The C-terminus of *Wheat streak mosaic virus* Coat Protein Is Involved in Differential Infection of Wheat and Maize through Host-Specific Long-Distance Transport

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Viral determinants and mechanisms involved in extension of host range of monocot-infecting viruses are poorly understood. Viral coat proteins (CP) serve many functions in almost every aspect of the virus life cycle. The role of the C-terminal region of *Wheat streak mosaic virus* (WSMV) CP in virus biology was examined by mutating six negatively charged aspartic acid residues at positions 216, 289, 290, 326, 333, and 334. All of these amino acid residues are dispensable for virion assembly, and aspartic acid residues at positions 216, 333, and 334 are expendable for normal infection of wheat and maize. However, mutants D₂₈₉N, D₂₈₉A, D₂₉₀A, DD_{289/290}NA, and D₃₂₆A exhibited slow cell-to-cell movement in wheat, which resulted in delayed onset of systemic infection, followed by a rapid recovery of genomic RNA accumulation and symptom development. Mutants D₂₈₉N, D₂₈₉A, and D₃₂₆A inefficiently infected maize, eliciting milder symptoms, while D₂₉₀A and DD_{289/290}NA failed to infect systemically, suggesting that the C-terminus of CP is involved in differential infection of wheat and maize. Mutation of aspartic acid residues at amino acid positions 289, 290, and 326 severely debilitated virus ingress into the vascular system of maize but not wheat, suggesting that these amino acids facilitate expansion of WSMV host range through host-specific long-distance transport.

The ability of plant viruses to systemically infect a wide range of host plants depends on successful interactions between viral and host factors for replication, suppression of host defense mechanisms, and movement (Nelson and Citovsky 2005; Voinnet et al. 1999). Cell-to-cell movement of plant viruses from initially infected cells to adjacent cells occurs through a specialized intercellular connection, the plasmodesmata (Benitez-Alfonso et al. 2010; Lucas et al. 2009; Maule 2008). Infection foci form with continuous cell-to-cell movement until the virus encounters the vascular tissue, followed by loading into phloem-related cells for long-distance transport. Long-distance movement of viruses through phloem-associated cells is more complex, involving the passive transport of viruses along with photosynthates from source to sink tissues and unloading at distal ends through veins, followed by cell-to-cell movement (Carrington et al. 1996; Harries et al. 2010; Hipper

et al. 2013). Successive cycles of cell-to-cell and long-distance translocation of viruses are needed to establish successful systemic infection throughout the plant.

Cell-to-cell movement of plant viruses is an active process mediated by virus-encoded movement proteins (MP) through interactions with host factors (Benitez-Alfonso et al. 2010; Harries and Ding 2011; Waigmann et al. 2004; Wang et al. 1998; Xoconostle-Cazares et al. 2000). Plant viruses encode a diverse range of MP with differences in the mode of virus transport. The tobamovirus 30K-like MP bind virions or viral ribonucleoprotein complexes and then translocate by increasing the size exclusion limit of plasmodesmata without structural reorganization (Kawakami et al. 2004). In contrast, the MP of members of families *Secoviridae*, *Bromoviridae*, and *Caulimoviridae* facilitate changes to the structural reorganization of plasmodesmata and form tubule-like structures that enable the movement of virus particles or ribonucleoproteins to adjacent cells (Benitez-Alfonso et al. 2010; Pouwels et al. 2004). In addition to MP, several viruses encode long-distance transport factors (LTF), which are required exclusively for long-distance transport (Cronin et al. 1995; Ding et al. 1995; Prokhnevsky et al. 2002; Ryabov et al. 1999; Tatineni et al. 2011b).

Plant viruses encode one or more coat proteins (CP) with the exception of umbraviruses, which do not encode any CP. The primary role of CP is protection of viral genetic information from nuclease degradation by encapsidating viral genomic RNAs or DNAs (Callaway et al. 2001). Additionally, the CP are involved in the translation of viral RNAs (Bol 2005), viral cell-to-cell and long-distance transport (Alzhanova et al. 2001; Callaway et al. 2001; Scholthof 2005), vector transmission (Callaway et al. 2001), disease induction (Qu and Morris 2002), and activation of resistance gene-mediated host defenses and suppression of host defense mechanisms (Culver 2002; Qu et al. 2003; Soosaar et al. 2005). Thus, CP are multifunctional, with roles in almost every aspect of the virus life cycle.

Though members of family *Potyviridae* do not encode a dedicated MP, several potyviral proteins have been implicated in virus intercellular movement with at least one other function in the virus life cycle. Potyviral helper component-proteinase (HC-Pro) (Kasschau et al. 1997; Rojas et al. 1997), CP (Dolja et al. 1994, 1995; Hofius et al. 2007), genome-linked protein (VPg) (Dunoyer et al. 2004), and cylindrical inclusion protein (Carrington et al. 1998; Roberts et al. 1998; Wei et al. 2010) have been implicated in cell-to-cell movement of potyvirus species. Recently, the P3N-PIPO, a small open reading frame (ORF) found within the P3 cistron (Chung et al. 2008), was implicated in cell-to-cell movement of potyvirus species (Choi et al. 2005; Vijayapalani et al. 2012; Wei et al. 2010; Wen and

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Hajimorad 2010). The core domain of *Tobacco etch potyvirus* (TEV) CP is required for cell-to-cell movement and the N- and C-terminal regions are required for long-distance transport (Dolja et al. 1994, 1995). So far, the roles of viral proteins in virus movement among members of the *Potyviridae* family have been studied only for those of the *Potyvirus* genus.

Wheat streak mosaic virus (WSMV) is an economically important wheat virus in the Great Plains region of the United States with estimated annual yield losses of 2 to 5% with 100% losses in localized infections (Brakke 1987; French and Stenger 2004). WSMV is the type species of the genus *Tritimovirus* within the family *Potyviridae* (Stenger et al. 1998) with a single-stranded positive-sense genomic RNA of 9,384 nucleotides [excluding the 3' poly (A) tail] encapsidating in flexuous filamentous 690- to 700-nm × 10- to 15-nm virions. The genomic organization of WSMV is similar to members of the aphid-transmitted *Potyvirus* and mite-transmitted *Rymovirus* genera of the family *Potyviridae*, with a single large ORF encoding a polyprotein of approximately 350 kDa (Fig. 1). The polyprotein is processed into 10 mature proteins, analogous to potyviruses, by three virus-encoded proteinases, namely, P1, HC-Pro, and NIa-Pro. However, WSMV and potyviruses differ from each other with regard to the functions of P1 and HC-Pro in the virus life cycle. HC-Pro of WSMV is dispensable for systemic infection of wheat in contrast to the multifunctional HC-Pro of the *Potyvirus* genus (Anandalakshmi et al. 1998; Atreya and Pirone 1993; Cronin et al. 1995; Kasschau and Carrington 1998; Stenger et al. 2005a). The P1 of WSMV is the suppressor of RNA silencing in contrast to HC-Pro of members of the *Potyvirus* genus (Kasschau and Carrington 1998; Young et al. 2012). The wheat curl mite (*Aceria tosichella* Keifer) transmits WSMV in a semipersistent manner (Slykhuis 1955), and HC-Pro has been implicated as a viral determinant for mite transmission (Stenger et al. 2005b, 2006).

The role of WSMV proteins in cell-to-cell and long-distance movement is not known, except that synonymous substitutions in the P3 cistron, which either mutated amino acid residues or introduced stop codons in the overlapping PIPO ORF (Chung et al. 2008), severely affected cell-to-cell movement (Choi et

al. 2005). The WSMV CP is 349 amino acid residues long and the N-terminal region is involved in host- and strain-specific LTF in maize (*Zea mays* L.) inbred line SDp2 (Tatineni et al. 2011b). However, no information is available on the role of the C-terminal region of CP in virus biology. The WSMV CP contains seven aspartic acid (D) residues, the smallest negatively charged amino acid, in the C-terminal 69 amino acid residues (Fig. 1). We studied the role of the C-terminal region of WSMV CP in virus movement and host specificity by arbitrarily mutating aspartic acid residues at amino acid positions 216, 289, 290, 326, 333, and 334 and examining the ability of these mutants to infect wheat and SDp2 maize. We found that these amino acids are dispensable for virion assembly, but mutation of aspartic acid residues at positions 289, 290, and 326 debilitated long-distance transport in maize but not in wheat. These amino acids facilitate expansion of WSMV host range through host-specific long-distance movement by allowing virus ingress into the vascular system of maize.

RESULTS

The role of the C-terminal region of WSMV CP in virus biology was examined by mutating negatively charged aspartic acid residues at position 216, 289, 290, 326, 333, or 334 to alanine, and the aspartic acid residue at position 289 was also mutated to asparagine (Fig. 1). Additionally, two dual-substitution mutants were created by substituting aspartic acid residues at positions 289/290 and 333/334 with asparagine/alanine and alanine/alanine, respectively. As a control, a glutamic acid residue, another negatively charged amino acid at position 337, was mutated to alanine (Fig. 1).

Mutation of aspartic acid residues at positions 289, 290, and 326 delayed the onset of systemic symptoms in wheat.

In vitro-generated RNA transcripts of WSMV with mutation of aspartic acid residues in the CP were inoculated to wheat cv. Tomahawk seedlings at the single-leaf stage as described by Tatineni and associates (2011a). Inoculated wheat plants were

Fig. 1. Genomic organization of *Wheat streak mosaic virus* (WSMV) and mutations introduced at the C-terminal region of coat protein (CP). Locations of proteins encoded by the WSMV genome are indicated and the positions of cleavage sites in the genome are indicated with vertical lines inside the large open reading frame (ORF) encoding for a polyprotein. The P3N-PIPO ORF is indicated with a separate box above the P3 cistron. The C-terminal region of the CP is enlarged below the genomic organization with mutated aspartic acid or glutamic acid residues (underlined). The positions and nature of introduced mutations in the C-terminal region of CP are indicated at the right.

incubated in a greenhouse at 20 to 27°C and systemic symptoms were observed at 7, 9, 11, and 15 days postinoculation (dpi).

At 7 dpi, wheat seedlings inoculated with mutants D₂₁₆A, D₃₃₃A, D₃₃₄A and DD_{333/334}AA elicited mild chlorotic streaks and spots and mosaic symptoms in 80 to 94% of plants, similar to WSMV-Sidney (wild-type) (Table 1; Fig. 2A). At 11 to 15 dpi, these mutants infected 94 to 100% of plants causing severe chlorotic streaks, leaf chlorosis, and mosaic symptoms similar to those of the wild-type virus (Fig. 2A). In contrast, at 7 dpi, mutants D₂₈₉N and D₂₉₀A elicited only a few chlorotic streaks and spots in 19 to 28% of plants, and mutants D₂₈₉A, DD_{289/290}NA, and D₃₂₆A did not induce visible symptoms (Table 1; Fig. 2A). The latter mutants induced the first visible symptoms of a few chlorotic streaks per leaf in 11 to 22% of plants at 9 dpi (Table 1). The infection rates of all aspartic acid mutants increased drastically to 87 to 100% by 11 dpi and elicited severe yellowing of lower leaves due to coalescence of chlorotic streaks and mosaic symptoms similar to the wild-type virus (Table 1; Fig. 2A). By 15 dpi, all aspartic acid mutants elicited symptoms approximately similar to those of the wild-type virus (Fig. 2A). The control mutant E₃₃₇A induced symptoms similar to those of the wild-type virus with no differences in the onset and severity (Table 1; Fig. 2A). These results indicate that aspartic acid residues at positions 289, 290, and 326 in WSMV CP are required for efficient systemic infection of wheat. Moreover, nucleotide sequence analysis of the progeny aspartic acid mutants from wheat revealed that all the mutant viruses retained introduced mutations, suggesting that severe symptoms induced by aspartic acid mutants by 15 dpi are not due to reversion of introduced mutations.

Attenuation of symptoms in wheat by aspartic acid mutants is correlated with reduced accumulation of CP and genomic RNA.

The above experiments suggested that WSMV with mutation of aspartic acid residues at positions 289, 290 and 326 in the CP attenuated symptoms during the early stages of infection in wheat (Table 1; Fig. 2A). We next examined accumulation of CP and genomic RNA in aspartic acid mutant-infected

wheat at 9 and 15 dpi. At 9 dpi, 45 kDa CP and two minor proteins of 31 and 29 kDa accumulated in WSMV-Sidney 81 (wild-type virus)-infected samples (Fig. 2B). The latter two minor proteins were attributed to unspecified *in vivo* proteolytic cleavage of CP in wheat leaves due to senescence (Brakke et al. 1990). At 9 dpi, CP accumulated at much reduced levels in mutant D₂₈₉N-, D₂₈₉A-, D₂₉₀A-, DD_{289/290}NA-, or D₃₂₆A-infected wheat, particularly in the latter two mutants, compared with wild-type virus (Fig. 2B, top panel). Conversely, the CP accumulated at levels similar to those of the wild-type virus in mutant D₂₁₆A-, D₃₃₃A-, D₃₃₄A-, DD_{333/334}AA-, or E₃₃₇A-infected wheat (Fig. 2B, top panel). At 15 dpi, CP accumulated in all aspartic acid mutants- and wild-type virus-infected wheat at approximately similar levels (Fig. 2B, bottom panel). CP from mutant-infected plants migrated slightly faster than that from the wild-type virus, due to either size differences between aspartic acid or glutamic acid and alanine residues, or conformational changes due to substituted amino acids, or both. The two truncated CP bands also migrated slightly faster than the corresponding bands of wild-type virus, suggesting that these truncated CP comprised the C-terminus of CP (Fig. 2B).

Accumulation of genomic RNAs in aspartic acid mutant-infected wheat was examined by real-time reverse transcription-polymerase chain reaction (PCR) at 9 and 15 dpi. The genomic RNA in mutant D₂₈₉N-, D₂₈₉A-, D₂₉₀A-, DD_{289/290}NA-, or D₃₂₆A-infected wheat accumulated at only 1 to 14% of the wild-type virus at 9 dpi (Table 2). Particularly, the mutants DD_{289/290}NA and D₃₂₆A accumulated at much reduced levels of 1 and 3%, respectively, of the wild-type virus. In contrast, viral genomic RNA in mutant D₂₁₆A-, D₃₃₃A-, D₃₃₄A-, or DD_{333/334}AA-infected wheat accumulated at moderate levels of 34 to 79%, as compared with wild-type virus (Table 2). At 15 dpi, accumulation of genomic RNAs had generally recovered in all mutant-infected plants. In mutant D₂₈₉N- or D₂₈₉A-infected wheat, genomic RNAs accumulated at 84% and 114%, respectively, of the wild-type virus (Table 2). The genomic RNA in mutant D₂₉₀A-, DD_{289/290}NA-, or D₃₂₆A-infected wheat recovered to a lesser degree, with levels of 46, 31, and 43%

Table 1. Effect of aspartic acid mutations at the C-terminus of the coat protein of *Wheat streak mosaic virus* (WSMV) on systemic infection of wheat cv. Tomahawk^a

Mutant	Experiment	7 dpi ^b	9 dpi ^b	11 dpi ^b	15 dpi ^b
WSMV-Sidney (WT)	1	12/15 (80)	14/15 (93)	15/15 (100)	15/15 (100)
	2	15/16 (94)	16/16 (100)	16/16 (100)	16/16 (100)
D ₂₁₆ A	1	16/18 (89)	17/18 (94)	17/18 (94)	17/18 (94)
	2	14/15 (93)	15/15 (100)	15/15 (100)	15/15 (100)
D ₂₈₉ N	1	4/18 (22)	10/18 (56)	18/18 (100)	18/18 (100)
	2	3/16 (19)	7/16 (44)	16/16 (100)	16/16 (100)
D ₂₈₉ A	1	0/18 (0)	4/18 (22)	16/18 (89)	18/18 (100)
	2	0/15 (0)	2/15 (13)	13/15 (87)	14/15 (100)
D ₂₉₀ A	1	4/16 (25)	12/16 (75)	15/16 (94)	16/16 (100)
	2	5/18 (28)	14/18 (78)	18/18 (100)	18/18 (100)
DD _{289/290} NA	1	0/17 (0)	2/17 (12)	17/17 (100)	17/17 (100)
	2	0/16 (0)	2/16 (13)	16/16 (100)	16/16 (100)
D ₃₂₆ A	1	0/18 (0)	2/18 (11)	16/18 (89)	18/18 (100)
	2	0/15 (0)	2/15 (13)	14/15 (93)	15/15 (100)
D ₃₃₃ A	1	14/16 (88)	14/16 (88)	16/16 (100)	16/16 (100)
	2	17/18 (94)	18/18 (100)	18/18 (100)	18/18 (100)
D ₃₃₄ A	1	12/15 (80)	15/15 (100)	15/15 (100)	15/15 (100)
	2	14/16 (88)	16/16 (100)	16/16 (100)	16/16 (100)
DD _{333/334} AA	1	14/17 (82)	16/17 (94)	17/17 (100)	17/17 (100)
	2	15/18 (83)	18/18 (100)	18/18 (100)	18/18 (100)
E ₃₃₇ A	1	13/16 (81)	16/16 (100)	16/16 (100)	16/16 (100)
	2	17/18 (94)	18/18 (100)	18/18 (100)	18/18 (100)

^a In vitro transcripts prepared from 1 µg of linearized cDNA in a 40-µl reaction were diluted with an equal volume of 2% sodium pyrophosphate in the presence of 2% Celite and were directly inoculated onto wheat seedlings at the single leaf stage. Inoculated wheat plants were incubated in a greenhouse at 20 to 27°C. Wheat seedlings inoculated with buffer did not develop any symptoms.

^b Number of plants infected to the number of plants inoculated at the indicated days postinoculation (dpi); % of infection is shown in parentheses.

compared with 6, 1, and 3% of the wild-type virus at 9 dpi, respectively. In contrast, genomic RNAs of mutants D₂₁₆A, D₃₃₃A, D₃₃₄A, and DD_{333/334}AA accumulated at 73 to 134% of wild-type virus (Table 2). These data suggested that mutation of aspartic acid residues at position 289, 290, or 326 in the CP severely affected the accumulation of virus at 9 dpi, followed by a substantial recovery by 15 dpi.

The delayed systemic infection in wheat by aspartic acid mutants is due to defective cell-to-cell movement.

Recently, we developed green fluorescent protein (GFP)-tagged WSMV variants with different cleavage sites at the 3' end of the GFP cistron, which served as excellent tools to monitor virus spread and distribution in wheat and maize (Tatineni

Fig. 2. A, Effect of mutation of aspartic acid residues at the C-terminus of coat protein (CP) of *Wheat streak mosaic virus* (WSMV) on symptom induction in wheat cv. Tomahawk. In vitro transcripts of cDNA clones with aspartic acid mutations in the CP were inoculated to wheat seedlings at the single-leaf stage, and symptoms were photographed at 7 and 15 days postinoculation (dpi). **B**, Western blot analysis of total proteins from wheat leaves infected with in vitro transcripts as shown in A at 9 and 15 dpi. Full-length (45 kDa) and truncated 31- and 29-kDa CP that reacted positively with WSMV antibodies are indicated with arrows, arrow heads, and asterisks, respectively. The large subunit of Rubisco protein of wheat in Coomassie Brilliant blue-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used as a control for the amount of protein loaded per well. The protein size markers used in SDS-PAGE are indicated on the right. Note that WSMV with aspartic acid mutations at amino acid position 289, 290, or 326 substantially affected symptom induction and accumulation of CP in wheat at 7 and 9 dpi, respectively.

et al. 2011a and b, 2013). The effect of aspartic acid mutations at the C-terminus of the CP on cell-to-cell and long-distance movement of WSMV was examined by transferring selective mutations into GFP-tagged WSMV, WSMV-S81-GFP-6K1/CI (7aa) (Tatineni et al. 2011a).

In vitro transcripts of GFP-tagged aspartic acid mutants were inoculated to wheat seedlings at the single-leaf stage and the GFP fluorescence was examined in upper fully expanded non-inoculated leaves for systemic virus spread under a Zeiss Stereo Discovery V12 fluorescence microscope at 7, 10, 15, and 22 dpi (Fig. 3). At 7 dpi, a large number of GFP foci along the veins covering a majority of the leaf lamina was observed in mutant D₂₁₆A-, DD_{333/334}AA-, and E₃₃₇A-infected leaves, similar to wild-type virus. In contrast, a moderate number of foci (15 to

30) per leaf in 20 to 30% of D₂₈₉N-infected leaves and only 1 to 3 foci per leaf in 5 to 10% of D₂₉₀A-, DD_{289/290}NA-, or D₃₂₆A-infected leaves were observed (Fig. 3, 7 dpi panel). At 10 dpi, GFP fluorescence covered most of the leaf lamina in mutant D₂₁₆A-, DD_{333/334}AA-, or E₃₃₇A-infected wheat, similar to wild-type virus, suggesting efficient systemic infection by these mutant viruses. However, in mutant D₂₈₉N-, D₂₉₀A-, DD_{289/290}NA-, or D₃₂₆A-infected plants, 20 to 70 foci per leaf were observed in 60 to 80% of plants (Fig. 3, 10 dpi panel). At 15 dpi, efficient distribution of GFP fluorescence was observed along the veins of a majority of leaf lamina of mutant D₂₈₉N-, D₂₉₀A-, DD_{289/290}NA-, or D₃₂₆A-infected plants (Fig. 3, 15 dpi panel). Distribution of GFP fluorescence in all mutant-infected plants was approximately similar to wild-type virus by 22 dpi

Table 2. Quantification of genomic RNAs of wild-type *Wheat streak mosaic virus* (WSMV) and aspartic acid mutants at the C-terminus of coat protein from systemically infected wheat leaves

Mutation	9 days postinoculation (dpi)		15 dpi	
	Genomic RNA copies ^a ± SE	% accumulation ^b	Genomic RNA copies ± SE	% accumulation ^b
WSMV-Sidney 81	$7.49 \times 10^5 \pm 2.44 \times 10^4$	100	$4.00 \times 10^6 \pm 2.79 \times 10^5$	100
D ₂₁₆ A	$5.15 \times 10^5 \pm 1.26 \times 10^4$	69	$4.68 \times 10^6 \pm 1.77 \times 10^5$	117
D ₂₈₉ N	$1.02 \times 10^5 \pm 3.67 \times 10^3$	14	$3.35 \times 10^6 \pm 9.29 \times 10^4$	84
D ₂₈₉ A	$5.77 \times 10^4 \pm 1.09 \times 10^4$	8	$4.54 \times 10^6 \pm 2.68 \times 10^5$	114
D ₂₉₀ A	$4.16 \times 10^4 \pm 5.88 \times 10^2$	6	$1.84 \times 10^6 \pm 8.32 \times 10^4$	46
DD _{289/290} NA	$7.58 \times 10^3 \pm 1.08 \times 10^3$	1	$1.22 \times 10^6 \pm 7.14 \times 10^4$	31
D ₃₂₆ A	$2.23 \times 10^4 \pm 1.05 \times 10^3$	3	$1.70 \times 10^6 \pm 1.22 \times 10^5$	43
D ₃₃₃ A	$2.58 \times 10^5 \pm 3.81 \times 10^4$	34	$2.92 \times 10^6 \pm 5.38 \times 10^4$	73
D ₃₃₄ A	$5.89 \times 10^5 \pm 3.66 \times 10^4$	79	$4.67 \times 10^6 \pm 6.29 \times 10^5$	117
DD _{333/334} AA	$2.74 \times 10^5 \pm 1.85 \times 10^4$	37	$5.36 \times 10^6 \pm 1.07 \times 10^6$	134
Healthy	ND	—	ND	—

^a The number of genomic RNA copies was detected in triplicate samples for each mutant by a real-time polymerase chain reaction method as described by Tatineni and associates (2010). Average number of genomic RNA copies per 2.5 ng of total RNA ± standard error (SE).

^b Percent accumulation over the wild-type virus.

Fig. 3. *Wheat streak mosaic virus* with aspartic acid mutations in the coat protein (CP) at amino acid positions 289, 290, and 326 delayed the onset of systemic infection in wheat cv. Tomahawk. In vitro transcripts of green fluorescent protein (GFP)-tagged aspartic acid mutants were inoculated to wheat seedlings at the single-leaf stage, and upper fully expanded leaves were observed under a Zeiss Stereo Discovery V12 fluorescence microscope at 7, 10, 15, and 22 days postinoculation (dpi). Bars represent 500 μm. Note that a few systemic foci were observed at 7 and 10 dpi in upper fully expanded leaves of wheat infected with GFP-tagged aspartic acid mutants at amino acid position 289, 290, or 326.

(Fig. 3, 22 dpi panel). These results suggest that the delayed onset of systemic symptoms by mutants D₂₈₉N, D₂₉₀A, DD_{289/290}NA, and D₃₂₆A in wheat could be due to defects in one or both cell-to-cell and long-distance transport.

Cell-to-cell movement of aspartic acid mutants was examined by inoculating wheat seedlings at the single-leaf stage with in vitro transcripts of GFP-tagged mutant viruses and observing inoculated leaves, at 4 to 5 dpi, under a Zeiss Stereo Discovery V12 fluorescence microscope for foci sizes. Mutants D₂₁₆A and E₃₃₇A formed foci similar in size to those of the wild-type virus on inoculated wheat leaves (2.93 to 2.99 mm² in experiment 1 and 1.91 to 2.15 mm² in experiment 2) (Table 3), suggesting that these amino acids do not affect CP function when exchanged with alanine residues and thus are compatible for cell-to-cell movement. Mutant DD_{333/334}AA formed local foci of 2.06 mm² in experiment 1 and 1.88 mm² in experiment 2 that are slightly smaller than the wild-type virus foci of 2.98 and 2.08 mm², respectively (Table 3). In contrast, mutants D₂₈₉N, D₂₉₀A, DD_{289/290}NA, and D₃₂₆A formed substantially smaller foci of 1.09 to 1.66 mm² (experiment 1) and 0.75 to

1.27 mm² (experiment 2) compared with 2.98 mm and 2.08 mm², respectively, of the wild-type virus (Table 3). These results suggested that aspartic acid residues at amino acid positions 289, 290, and 326 are required for efficient cell-to-cell movement; thus, defects in cell-to-cell transport might have contributed to delayed onset of systemic symptoms in wheat.

The C-terminus aspartic acid residues of CP are required for systemic infection of maize.

Previously, we reported the N-terminal region of WSMV CP as a host- and strain-specific long-distance transport factor for systemic infection of maize inbred line SDp2 (Tatineni et al. 2011b). Here, we examined whether the C-terminal aspartic acid residues of CP are required for systemic infection of SDp2 maize. In vitro transcripts of wild-type cloned virus infected only 10% of maize seedlings at 30 dpi compared with 100% infection with a 1:20 diluted crude sap of infected wheat at 8 to 10 dpi, suggesting that virions in crude sap are highly infectious to maize. Hence, SDp2 maize seedlings were mechanically inoculated at the two- to three-leaf stage with crude sap at a 1:10 dilution from 18 dpi wheat leaves infected with in vitro transcripts, and symptom development was observed from 12 dpi onwards.

Mutants D₂₁₆A, D₃₃₃A, D₃₃₄A, and DD_{333/334}AA systemically infected 100% of maize seedlings by 12 dpi. However, mutants D₂₈₉N, D₂₈₉A, D₂₉₀A, DD_{289/290}NA, and D₃₂₆A failed to infect maize at 12 dpi (Fig. 4; Table 4). At 18 dpi, maize seedlings inoculated with D₂₈₉N, D₂₈₉A, and D₃₂₆A elicited a few chlorotic streaks and spots per leaf in 20 to 56% of inoculated plants (Table 4). These chlorotic streaks and spots were restricted to smaller areas, sometimes to one half of the leaf (Fig. 4). Such mild symptoms continued even after 30 to 35 dpi, suggesting that aspartic acid residues located at amino acid positions 289 and 326 are required for efficient systemic infection of maize. In contrast, mutants D₂₉₀A and DD_{289/290}NA failed to infect maize systemically even at 35 to 45 dpi, suggesting that the aspartic acid residue at position 290 is critical for systemic infection of maize. The wild-type virus and E₃₃₇A infected 100% of inoculated maize seedlings at 12 dpi (Table 4; Fig. 4).

Table 3. Infection foci sizes of green fluorescent protein (GFP)-tagged wild-type *Wheat streak mosaic virus* (WSMV) and coat protein aspartic acid mutants in inoculated leaves of wheat^a

Mutant	Foci sizes ^b	
	Experiment 1	Experiment 2
WSMV-GFP-6KI/CI(7aa) (wild-type)	2.98 ± 0.36	2.08 ± 0.80
D ₂₁₆ A	2.99 ± 0.74	1.91 ± 0.45
D ₂₈₉ N	1.66 ± 0.48	1.27 ± 0.44
D ₂₉₀ A	1.44 ± 0.42	1.18 ± 0.47
DD _{289/290} NA	1.09 ± 0.34	0.75 ± 0.26
D ₃₂₆ A	1.26 ± 0.38	1.05 ± 0.35
DD _{333/334} AA	2.06 ± 0.38	1.88 ± 0.59
E ₃₃₇ A	2.93 ± 0.39	2.15 ± 0.66

^a The sizes of infection foci were estimated at 5 days postinoculation (dpi) for experiment 1 and at 4 dpi for experiment 2 under a Zeiss Stereo Discovery V12 fluorescence microscope.

^b In square millimeters ± standard deviation. Average foci sizes were estimated from 20 to 30 individual foci.

Fig. 4. Aspartic acid residues at positions 289, 290, and 326 in the coat protein of *Wheat streak mosaic virus* are required for systemic infection of maize (*Zea mays* L.) inbred line SDp2. Maize seedlings at the two to three-leaf stage were mechanically inoculated with 1:10 diluted crude sap of systemically infected wheat leaves at 18 days postinoculation (dpi). Inoculated plants were incubated in a growth chamber at 24°C maximum and 20°C minimum temperature with a 14-h photoperiod. Note that mutants D₂₉₀A and DD_{289/290}NA failed to infect maize, while mutants D₂₈₉N, D₂₈₉A and D₃₂₆A induced mild chlorotic spots and streaks, and E₃₃₇A and wild-type (WT) virus efficiently infected at 12 dpi.

Aspartic acid residues at positions 289, 290, and 326 are required for virus ingress into maize vascular system.

The inability of mutants D₂₈₉N, D₂₈₉A, D₂₉₀A, DD_{289/290}NA, and D₃₂₆A to systemically infect SDp2 maize at 12 dpi prompted us to monitor the movement of GFP-tagged mutant viruses in inoculated and upper noninoculated leaves of maize. Crude sap diluted 1:10 (wt/vol) from 18-dpi infected wheat leaves was used to mechanically inoculate two sets of SDp2 seedlings. One set of inoculated plants was used to examine the size of local foci at 7 dpi, and the other set of plants was used to follow the development of systemic infection.

In two independent experiments, mutants D₂₁₆A, DD_{333/334}AA, and E₃₃₇A formed local foci and systemically infected maize seedlings similar to wild-type virus (Table 5; Fig. 5A). However, mutants D₂₈₉N, D₂₉₀A, DD_{289/290}NA, and D₃₂₆A formed smaller foci of 0.44 to 0.99 mm², compared with foci of 1.15 to 1.80 mm² formed by the wild-type virus (Table 5), and failed to form systemic foci in upper noninoculated leaves at 15 dpi (Fig. 5A). The smallest local foci were observed with mutant D₃₂₆A with 0.44 to 0.49 mm², followed by DD_{289/290}NA, D₂₉₀A, and D₂₈₉N with 0.51 to 0.53, 0.68 to 0.76, and 0.81 to 0.99 mm², respectively (Table 5). Mutants D₂₈₉N and D₃₂₆A infected 10 to 20% of SDp2 seedlings with a few systemic foci per leaf at 21 to 30 dpi (Fig. 5A). However, mutants D₂₉₀A and DD_{289/290}NA failed to elicit detectable systemic foci in upper noninoculated leaves, even at 40 dpi, further confirming that the aspartic acid residue at position 290 in the CP is critical for loading or unloading the virus into or out of the vascular system of maize or for both of these functions.

The role of aspartic acid residues at positions 289, 290, and 326 in virus entry into the vascular system of maize was examined by inoculating the upper half of the third leaf of 10 to 15 SDp2 maize seedlings with 1:10-diluted crude sap of the respective GFP-tagged mutant virus-infected wheat leaves. The upper halves of inoculated leaves were observed for the development of local infection foci and the lower noninoculated halves were observed for the presence of virus in the veins at 30 dpi. The sizes of local infection foci in D₂₈₉N, D₂₉₀A, DD_{289/290}NA, and D₃₂₆A inoculated areas of maize leaves increased with time and coalesced at 30 dpi (Fig. 5B), suggesting that these mutants were competent in cell-to-cell movement, although at reduced levels compared with wild-type virus. GFP fluorescence was found in the veins of lower noninoculated areas of the wild-type virus- and mutant DD_{333/334}AA-inoculated leaves but not in D₂₉₀A- or DD_{289/290}NA-inoculated maize leaves at 30 dpi (Fig. 5B). GFP fluorescence was not observed in the veins, even though infection foci developed next to the veins in mutant D₂₉₀A- or DD_{289/290}NA-inoculated leaves of maize. In contrast, weak GFP fluorescence was observed in

the veins next to a few infection foci in 10% of mutant D₂₈₉A- or D₃₂₆A-inoculated leaves at 30 dpi (Fig. 5B), which is consistent with weak and delayed systemic infection by these mutants in maize. These data demonstrated that aspartic acid residues at positions 289, 290, and 326 are required for virus ingress into the vascular system of maize in order to extend the host range of WSMV.

WSMV CP aspartic acid mutants are competent to form virions.

Point mutations in viral CP may affect virion formation as reported for several plant viruses (Dawson et al. 1988; Dolja et al. 1994, 1995; Park et al. 2012; Rao and Grantham 1996). The ability to form virions by aspartic acid mutants was examined by partially purifying virions from systemically infected wheat leaves at 18 dpi, as described by Tatineni and associates (2011b). A large number of flexuous filamentous virus particles were observed in partially purified virus preparations of all aspartic acid mutants, and no obvious morphological differences were observed between the mutants and wild-type virions (Fig. 6A). Western blot analysis of partially purified virions from systemically infected wheat leaves further revealed that aspartic acid mutants accumulated virions at approximately the same amounts as those of the wild-type virus at 18 dpi (Fig. 6B). These data suggested that mutation of aspartic acid residues at position 289, 290, 326, 333, or 334 in the CP did not significantly affect WSMV virion formation. However, we cannot

Table 5. Infection foci sizes of green fluorescent protein-tagged *Wheat streak mosaic virus* coat protein aspartic acid mutants in inoculated leaves of maize inbred line SDp2^a

Mutant	Foci sizes ^b	
	Experiment 1	Experiment 2
WSMV-GFP-6KI/CI(7aa) ^c	1.80 ± 0.43	1.15 ± 0.39
D ₂₁₆ A	1.72 ± 0.34	1.32 ± 0.44
D ₂₈₉ N	0.99 ± 0.23	0.81 ± 0.24
D ₂₉₀ A	0.68 ± 0.16	0.76 ± 0.25
DD _{289/290} NA	0.51 ± 0.17	0.53 ± 0.29
D ₃₂₆ A	0.49 ± 0.14	0.44 ± 0.13
DD _{333/334} AA	1.98 ± 0.51	1.04 ± 0.24
E ₃₃₇ A	1.81 ± 0.47	1.09 ± 0.35

^a Maize inbred line SDp2 seedlings were inoculated at the two-leaf stage with a 1:10 dilution of crude extracts prepared from 18-days postinoculation (dpi) infected wheat leaves. Inoculated SDp2 seedlings were incubated in a growth chamber at 24°C maximum and 20°C minimum temperatures with 14 h of light. Average sizes of foci estimated from 20 to 25 individual foci.

^b In square millimeters ± standard deviation at 7 dpi.

^c Wild-type virus.

Table 4. Effect of *Wheat streak mosaic virus* (WSMV) coat protein aspartic acid mutants on systemic infection of maize inbred line SDp2^a

Mutant	Experiment 1 ^b		Experiment 2 ^b	
	12 dpi	18 dpi	12 dpi	18 dpi
WSMV-Sidney (wild-type virus)	6/6	6/6	7/7	7/7
D ₂₁₆ A	7/7	7/7	8/8	8/8
D ₂₈₉ N	0/8	3/8	0/5	1/5
D ₂₈₉ A	0/8	3/8	1/4	2/4
D ₂₉₀ A	0/7	0/7	0/8	0/8
DD _{289/290} NA	0/8	0/8	0/7	0/7
D ₃₂₆ A	0/9	5/9	0/7	2/7
D ₃₃₃ A	10/10	10/10	6/6	6/6
D ₃₃₄ A	7/7	7/7	8/8	8/8
DD _{333/334} AA	7/7	7/7	7/7	7/7
E ₃₃₇ A	7/7	7/7	6/6	6/6

^a Inoculum was prepared from 18 days postinoculation (dpi) infected wheat leaves in 20 mM sodium phosphate buffer, pH 7.0, at a 1:10 dilution. Inoculated plants were incubated in a growth chamber at 24°C maximum and 20°C minimum temperatures with 14 h of light.

^b Number of plants infected to the number of plants inoculated.

exclude the possibility of mutation of aspartic acid residues might have affected the efficiency of virion formation. Virion assembly assays in wheat can provide information on virion encapsidation if mutant viruses infect similarly to wild-type virus but not from mutants that are inefficiently infected due to defects in cell-to-cell movement but not in virion assembly.

Because aspartic acid mutants differentially affected cell-to-cell movement in wheat, it is not possible to determine whether mutation of aspartic acid residues in the CP might have affected the efficiency of virion assembly in wheat. It is also possible that reduced virion assembly of aspartic acid mutants might have affected cell-to-cell movement in wheat. Previously, we have shown that WSMV replicates weakly and forms virions without cell-to-cell or long-distance movement in agroinfiltrated *Nicotiana benthamiana* leaves (Tatineni et al. 2011a, 2013). We took advantage of this movement-independent virion assembly assay to examine virion assembly efficiency of selective aspartic acid mutants.

Mutants D₂₈₉N, D₂₉₀A, DD_{289/290}NA, D₃₂₆A, and DD_{333/334}AA were transferred to pCAM-WSMV-S81-GFP-6K1/CI(7aa)

(Tatineni et al. 2011a), followed by transformation into *Agrobacterium tumefaciens* EHA105. As a control, arginine at amino acid position 237 in WSMV CP, a conserved amino acid in all flexuous filamentous plant viruses required for virion formation (Dolja et al. 1991, 1994), was mutated to alanine. *Agrobacterium* suspensions (1.0 optical density at 600 nm [OD₆₀₀]) harboring WSMV-S81-GFP-6K1/CI(7aa) or arginine or aspartic acid mutants were mixed with equal volumes of 1.0 OD₆₀₀ agrosuspension harboring a binary vector containing *Tomato bushy stunt virus* p19, a suppressor of RNA silencing (Qu and Morris 2002) and infiltrated into *N. benthamiana* leaves as described by Tatineni and associates (2012). Partially purified virions were prepared from 7 g of 9 days–postagroinfiltrated *N. benthamiana* leaves as described by Tatineni and associates (2011b), followed by Western blot using WSMV CP polyclonal antibodies (Fig. 6C). Mutants D₂₈₉N, D₂₉₀A, DD_{289/290}NA, D₃₂₆A, and DD_{333/334}AA encapsidated virions at approximately similar or higher levels than that of the wild-type virus, and the assembly-null mutant R₂₃₇A failed to form detectable virions (Fig. 6C). These results demonstrated that

Fig. 5. A, Green fluorescent protein (GFP)-tagged *Wheat streak mosaic virus* (WSMV) with mutation of aspartic acid residues at position 289, 290, or 326 in the coat protein (CP) displayed profound defects in systemic transport in SDp2 maize. Fully expanded upper noninoculated leaves were observed under a fluorescence microscope for the presence of GFP fluorescence at 15, 21, and 30 days postinoculation (dpi). Note that mutant D₂₈₉N failed to induce systemic foci at 15 dpi and produced very few foci per leaf at 21 and 30 dpi; similar infection foci were induced by mutant D₃₂₆A (not shown in picture). Mutants D₂₉₀A and DD_{289/290}NA failed to infect SDp2 maize systemically, even at 30 dpi. Wild-type virus and mutant DD_{333/334}AA efficiently infected maize at 15 dpi. **B**, Aspartic acid residues at positions 289, 290, and 326 in the CP are crucial for WSMV to enter the vascular system for systemic infection in maize. The upper half of the third leaf of maize was mechanically inoculated with crude sap of GFP-tagged aspartic acid mutant–infected wheat leaves. The upper inoculated and lower noninoculated areas of the third leaf were observed under a fluorescence microscope at 30 dpi. Note that mutants D₂₉₀A and DD_{289/290}NA failed to enter the lower veins of inoculated leaves. Mutant D₂₈₉N inefficiently entered the vascular system, as weak fluorescence was observed in lower veins of a few noninoculated areas of maize; similar results were observed with mutant D₃₂₆A (not shown in picture). In contrast, the wild-type virus and mutant DD_{333/334}AA efficiently entered the vascular system, as strong fluorescence was observed in lower veins of noninoculated areas. Bars represent 500 μ m.

aspartic acid residues at positions 289, 290, 326, 333, and 334 in CP are dispensable for WSMV virion assembly.

DISCUSSION

The role of the C-terminal region of CP in virion formation, disease induction, host specificity, and cell-to-cell and long-distance movement of WSMV was examined by arbitrarily mutating negatively charged aspartic acid residues. The aspartic acid mutants induced two symptom phenotypes in wheat: i) mutants $D_{216}A$, $D_{333}A$, $D_{334}A$, and $DD_{333/334}AA$ do not have a

significant effect on symptom phenotype, and ii) mutants $D_{289}N$, $D_{289}A$, $D_{290}A$, $DD_{289/290}NA$, and $D_{326}A$ induced delayed or mild systemic symptoms during the early stages of infection, followed by severe symptoms similar to those of the wild-type virus. Mutation of aspartic acid at position 289 to asparagine or alanine induced different symptom phenotypes on wheat (Table 1), suggesting that substituted amino acids interacted differently with either host proteins, or other viral proteins, or both. Our data suggest that aspartic acid residues at positions 289, 290, and 326 are required for early events of the infection process in wheat, such as efficient encapsidation

Fig. 6. Aspartic acid residues at the C-terminus of coat protein (CP) are dispensable for virion assembly of *Wheat streak mosaic virus* (WSMV). **A**, Electron micrographs of partially purified virions of WSMV aspartic acid mutants. Virions were partially purified from systemically infected wheat leaves at 18 days post-inoculation. Bars represent 500 nm. **B**, Western blot analysis of partially purified virions from wheat leaves infected with aspartic acid mutants as indicated. **C**, Western blot analysis of partially purified virions from agroinfiltrated *Nicotiana benthamiana* leaves at 7 days postagroinfiltration. Note that all WSMV CP aspartic acid mutants accumulated encapsidated CP equally or more than the wild-type virus in a movement-independent virion assembly assay in agroinfiltrated *N. benthamiana* leaves. The full-length 45-kDa and truncated 31- and 29-kDa CP bands are indicated with arrows, arrow heads, and asterisks, respectively. The protein size markers used in sodium dodecyl sulfate-polyacrylamide gel electrophoresis in B and C are indicated on the left and right, respectively.

and cell-to-cell movement. Moreover, the WSMV CP is not required for replication (Tatineni et al. 2013), and it has not been demonstrated to have host RNA silencing suppressor activity (Young et al. 2012). Hence, it is unlikely that mild symptom phenotype of aspartic acid mutants is due to defects in virus replication or suppression of host defense mechanisms.

The aspartic acid residues at positions 289, 290, and 326 in the CP are required for efficient establishment of systemic infection of WSMV in wheat. It is not clear why mutation of these amino acid residues resulted in a drastic shift in symptom induction in wheat. Perhaps the C-terminus of CP is a dedicated cell-to-cell movement determinant of WSMV and mutation of aspartic acid residues may have affected the efficiency of cell-to-cell movement, thus causing a delay in the onset of systemic infection. The increased symptom severity phenotype of aspartic acid mutants at 15 dpi is also corroborated with a substantial increase in the accumulation of CP and genomic RNAs as compared with 7 dpi. At 15 dpi, mutants D₂₉₀A, DD_{289/290}NA, and D₃₂₆A elicited severe symptoms similar to wild-type virus with only 31 to 46% of genomic RNA accumulation of the wild-type virus. These data suggest that there is no linear relation between virus concentration and severity of symptoms, as has been observed in other virus-host combinations (Kassanis and Bastow 1971; Técsi et al. 1994).

The C-terminal region of TEV CP is required for long-distance transport with little effect on cell-to-cell movement (Dolja et al. 1995). In contrast, aspartic acid residues at positions 289, 290, and 326 at the C-terminal region of WSMV CP are required for efficient cell-to-cell movement with little or no effect on long-distance transport in wheat. Efficient systemic infection, though delayed, by aspartic acid mutants, suggests that these amino acids have little or no role in long-distance movement of WSMV in wheat. The delayed onset of systemic infection by aspartic acid mutants might be due to longer periods required for the virus to come across the vascular system due to defective cell-to-cell movement. Cell-to-cell movement of TEV is likely facilitated in the form of virions (Dolja et al. 1994, 1995), and virion formation is a minimal requirement for cell-to-cell movement of WSMV (Tatineni et al. 2013). It is possible that inefficient cell-to-cell movement of aspartic acid mutants in wheat could be due to their defects in virion formation. However, two lines of evidence, virions in systemically infected wheat and movement-independent virion formation in *N. benthamiana*, suggest that aspartic acid mutants are not defective in virion formation. Our data suggest that aspartic acid residues at positions 289, 290, and 326 have a role in cell-to-cell movement of WSMV in wheat independent of virion formation.

The aspartic acid mutants elicited two symptom phenotypes in maize, with residues at positions 216, 333, and 334 being dispensable for systemic infection and residues at positions 289, 290, and 326 being required for systemic infection. Mutants D₂₉₀A and DD_{289/290}NA failed to infect maize, while D₂₈₉A, D₂₈₉N, and D₃₂₆A infected poorly, with mild and delayed symptoms, suggesting that aspartic acid residues at positions 289, 290, and 326 have an important role in interactions with maize proteins. It is possible that failure of aspartic acid mutants to infect maize is due to their defects in one or both cell-to-cell or long-distance movement. Aspartic acid mutants that failed to infect or poorly infected maize formed smaller sized local foci as compared with wild-type virus. Mutants D₂₉₀A and DD_{289/290}NA formed larger local foci as compared with mutant D₃₂₆A but failed to infect maize systemically, suggesting that failure to infect maize by aspartic acid mutants is not exclusively due to defects in cell-to-cell movement.

GFP-tagged D₂₈₉N and D₃₂₆A mutants produced a few small fluorescent foci along the veins in upper noninoculated leaves of maize at 21 dpi, suggesting that these mutants are defective

in either entering into or exiting from the vascular system. Failure to ingress by mutants D₂₉₀A and DD_{289/290}NA and inefficient ingress by mutants D₂₈₉N and D₃₂₆A into the vascular system of maize was correlated with failure to infect and inefficient infection of maize, respectively. These data revealed that mutants D₂₉₀A and DD_{289/290}NA are defective in loading the virus into the vascular system, thus failing to infect maize systemically. Though it is not known for WSMV CP, the N- and C-terminal amino acids in potyviral CP are exposed on the virion surface (Allison et al. 1985; Shukla et al. 1988). It is possible that the N- and C-terminal amino acids are potential targets for interactions with host factors, and the aspartic acid residues at positions 289, 290, and 326 might be such targets. Our data also suggest that aspartic acid residues at positions 289, 290, and 326 interacted differently with maize and wheat proteins, thus causing differential infection in these two hosts. The requirement of the N-terminal region for differential infection of maize by WSMV isolates (Tatineni et al. 2011b) and the C-terminal region for virus entry into the vascular system of maize (present study) suggest that the N- and C-terminal regions of CP may be involved in interactions with the host proteins for a successful invasion of hosts by WSMV. Viral determinants for host-specific infections of members of the *Potyvirus* genus have been studied widely, and P1, P3, 6K2, NIa-VPg, NIa-Pro, and CP have been reported to be involved in differential infection of certain hosts (Carbonell et al. 2013; Chen et al. 2008; Decroocq et al. 2009; Rajamäki and Valkonen 1999; Salvador et al. 2008; Schaad et al. 1997). The CP of tobamovirus also functions in host-specific determinant of long-distance transport and the production of *Tobacco mosaic virus* CP was implicated in symptom development (Dawson et al. 1988; Hilf and Dawson 1993).

In contrast to aspartic acid residues at positions 289, 290, and 326, mutation of aspartic acid residues at positions 216, 333, and 334 has no measurable effect on cell-to-cell and long-distance transport, symptom induction, and ability to infect maize. However, these mutants accumulated genomic RNAs at slightly lower levels than the wild-type virus, especially during the early stages of infection. These data suggest that amino acids at positions 216, 333, and 334 provide some important functions enhancing viral fitness, particularly during the early stages of infection.

Cell-to-cell and long-distance movement of eudicot-infecting viruses, including members of the family *Potyviridae* has been studied widely (Benitez-Alfonso et al. 2010; Carrington et al. 1996; Harries et al. 2010; Lucas 2006), and several viral proteins have been implicated in long-distance movement of viruses (Ding et al. 1995; Prokhnovsky et al. 2002; Ryabov et al. 1999; Schaad et al. 1997). In contrast, long-distance movement of monocot-infecting plant viruses is poorly understood. Here, we provide evidence that aspartic acid residues at positions 289, 290, and 326 at the C-terminus of CP extended WSMV host range through differential long-distance transport. These amino acids play a critical role in long-distance transport of WSMV in maize with little or no effect in wheat. Our results provide evidence that the C-terminus of WSMV CP is involved in extension of virus host range by specifically allowing the virus into the vascular system of maize. Further studies on the requirement of host proteins and their interactions with CP would help decipher the roles of host proteins in WSMV host range and long-distance transport.

MATERIALS AND METHODS

Generation of mutants.

Infectious cDNA clones of WSMV-Sidney 81, pSP6-WSMV-S81 (Choi et al. 1999), pSP6-WSMV-S81-GFP-6K1/CI(7aa),

and pCAM-WSMV-S81-GFP-6K1/CI(7aa) (Tatineni et al. 2011a) were used as base clones to introduce mutations at the C-terminal region of CP. Mutations were introduced into oligonucleotides, and overlap extension PCR was performed (Ho et al. 1989) with oligonucleotides W-3 and W-89 (Tatineni et al. 2011b). The PCR products were ligated into pSP6-WSMV-S81 or pSP6-WSMV-S81-GFP-6K1/CI(7aa) between *Bst*EII (nt 6,319) and *Nor*I (at the 3' end) restriction endonuclease sites as described previously (Tatineni et al. 2011b).

A *Pst*I-*Nor*I restriction fragment (nt 4816-3' end) from pSP6-WSMV-S81 or pSP6-WSMV-S81-GFP-6K1/CI(7aa) constructs harboring aspartic acid mutations in the CP was ligated into similarly digested pCAM-WSMV-S81-GFP-6K1/CI(7aa) (Tatineni et al. 2011a). PCR, overlap extension PCR, and ligations and transformations were performed using standard molecular biology methods as described by Sambrook and Russell (2001). *Escherichia coli* JM109 was used to transform cDNA clones of WSMV, and plasmid DNA was prepared from an overnight-grown 40-ml culture using the Bio-Rad plasmid midiprep kit (Bio-Rad, Hercules, CA, U.S.A.).

The presence of introduced mutations in cDNA clones was confirmed by nucleotide sequencing. Three independent clones per mutant were used in phenotypic studies in two to three experiments and the results of one independent clone per mutant were presented.

Inoculation of wheat seedlings with in vitro transcripts.

In vitro transcripts were prepared from 1.0 µg of *Nor*I- or *Spe*I-linearized plasmid DNA in 40-µl reactions as described by Tatineni and associates (2011a). An equal volume of 2% sodium pyrophosphate, pH 9.0, containing 1.0% baked Celite was added to freshly prepared in vitro transcripts and was inoculated to wheat seedlings at the single-leaf stage using a gloved finger. Wheat seedlings were washed 5 min after inoculation with distilled water and were incubated in a greenhouse at 20 to 27°C or in a growth chamber at 24°C maximum and 20°C minimum temperature with a 14-h photoperiod for symptom development. Total RNA and proteins were extracted from 9- and 15-dpi wheat leaves as described by Tatineni and associates (2010, 2011a).

Inoculation of SDp2 maize.

Wheat leaves at 18 dpi were used to prepare crude sap at a 1:10 dilution in 20 mM sodium phosphate buffer, pH 7.0, and the sap was used to inoculate maize inbred line SDp2 at the two- to three-leaf stage. Inoculated SDp2 maize seedlings were incubated in a growth chamber at 24°C maximum and 20°C minimum temperature with a 14-h photoperiod. Symptom development on maize was observed from 12 dpi onwards, and symptoms were photographed at 12 and 18 dpi.

Examining virus movement.

Wheat and SDp2 maize seedlings were inoculated with in vitro transcripts of GFP-tagged seedlings and crude sap at a 1:10 (wt/vol) dilution from 18-dpi wheat leaves infected with GFP-tagged viruses, respectively. Cell-to-cell movement was observed by examining inoculated leaves for the development of infection foci under a Zeiss Stereo Discovery V12 fluorescence microscope (Carl Zeiss MicroImaging, Inc., New York), using a GFP narrow band filter. The GFP fluorescence pictures were taken using an AxioCam MRc5 camera attached to a V12 fluorescence microscope. The foci sizes were estimated by measuring the total area of each focus by a program provided with the AxioCam MRc5 camera. The long-distance movement of viruses was examined in upper noninoculated leaves by observing the presence of GFP fluorescence.

Analysis of accumulation of WSMV CP.

Total proteins, extracted from 9- and 15-dpi leaves, and partially purified virions (described below) were separated through 4 to 20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Invitrogen, Carlsbad, CA, U.S.A.), followed by a transfer onto a polyvinylidene difluoride (PVDF) membrane using an iBlot apparatus (Invitrogen). The blots were developed using a 1:15,000 dilution of anti-WSMV serum as a primary antibody and horseradish peroxidase-labeled goat anti-rabbit immunoglobulin G (at 1:50,000) as a secondary antibody. The PVDF membranes were developed using Immobilon Western blot substrate (Millipore, Billerica, MA, U.S.A.), and images of immuno-reactive protein bands were captured using the Molecular Imager ChemiDoc XRS+ with Image Lab Software system (Bio-Rad, Hercules, CA, U.S.A.).

Quantification of genomic RNA copies.

One microgram of total RNA from 9- and 15-dpi infected plants was used to synthesize the first-strand cDNA, using random primers. One microliter of 1:10-diluted first-strand cDNA reaction was used for real-time PCR, using primers and probe as described by Tatineni and associates (2010).

Virus purification and electron microscopy of virions.

Virions were partially purified from 4 to 6 g of 18-dpi wheat tissue or 7 g of agroinfiltrated *N. benthamiana* leaves as described by Tatineni and associates (2009). Briefly, frozen infected tissue was ground in 0.1 M sodium citrate buffer (SCB), pH 6.5, containing 0.1% β-mercaptoethanol (3 ml per gram of tissue), using an electric blender (for wheat tissue) or in a mortar and pestle (for *N. benthamiana* tissue). The extract was filtered through four layers of muslin cloth and clarified at 7,700 × g for 10 min. The supernatant was treated with 2% Triton-X 100 at 4°C for 10 to 15 min and was layered on 5 ml of 20% sucrose (wt/vol) in SCB. The virus particles were concentrated by centrifugation at 118,000 × g for 1.5 h in a Beckman 50.2 Ti rotor. The virus pellet was suspended in 270 µl of 40 mM sodium phosphate buffer, pH 7.0, plus 5% sucrose overnight at 4°C. Purified virus was mixed by pipetting up and down several times and was clarified at 3,000 × g for 5 min at 4°C. Twenty microliters of purified virion was used for electron microscopy grid preparation. Virions of wild-type and mutant viruses were observed under a Hitachi H-7500 transmission electron microscope. Purified virions were mixed with equal volumes of 2× sample buffer (100 mM Tris-Cl, pH 6.8, 4% SDS, 20% glycerol, 5% β-mercaptoethanol and 0.02% bromophenol blue) and were incubated in a boiling water bath for 3 min, and 20 µl were used for SDS-PAGE, followed by immunoblot hybridization as described above.

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A corrected version of Fig. 6 with the labels of some of the images in part A changed was published online on January 10, 2014.