV-S81 (WT)	1/5, 3/5, 2/5, 3/5, 3/5: **12/25**	48%	6/10, 7/10, 7/10: **20/30**	67%	58% a[d]
WSMV-CP-D289A	4/5, 2/5, 1/5, 0/5, 1/5: **8/25**	32%	6/10, 2/10, 4/10: **12/30**	40%	36% ab
WSMV-CP-D289A + R131C	1/5, 0/5, 2/5, 1/5, 1/5: **5/25**	20%	4/10, 3/10, 6/10: **13/30**	43%	33% b
WSMV-CP-D326A	1/5, 0/5, 1/5, 1/5, 0/5: **3/25**	12%	0/10, 1/10, 6/10: **7/30**	23%	18% b
WSMV-CP-D326A + N296H	1/5, 1/5, 1/5, 3/5, 0/5: **6/25**	24%	4/10, 4/10, 6/10: **14/30**	47%	36% ab

[a] Wheat curl mite transmissions were performed using a single viruliferous mite per test plant. All source and test plants were assayed for WSMV infection by DAC-ELISA.
[b] Number of plants infected/number plants infested per source plant. The total number of plants infected/number of plants infested from all source plants was indicated in bold.
[c] Average mite transmission of experiment 1 and 2.
[d] Different letters indicate significant differences between mutants at $P < 0.05$.

experiments were combined for the analysis. Mite presence did not differ between treatments ($F = 0.22$; df = 4, 35; $P = 0.9270$) indicating that transmission differences were not due to mite establishment and survival. Virus transmission differed between treatments ($F = 6.15$; df = 4, 35; $P = 0.0232$); however, t-tests showed that many of the paired comparisons were only approaching significance. WSMV-S81 (58%; 32 out of 55 test plants infected) had the greatest transmission when compared to double mutant D289A + R131C (33%, 18 out of 55 test plants infected; $t = -1.82$; df = 35; $P = 0.0776$) or D326A + N296H (36%, 20 out of 55 test plants infected; $t = -1.89$; df = 35; $P = 0.0677$). Mite transmission of the double mutant D289A + R131C was not different ($t = 0.40$; df = 35; $P = 0.6912$) when compared to the parental mutant D289A (Table 4), indicating that the second-site mutation in the mutant D289A did not increase vector transmission. In contrast, mite transmission of the double mutant D326A + N296H (36%) was marginally greater ($t = -1.79$; df = 35; $P = 0.0825$) than the parental mutant D326A (18%), indicating that the N296H second-site mutation in mutant D326A might have facilitated an increase in mite transmission efficiency (Table 4). Nucleotide sequencing of RT-PCR products of CP cistrons of mutants D289A + R131C or D326A + N296H from four wheat plants infected through mite transmission revealed no other mutations.

3. Discussion

This study demonstrated that CP amino acids 58–100 are required for WSMV transmission by wheat curl mites, while amino acids 6–27, 36–57, and C-terminal 14 amino acids are expendable. Previously, we reported that deletion of CP amino acids 58–84 did not affect virion formation and cell-to-cell and long-distance movement of WSMV, and these deletions resulted in increased virus titer compared to wild-type virus (Table 5; Tatineni et al., 2014, 2017). These data suggest that failure of mite transmission of mutants lacking CP amino acids 58–84 are not due to the effect of these deletions on virion formation and/or movement. CP amino acids 85–100 are required for efficient virion formation and cell-to-cell movement (Tatineni et al., 2014); however, WSMV-CPΔ85-100aa efficiently accumulated in wheat (Table 5), suggesting that reduced virion assembly may not be the primary reason for inability to be transmitted by wheat curl mites. Moreover, wheat curl mites efficiently propagated on wheat plants infected by all deletion mutants, suggesting that lack of mite transmission is not due to failure to establish mite populations. Taken together, these data revealed that complete loss of mite transmission of WSMV mutants lacking CP amino acids 58–100 provides the first evidence that CP is required for vector transmission of a potyvirid species outside of the genus *Potyvirus* in the family *Potyviridae*.

The amino and carboxy termini of CP of potyviruses have been shown to be exposed at the virion surface (Shukla et al., 1988) and the DAG motif located near the N-terminus of CP interacts with HC-Pro for successful aphid transmission (Blanc et al., 1997). In contrast, WSMV

Table 5
Accumulation of *Wheat streak mosaic virus* (WSMV) coat protein (CP) deletion mutants in wheat cv. Tomahawk.

Mutant	ELISA[a] OD_{405}
WSMV-S81 (WT)[b]	2.06 ± 0.01[c]
WSMV-CPΔ58–84aa[b]	2.26 ± 0.01
WSMV-CPΔ49–83aa[b]	2.15 ± 0.14
WSMV-CPΔ36–84aa[b]	2.33 ± 0.02
WSMV-CPΔ85–100aa[d]	1.86 ± 0.01
WSMV-CPΔ36–100aa[d]	1.66 ± 0.00
WSMV-CPΔN6–27aa[d]	1.26 ± 0.00
WSMV-CPΔC14aa[d]	1.93 ± 0.06
Healthy	0.28 ± 0.01

Wheat seedlings inoculated at the single-leaf stage with crude sap at 1:20 dilution.
[a] DAC-ELISA performed with polyclonal antibodies.
[b] Plants tested at 12 days postinoculation (dpi).
[c] ELISA values are average of three wells ± standard error.
[d] Plants tested at 18 dpi.

CP lacks the DAG motif (Stenger et al., 1998) and N-terminal 6–27 amino acids are dispensable for mite transmission. Additionally, the carboxy-terminal region of CP also is dispensable for mite transmission as WSMV with a deletion comprising the C-terminal 14 amino acids transmitted at 10%. The reduced mite transmission of WSMV with a deletion at the amino-proximal or carboxy-terminal regions of CP might have been due to substantially reduced virion assembly and/or cell-to-cell movement by these mutants (Table 1 and 5; Tatineni et al., 2014). These data suggest that in contrast to aphid-transmitted potyviruses, the amino-proximal region of WSMV CP is dispensable for vector transmission.

Mutation of aspartic acid residues at amino acid positions 289 or 326 to alanine in the carboxy-proximal region of CP dramatically reduced mite transmission efficiency to 13–15% compared to 55% by the wild-type virus. In contrast, mutation of aspartic acid or glutamic acid residues respectively at amino acid positions 290 or 337 to alanine did not significantly affect mite transmission. These data indicate that aspartic acid residues at 289 and 326 are crucial for efficient mite transmission. Remarkably, every wheat plant infected via mite transmission of mutants D289A or D326A contains a second-site mutation of R131C and N275H, respectively. However, these second-site mutations were not found in 15 sequenced clones from wheat infected with *in vitro* transcripts of mutants D289A or D326A. These data suggest that mutation of D289A and D326A in CP might have affected interaction with other viral and/or mite proteins. Thus, under selection pressure for protein-protein interaction for mite transmission, the CP with D289A or D326A mutation might have acquired a compensatory second-site mutation. This hypothesis was further supported by the fact that a second-site mutation was found in all mite-transmitted plants of mutant D289A, but not in mutants D289N, DD289/90NA, and D289A + R131C. These data also suggest that mutation of aspartic acid residue at amino

acid position 289 to asparagine is more tolerable compared to alanine.

Mutant D326A with a second-site mutation caused increased mite transmission compared to parental mutant (18% vs 36%). In contrast, the second-site mutation in D289A did not affect vector transmission efficiency (32% vs 36%). However, neither of these mutants with a second-site mutation restored mite transmission to wild-type level, suggesting that mutation of aspartic acid residues at amino acid positions 289 or 326 to alanine might have caused perturbation of a structural motif required for mite transmission, while the second-site mutation might have at least partially compensated for these structural changes. Since second-site mutations were not detected in wheat plants infected by *in vitro* transcripts, it is conceivable that these mutations might have occurred during replication of virus in mites. Recently, we found that WSMV in wheat curl mites exhibited 0.3% sequence diversity in 0.4 to 4.2% of virus-specific RNA-seq reads, suggesting that sequence diversity found in WSMV genome might have resulted during viral replication in wheat curl mites (A. K. Gupta, G.L. Hein, and S. Tatineni, unpublished data). Thus, the observed second-site mutations in CP might have compensated for the introduced mutations during WSMV replication in wheat curl mites and facilitated mite transmission. Alternately, wheat curl mites might have acquired a rare *in planta* mite-transmissible double-mutant under strong selection pressure. Second-site mutations in the CP of *Cucumber mosaic virus* also have been reported (Liu et al., 2002; Suzuki et al., 1995); however, these second-site mutations were found *in planta* prior to aphid transmission.

This report and studies by Stenger et al. (2005b, 2006) and Young et al. (2007) revealed that both CP and HC-Pro are determinants of WSMV transmission by wheat curl mites, similar to aphid-transmitted potyviruses (Atreya et al., 1991; Atreya and Pirone, 1993). However, potyviruses and tritimoviruses differ in the mode of vector transmission. In nonpersistently transmitted potyviruses, the interaction between HC-Pro and CP is essential for the bridge hypothesis that would facilitate virion attachment to aphid stylet; thus, HC-Pro provides a link between virus particles and aphid stylets (Peng et al., 1998; Pirone and Blanc, 1996). In potyvirus bridge model, the PTK motif in HC-Pro and the DAG motif in CP determine the HC-Pro-CP interaction (Atreya et al., 1992; Blanc et al., 1997, 1998; Granier et al., 1993), while the KITC motif of HC-Pro mediates interaction with aphid stylet (Huet et al., 1994; Peng et al., 1998; Wang et al., 1996). However, these motifs are not present in HC-Pro and CP of WSMV-S81 and the interaction between HC-Pro and CP of WSMV has not been observed in the yeast two-hybrid system and pull-down assays (Choi et al., 2000). This indicates that the mechanism of HC-Pro- and CP-mediated vector transmission of WSMV is different from that of aphid-transmitted potyviruses because WSMV is maintained through the molt of the mite and thus, it is not stylet borne. The present study opens the door for additional experiments to further dissect the mechanisms of wheat curl mite-WSMV interactions required for vector transmission.

4. Materials and methods

4.1. WSMV CP mutants

WSMV isolate Sidney 81 (WSMV-S81) was obtained by inoculating wheat seedlings (cv. Tomahawk) at the single-leaf stage with *in vitro* transcripts from pSP6-WSMV (Choi et al., 1999). Wheat leaves infected with WSMV-S81 were stored at −20 °C for future inoculations or directly inoculated onto source plants raised in cone-tainers (Stuewe & Sons, Inc.) that were covered with plastic cages.

WSMV CP deletion and point mutants were previously described in Tatineni and French (2014) and Tatineni et al., (2014, 2017). The CP deletion mutants WSMV-CPΔ36-84aa, WSMV-CPΔ85-100aa, WSMV-CPΔ36-100aa, WSMV-CPΔ6-27aa, and WSMV-CPΔC14aa were described in Tatineni et al. (2014); and WSMV-CPΔ36-40aa, WSMV-CPΔ43-47aa, WSMV-CPΔ53-57aa, WSMV-CPΔ36-57aa, WSMV-CPΔ58-84aa, and WSMV-CPΔ49-83aa were described in Tatineni et al. (2017). Point mutations, WSMV-CP-D289A, WSMV-CP-D289N, WSMV-CP-DD289/90NA, WSMV-CP-D326A, and WSMV-CP-E337A, at the carboxy-proximal region of CP were described in Tatineni and French (2014). All plasmid DNAs were transformed into *Escherichia coli* strain JM109 (Promega Corporation, Madison, WI), and plasmid DNAs were prepared from 40 ml cultures grown overnight by using a Bio-Rad plasmid midiprep kit (Bio-Rad, Hercules, CA). Linearized plasmid DNAs were used to generate *in vitro* RNA transcripts and inoculated onto wheat seedlings at the single-leaf stage as described previously (Tatineni et al., 2011b). Wheat leaves infected with WSMV-S81 or WSMV mutants with a deletion or point mutations in the CP were collected at 18 dpi, and stored at −20 °C for inoculation onto wheat seedlings raised in cone-tainers as mite transmission source plants.

4.2. Mite transmission assays

Wheat curl mite transmission assays of wild-type WSMV and CP deletion and point mutants were performed as described in McMechan et al. (2014). Briefly, three wheat seedlings (cv. Millennium) per container were raised in a greenhouse in 4-cm diameter cone-tainers. The cone-tainers were covered with plastic cylindrical cages 5-cm in diameter and 50-cm height with two to three Nytex screen covered vents for aeration. Wheat seedlings at the single-leaf stage were inoculated with crude extracts of WSMV-S81 or WSMV CP deletion or point mutants at 1:20 dilution in 20 mM sodium phosphate buffer, pH 7.0, and incubated in a greenhouse. At 14 dpi, a single symptomatic source plant was retained per cone-tainer. Individual source plants were infested with 5–10 non-viruliferous Type 2 wheat curl mites (Hein et al., 2012). When infesting source plants, a randomized complete block design was used, and within replicates, infestation order was determined randomly. Source plants were incubated in a growth chamber at 24–26 °C max and 20–22 °C min with a 14 h photoperiod for rearing mites on infected wheat. Three weeks after infestation, wheat curl mites from source plants were transferred to test plants (one or five mites per test plant) at the two-leaf stage under the microscope with the aid of an eyelash attached to a wooden dowel. Infestation order of test plants was randomized using SAS (version 9.4; SAS Institute 2012) with the random number function set to 0, allowing the internal clock of computer to initialize the stream of random numbers. Infested test plants were immediately caged, and maintained in the growth chamber until harvest. For each mutant virus, 3–5 source plants with 5–10 test plants per source plant were used. In total, 15–40 test plants were used per mutant virus. Test plants were harvested at 25 days after infestation (dai) except for mutants WSMV-CPΔ85-100aa, WSMV-CPΔ36-100aa, WSMV-CPΔC14aa and WSMV-CPΔN6-27aa, which were harvested at 32 dai. Each test plant at 25 or 32 dai was examined under dissecting microscope for the presence of mites and evaluated for infection by direct antigen coating enzyme-linked immunosorbent assay (DAC-ELISA) and/or RT-PCR.

4.3. Reverse transcription-polymerase chain reaction (RT-PCR) of CP region

Total RNA was extracted from virus source and select test plants of mite transmission experiments as described in McNeil et al. (1996). First-strand cDNA was synthesized from total RNA in a 10-µl volume using random primers (Promega) as described previously (Tatineni et al., 2010). One microliter of first-strand cDNA reaction was used for PCR in a 25-µl volume with a forward primer XV1 and a reverse primer XC1 (Tatineni, 2017) by using Herculase II Fusion DNA polymerase (Agilent Technologies, Santa Clara, CA) as described in Tatineni et al. (2014). RT-PCR products were analyzed electrophoretically through 1.0% agarose gels in TAE (Tri-acetate-EDTA) buffer.

4.4. Nucleotide sequencing

RT-PCR products from virus source and select test plants were purified by separating through 1.0% agarose gels, followed by gel elution using GENECLEAN III kit (MP Biomedicals, Solon, OH). Gel-isolated RT-PCR products were directly sequenced with XV1 and XC1 primers, and pairwise sequence comparisons of CP sequences from source and test plants were carried out using the ALIGN program of online analysis tools at http://molbioltools.ca.

4.5. ELISA assays

Test plants were examined for virus presence by DAC-ELISA as described in Hobbs et al. (1987). WSMV polyclonal antiserum and goat anti-rabbit IgG-alkaline phosphatase conjugate (Sigma-Aldrich, St. Louis, MO) were used as primary and secondary antibodies at 1:5000 and 1:2000 dilution, respectively. After addition of primary and secondary antibodies to antigen coated plates, p-nitrophenyl phosphate (Sigma-Aldrich) was used as substrate at 1 mg/ml. ELISA plates were read at OD_{405} in a Spectra-Max plate reader (Molecular Devices, Sunnyvale, CA) at 30 and 60 min following the addition of substrate. Samples tested in ELISA were considered positive if OD values were at least three times higher than those of healthy values.

4.6. Statistical Analysis

Presence-absence data for virus transmission and mites were analyzed by using PROC GLIMMIX (version 9.4; SAS Institute 2012) with a binomial distribution. In these analyses of variance, treatment was included as a fixed effect, and experimental source plant (replicate) was included as a random effect. The possibility for interactions between experiments (runs) and treatments was analyzed when applicable and eliminated before combining experiments for the final analysis. When analysis of variance revealed significant differences between treatments, contrasts were used to separate treatment groups, and Tukey's studentized range tests were used to determine differences ($P < 0.05$) between specific treatments.

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