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Wheat Streak Mosaic Virus Infects Systemically Despite Extensive Coat Protein Deletions: Identification of Virion Assembly and Cell-to-Cell Movement Determinants

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ABSTRACT

Viral coat proteins function in virion assembly and virus biology in a tightly coordinated manner with a role for virtually every amino acid. In this study, we demonstrated that the coat protein (CP) of *Wheat streak mosaic virus* (WSMV) (genus *Tritimovirus*; family *Potyviridae*) is unusually tolerant of extensive deletions with continued virion assembly and/or systemic infection. A series of deletion and point mutations were created in the CP cistron of wild-type and/or GFP-tagged WSMV and examined the effects of these mutations on cell-to-cell and systemic transport and virion assembly of WSMV. Mutants with overlapping deletions comprising N-terminal amino acids 6 to 27, 36 to 84, 85 to 100, 48 to 100, 36 to 100, or the C-terminal 14 or 17 amino acids systemically infected wheat with different efficiencies. However, mutation of conserved amino acids in the core domain, which may be involved in a salt bridge, abolished virion assembly and cell-to-cell movement. The N-terminal amino acids 6 to 27 and 85 to 100 are required for efficient virion assembly and cell-to-cell movement, while the C-terminal 65 amino acids are dispensable for virion assembly but required for cell-to-cell movement, suggesting that the C-terminus of CP functions as a dedicated cell-to-cell movement determinant. In contrast, amino acids 36 to 84 are expendable with no obvious effects on systemic infection and virion assembly. In total, 152 amino acids (6 to 27, 36 to 100 and 65 amino acids at the C-terminus end) of 349 amino acids of CP are dispensable for systemic infection and/or virion assembly, which is rare for multifunctional viral CPs.

INTRODUCTION

Viral coat proteins (CPs) are multifunctional, with roles in almost every aspect of the virus life-cycle (1, 2). In addition to virion assembly and disassembly, the CPs have been attributed to multiple non-structural functions in virus biology such as symptom modulation (e.g., 3, 4), vector transmission (1), replication (5), viral RNA translation (6), virus translocation (1, 7), suppression of host RNA silencing (8, 9), and activation of R gene-mediated host defenses (10). To facilitate these multiple tasks, the CPs should function in a tightly coordinated manner with a role for virtually every amino acid (1, 2). In contrast to other virus-encoded proteins, the CPs possess several determinants with distinct and/or overlapping functions in virus biology (11). Hence, the CPs tolerate minimal or no mutations without having deleterious effects on virus biology (e.g., 4, 11-14).

Successful systemic infection of plants by viruses requires the virus to spread beyond the initially infected cells through specialized intercellular connections termed plasmodesmata (PD) until the virus comes in contact with the vascular system. The virus is then passively transported through phloem-associated cells and egressed at a distal place, followed by further cell-to-cell movement allowing the virus to spread systemically (15). Cell-to-cell movement of plant viruses is mediated by virus-encoded movement proteins (MPs) through interactions with host factors, which allow the virus to move through PD to adjacent cells in virion or nucleoprotein complex form (16, 17). Plant viruses can be divided into at least three types based on the characteristics of cell-to-cell movement. The first group of viruses encodes a single dedicated *Tobamovirus* 30K-like MP, which increases the size exclusion limit of PD to allow virions or ribonucleoprotein complexes to pass through modified PD channels (15, 18). The second group

of viruses includes icosahedral viruses such as the *Secoviridae*, *Bromoviridae* and *Caulimoviridae*, which mediate cell-to-cell movement with the involvement of both MPs and CPs through tubule-like structures (19, 20). The third group of viruses includes potex-, carla-, hordei-, and some furo-like viruses, which encode the triple gene block proteins that function collectively without forming tubule-like structures (21).

The family *Potyviridae* comprises the largest number of positive-stranded RNA viruses infecting a wide range of plant species (22). The cell-to-cell movement mechanism of potyvirids does not fall into any of the above three categories. Instead, several virus-encoded proteins have been reported to be involved in virus movement with no dedicated MP. The proteins involved in the movement function of potyvirids also contain at least one additional role in virus biology (23). Potyviral helper component-proteinase (HC-Pro) (24), genome-linked protein (25), cylindrical inclusion protein (26) and CP (11, 13, 24) were reported with a function in cell-to-cell movement of potyviruses. Recently, the P3N-PIPO, probably translates from the P3 coding region at a conserved shifty sequence at the 5' end of PIPO (27), was also implicated in cell-to-cell movement (28-31). Among potyvirid species, the role of the CP in virus biology has been well-studied for members of the *Potyvirus* genus. The CP has three distinct domains: the variable N- and C-terminal domains that are exposed on virion surface and susceptible to mild trypsin treatment, and the more conserved central core domain that forms the core subunit structure of the virion (32, 33). The N-terminal Asp-Ala-Gly (DAG) motif and its adjacent amino acids in the CP are required for aphid transmission of potyviruses by a specific interaction between the DAG and PTK-motif located in the HC-Pro (34-36). The elegant work of Dolja et al. (11, 13) identified distinct functions of CP in virion assembly and cell-to-cell movement of *Tobacco etch potyviru*s (TEV).

89 *Wheat streak mosaic virus* (WSMV), an economically important wheat virus, is the type
90 species of the genus *Tritimovirus* of the family *Potyviridae* with a 9,384-nucleotide (nt)
91 [excluding the 3'-poly (A) tail] single-stranded plus-sense RNA genome encapsidated in
92 flexuous filamentous particles of 690 to 700 nm X 11 to 15 nm (37). WSMV is transmitted by
93 the wheat curl mite (*Aceria tosichella* Keifer) in a semipersistent manner, and HC-Pro has been
94 implicated as a viral determinant for wheat curl mite transmission (38-40). The WSMV genome
95 contains a single large open reading frame encoding a polyprotein that is translated from the
96 genomic RNA (37). The polyprotein is cleaved co-translationally and *in trans* by three virus-
97 encoded proteinases, the P1, HC-Pro and NIa-Pro, into 10 mature proteins. In contrast to the
98 multifunctional potyviral HC-Pro (23), WSMV HC-Pro is dispensable for systemic infection
99 (41). The P1 of WSMV, not HC-Pro as in potyviruses, was identified as the suppressor of RNA
100 silencing (42).
101 The WSMV CP is 349 amino acid residues long, compared to 263 and 267 amino acids
102 of respective CPs of TEV and *Potato virus* Y (32, 37, 43). The CP determinants in virion
103 assembly, virus transport, pathogenicity, and vector transmission of WSMV are not known
104 except that the N-terminal region of CP was identified as a host- and strain-specific long-distance
105 transport factor (44). Recently, we demonstrated that the C-terminal aspartic acid residues of CP
106 are involved in host-specific virus movement with a role for efficient cell-to-cell movement in
107 wheat and a long-distance transport function in maize inbred line SDp2 (45). In the present
108 study, we identified a region between amino acids 155 and 285 of CP as the core domain through
109 predictive structural modeling. Additionally, we explored the roles of the CP in WSMV biology
110 by introducing point mutations in the central core domain and a series of deletions at the N- and
111 C-terminal regions and examining the effects of these mutations on cell-to-cell and long-distance

112 movement and virion assembly. In total, 152 amino acids (6 to 27, 36 to 100 and 65 amino acids
113 at the C-terminus end) of 349 amino acids of CP are dispensable for virion assembly and/or
114 systemic infection of WSMV albeit at reduced levels. Remarkably, 49 amino acid residues
115 comprising amino acids 36 to 84 of CP are expendable with no obvious effects on WSMV virion
116 assembly and systemic infection of wheat.
117
118 **MATERIALS AND METHODS**
119
120 **Construction of CP mutants.** An infectious cDNA clone of WSMV-Sidney 81, pSP6-WSMV-
121 S81 (46), was the basis for all the mutants generated in this study. Previously, we modified
122 pSP6-WSMV-S81 to express GFP as a marker protein in a pUC-based construct pSP6-WSMV-
123 S81-GFP-6K1/CI(7aa) and in T-DNA based pCAMBIA-construct pCAM-WSMV-S81-GFP-
124 6K1/CI(7aa) (47). Point mutations and inframe deletions in the CP cistron were created in pSP6-
125 WSMV-S81, pSP6-WSMV-S81-GFP-6K1/CI(7aa) and pCAM-WSMV-S81-GFP-6K1/CI(7aa)
126 using mutagenic oligonucleotides, followed by overlap extension PCR (48). The overlap
127 extension PCR was performed with oligonucleotides W-3 and W-89 (44), and ligated into pSP6-
128 WSMV-S81 or pSP6-WSMV-S81-GFP-6K1/CI(7aa) between *Bst*EII (nt 6319) and *Spe*I (at the
129 3' end) restriction endonuclease sites as described previously (44). Point or deletion mutations in
130 the CP cistron in pCAM-WSMV-S81-GFP-6K1/CI(7aa) were created by substituting a *Pst*I-*Not*I
131 restriction fragment (nt 4816-3' end) with the corresponding fragment from pSP6-WSMV-S81
132 or pSP6-WSMV-S81-GFP-6K1/CI(7aa) containing mutations in the CP cistron.
133 Standard molecular biology methods were used for PCR, overlap extension PCR,
134 ligations, and transformations as described in Sambrook and Russell (49). *Escherichia coli*

strain JM109 was used to transform cDNA clones of WSMV and plasmid DNA was prepared from 40 ml of culture grown overnight using the Bio-Rad plasmid midiprep kit (Bio-Rad, Hercules, CA). Presence of point or deletion mutations in cDNA clones was confirmed by nucleotide sequencing, and 3 independent clones per mutant were tested in phenotypic studies. Each mutant was examined in 2 to 3 independent experiments, and the results presented are from one independent clone.

Inoculation of wheat with *in vitro* transcripts and virion stability assay. One µg of *Not*I- or *Spe*I-linearized plasmid was used to prepare *in vitro* transcripts in a 40 µl reaction as described in Tatineni et al. (47). Freshly prepared *in vitro* transcripts were mixed with an equal volume of 2% sodium pyrophosphate, pH 9.0, containing 1.0% baked Celite and inoculated onto 13 to 20 wheat cv. Tomahawk seedlings at the single-leaf stage. Wheat seedlings were washed with distilled water 5 min after inoculation, and incubated in a greenhouse at 20 to 27ºC for symptom development. Wheat seedlings were observed for symptom development at 7 to 25 days post-inoculation (dpi).

Virion stability of WSMV CP deletion mutants was examined by preparing crude extract from symptomatic leaves of transcript-inoculated plants in 20 mM sodium phosphate buffer, pH 7.0 at a 1:20 dilution. The extracts were incubated at room temperature for 45 min, inoculated to wheat seedlings at the single-leaf stage, and observed for symptom development in a greenhouse for symptom development at 20 to 27ºC.

Reverse transcription (RT)-PCR assay. Total RNA extracted from upper fully expanded symptomatic wheat leaves of CP deletion mutants-infected plants (50) was used to

158 synthesize the first-strand cDNA in a 10 µl reaction volume with random primers as described
159 previously (51). One µl of first-strand cDNA was used for PCR in a 25 µl reaction volume with
160 plus- and minus-sense CP-specific primers with the following PCR program: 95˚C for 2 min,
161 followed by 35 cycles at 95˚C for 30 s, 54˚C for 30 s and 72˚C for 90 s and one cycle at 72˚C for
162 10 min. The RT-PCR products were analyzed through 1.0% agarose gels in TAE (Tris-acetate-
163 EDTA) buffer.
164
165 **Cell-to-cell movement of WSMV CP mutants.** *In vitro* transcripts of GFP-tagged
166 WSMV CP point/deletion mutants were inoculated to wheat seedlings at the single-leaf stage as
167 described above. Cell-to-cell movement of GFP-tagged wild-type and mutant viruses was
168 monitored by examining the formation of fluorescent foci on inoculated wheat leaves under a
169 Zeiss Stereo Discovery V12 Fluorescence Microscope (Carl Zeiss MicroImaging, Inc., New
170 York, NY) using a GFP narrow band filter at 4 and 14 dpi. The GFP fluorescence pictures were
171 taken using AxioCam MRc5 camera attached to a V12 Fluorescence Microscope, and the sizes
172 of foci were measured using a program provided with the AxioCam MRc5 camera.
173
174 **Agroinfiltration assays.** pCAM-WSMV-S81-GFP-6K1/CI(7aa) with point or deletion
175 mutations in the CP cistron were transformed into *Agrobacterium tumefaciens* strain EHA105.
176 Agrosuspensions containing WSMV constructs (1.0 OD_{600}) were mixed with an equal volume of
177 *Agrobacterium* harboring *Tomato bushy stunt virus* p19, a suppressor of RNA silencing (52) and
178 infiltrated into the abaxial side of *Nicotiana benthamiana* leaves using needleless 3 ml syringes.
179 The agroinfiltrated plants were incubated in a growth chamber at 24˚C maximum and 20˚C

minimum temperature with a 14-h photoperiod. *N. benthamiana* leaves were collected at 7 days post-agroinfiltration (dpa) for GFP fluorescence observation, followed by virion purification.

Purification of virions and electron microscopy. Virions were partially purified from 4 to 6 g of symptomatic wheat leaves, or 7 g of agroinfiltrated *N. benthamiana* leaves expressing GFP as described in Tatineni et al. (53). Briefly, frozen infected tissue was ground in 0.1 M sodium citrate buffer (SCB), pH 6.5, containing 0.1% β-mercaptoethanol (3 ml/g tissue). The extract was filtered through four layers of muslin cloth and clarified at 8,000 x g for 10 min. The supernatant was treated with 2% Triton-X 100 at 4ºC for 15 to 20 min, layered on 5 ml of 20% sucrose (w/v) in SCB, followed by centrifugation at 118,000 x g for 1.5 h in a Beckman 50.2 Ti rotor. The virus pellet was suspended in 300 µl of 40 mM sodium phosphate buffer, pH 7.0, containing 5% sucrose at 4˚C overnight. The purified virus was clarified at 3,000 x g for 5 min and 20 µl of purified virus preparation was used to prepare 400 mesh carbon coated copper grids and observed under a Hitachi H-7500 transmission electron microscope. For Western blots, purified virus was mixed with an equal volume of 2X sample buffer (100 mM Tris-Cl pH 6.8, 4% SDS, 20% glycerol, 5% β-mercaptoethanol and 0.02% bromophenol blue) and incubated in a boiling water bath for 3 min.

Virion assembly assays of WSMV CP mutants. The ability to form virions by CP mutants was examined by purifying virions from systemically infected wheat (movement-dependent virion assembly assay, MDVA assay) and from agroinfiltrated *N. benthamiana* leaves (movement-independent virion assembly assay, MIVA assay). The MDVA assay can provide information on virion assembly only if a mutant efficiently infects wheat in a similar manner to a

wild-type virus, but not from mutants that fail to infect or inefficiently infect due to defects in cell-to-cell movement but not in virion assembly. In contrast, the MIVA assay in *N. benthamiana* would facilitate examination of the virion assembly independent of virus movement (see below).

Recently, we found that *Agrobacterium* harboring a GFP-tagged variant of WSMV in a binary vector [pCAM-WSMV-S81-GFP-6K1/CI(7aa)] replicated weakly and formed infectious virions in agroinfiltrated *N. benthamiana* leaves without cell-to-cell and long-distance movement (47; data not shown), which would facilitate examination of virion assembly independent of virus movement. Purification of virions from agroinfiltrated leaves through a 20% sucrose cushion would separate encapsidated virions from that of nonencapsidated free CP. We utilized the MDVA and MIVA assays to examine the ability and efficiency of virion formation by CP mutants. Partially purified virions from wheat and *N. benthamiana* leaves were observed under an electron microscope and/or subjected to Western blot using an anti-WSMV serum.

Western blot assay. Partially purified denatured virions were subjected to SDS-PAGE on 4 to 20% gels (Invitrogen, Carlsbad, CA), followed by a transfer onto a 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 HRP-labeled goat anti-rabbit IgG (at 1:50,000) as a secondary antibody. The PVDF membranes were developed using Immobilon Western blot substrate (Millipore), and images of immuno-reactive protein bands were captured using the Molecular Imager ChemiDoc XRS+ with Image Lab Software system (Bio-Rad).

Predictive structural modeling. Since there is no three dimensional structure of the CP or a closely related protein, the I-TASSER (*I*terative *T*hreading *ASSE*mbly *R*efinement) server, a

widely used integrated platform for structure prediction, was used to model the structure of the WSMV CP both with and without user supplied restraint (54). The crystal structure of *Papaya mosaic virus* (PMV) CP (4DOX, chain A) (55) was used as a user restraint for generating models of full-length and a number of truncated versions of CP. The confidence score (C-score), based on the quality of threading template alignments and structural assembly simulation convergence parameters, along with the TM-score, a measurement of structural similarity between two structures, were used to assess the reliability of the predicted models.

RESULTS

The core domain of CP is estimated to extend from R155 to M285 based on predictive structural modeling. Initial models generated by the iTASSER server using the full CP sequence with and without user restraint had very low C-scores (-4.2 to -3.5) and TM-scores (0.27 to 0.33). For a predictive model to be considered reliable, it should have C-scores > -1.5 and TM-scores > 0.5 (54). These models failed on both scores. However, the models generated using the user restraint of the 4DOX pdb structure (55) did provide insight into the possibility of modeling a C-terminal core domain. The PMV CP crystal structure displays a C-terminal core domain with a short N-terminal domain consisting of a loop with a single helix that extends away from the core (55). This loop contains a Phe residue that is inserted into a hydrophobic pocket of an adjacent core domain. The entire 4DOX sequence only contains 173 amino acid residues and therefore cannot be used to model the full WSMV CP sequence of 349 amino acids. However, models generated using 4DOX as a restraint showed sequence and structural alignment of the CP C-terminal domain with the C-terminal core domain structure of the PMV CP crystal structure.

After recognition of this, models were generated using truncated CP starting at R155 (C-term-R155) that had acceptable C- (-1.53) and TM- (0.53) scores. Models generated for C-term-R155-Delta17 (minus the last 17 amino acids), C-term-R155-Delta35 (Fig. 1) and C-term-R155-Delta65 had C-scores of -0.82, -0.25 and 0.04 and TM-scores of 0.61, 0.68 and 0.72, respectively. These observations suggest that CP with progressive deletions from the C-terminus yielded better structural models for the core domain. Thus, we predict that the core domain extends approximately from amino acid R155 to M285, which suggests that the remaining amino acids are part of a separate C-terminal domain. Since all of these models were generated using 4DOX, they are similar in fold and are all α-helix.

All of the models revealed two salt-bridges between the critical amino acid residues R237, D282 and R307 with D282 in the middle (Fig. 1), which is experimentally supported by mutational analysis of R237 and D282 (see below). These residues are highly conserved in related viruses, and the residue R307 aligns with R223 in TEV CP. Previously, a salt bridge was predicted between R154 and D198 residues in the TEV CP (13).

The N-terminal domain (residues 1-155) was also modeled using iTASSER; reliable models for this domain were not found. The C and TM scores for all of the N-terminal deletions were quite low. The best scores obtained were for the full-length N-terminal domain (C-score= -1.79 and TM-score = 0.5) and they were still below the cutoff for what is considered trustworthy.

The core domain of WSMV CP is required for cell-to-cell movement and virion formation. The conserved tryptophan, arginine and aspartic acid residues at positions 165, 237 and 282, respectively, in the CP were individually mutated to alanine residues in pSP6-WSMV-S81-GFP-6K1/CI(7aa) to obtain $W_{165}A$, $R_{237}A$ and $D_{282}A$ (Fig. 2A). These three amino acid

residues are conserved among the CPs of a majority of potyvirid species, and R237 and D282 are conserved among the CPs of plant filamentous viruses and are implicated in cell-to-cell movement and virion assembly of TEV (13, 56). *In vitro* transcripts of GFP-tagged wild-type virus and CP mutants were inoculated to wheat seedlings at the single-leaf stage. The wild-type virus WSMV-S81-GFP-6K1/CI(7aa) systemically infected wheat at 7 dpi, while mutant viruses failed to infect wheat even at 25 dpi (Table 1). At 9 dpi, the wild-type virus induced large foci spreading throughout most of the inoculated leaf lamina; in contrast, mutant viruses restricted spreading to 1 to 3 cells (Fig. 2B), indicating that these mutations debilitated cell-to-cell movement of WSMV.

Virion assembly competency of these mutants was examined in an MIVA assay by transferring CP point mutations to pCAM-WSMV-S81-GFP-6K1/CI(7aa) (47). As a control, a ΔGDD mutant was created in pSP6-WSMV-S81-GFP-6K1/CI(7aa) and pCAM-WSMV-S81-GFP-6K1/CI(7aa) by deleting the GDD motif plus five amino acids on either side of the GDD motif in the NIb cistron. As expected, *in vitro* transcripts of pSP6-WSMV-S81-GFP-6K1/CI(7aa) with ΔGDD mutation failed to form detectable fluorescent foci in wheat at 9 dpi, and failed to infect wheat systemically at 25 dpi (Table 1; Fig. 2B). The point and ΔGDD mutants in pCAM-WSMV-S81-GFP-6K1/CI(7aa) were transformed into *A. tumefaciens* strain EHA105, and agrosuspensions harboring CP or GDD mutants infiltrated into *N. benthamiana* leaves. At 7 dpa, GFP fluorescence was observed similarly in point mutants and wild-type virus (Fig. 2C). Surprisingly, the GFP fluorescence was also observed in ΔGDD mutant-infiltrated leaves (Fig. 2C), suggesting that constitutively produced viral transcripts under the 35S promoter most likely translated and the GFP protein might have been released from the polyprotein by viral proteinases. Moreover, GFP expression in agroinfiltrated leaves served as a good marker

295 for the expression of polyprotein and also as an indication of the virus-encoded RNA silencing
296 suppressor protein effectively counteracting the host defense system.

297 Partially purified virions from agroinfiltrated *N. benthamiana* leaves at 7 dpa were
298 subjected to Western blot using antibodies against WSMV. A WSMV CP of 45 kDa, truncated
299 31 and 29 kDa proteins were found in partially purified virion preparations of the wild-type virus
300 (Fig. 2D). Previously, it has been reported that three CP bands of 45, 31 and 29 kDa were found
301 in purified virion preparations of WSMV, which was attributed to *in vivo* proteolysis of CP due
302 to leaf senescence (57). However, the exact nature of multiple CP bands in purified virion
303 preparations is not known. In contrast to wild-type virus, CP did not accumulate at detectable
304 levels in purified virion preparations of mutants-infiltrated *N. benthamiana* leaves (Fig. 2D),
305 suggesting that W165, R237 and D282 in the CP are critical for virion assembly. Though the
306 GFP fluorescence was observed in ΔGDD-infiltrated leaves, CP did not accumulate at detectable
307 levels in partially purified virion preparations (Fig. 2D). This result suggests that failure to form
308 virions by a replication-deficient ΔGDD mutant could be due to lack of free CP at a threshold
309 level.

310

311 **WSMV CP is dispensable for replication.** The requirement of CP for WSMV
312 replication was examined by deleting codons encoding the CP amino acid residues except 5
313 amino acids each at the N- and C-termini in pSP6-WSMV-S81-GFP-6K1/CI(7aa) to obtain
314 pSP6-WSMV-GFP-ΔCP. *In vitro* transcripts of pSP6-WSMV-GFP-ΔCP elicited fluorescent foci
315 restricted to single cells in wheat similar to those of $R_{237}A$ and $D_{282}A$ (Fig. 2B). As expected, no
316 fluorescent foci were observed in ΔGDD-inoculated wheat seedlings (Fig. 2B). The development
317 of foci by WSMV-GFP-ΔCP similar to those of mutants $R_{237}A$ and $D_{282}A$ together with the

318 results of analogous CP mutants of TEV that are dispensable for replication (13), suggests that
319 deletion of CP did not appreciably affect WSMV replication in wheat but was required for cell-
320 to-cell movement.
321
322 **Amino acids 36 to 84 in the CP are dispensable for virion assembly and systemic**
323 **infection of wheat.** The requirement of the N-terminal region of WSMV CP for virion assembly
324 and systemic infection of wheat was examined by deleting codons encoding amino acid residues
325 36 to 84 in pSP6-WSMV-S81 to obtain pSP6-WSMV-CPΔ36-84aa (Fig. 3A). Wheat seedlings
326 inoculated with *in vitro* transcripts of this mutant developed systemic chlorotic streaks and
327 mosaic symptoms similar to wild-type virus at 9 dpi (Fig. 3B; Table 1), demonstrating that
328 amino acid residues 36 to 84 of CP are dispensable for systemic infection of wheat. Systemic
329 infection of wheat by WSMV-CPΔ36-84aa was further confirmed by RT-PCR amplification of
330 the CP cistron from deletion mutant-infected plants (Fig. 3C), followed by nucleotide sequencing
331 of PCR amplicons.
332 The ability to form virions by WSMV-CPΔ36-84aa was examined by partially purifying
333 virions from systemically infected wheat at 14 dpi, and found virus particles with no obvious
334 morphological differences from those of the wild-type virus under electron microscopy (Fig.
335 3D). Western blot analysis using an anti-WSMV serum suggested that the CP of 35 kDa was
336 detected in virion preparations of WSMV-CPΔ36-84aa in similar amounts to that of the wild-
337 type virus (Fig. 3E, compare lane 2 with lane 1). In addition to full-length CP, this mutant virus
338 also accumulated a truncated form of 29 kDa protein but lacked a 31 kDa protein (Fig. 3E).
339

340 **Amino acids 85 to 100 of CP are required for efficient systemic infection of wheat.**
341 We next examined the requirement of amino acid residues 85 to 100 for systemic infection by
342 deleting corresponding codons in pSP6-WSMV-S81 to obtain pSP6-WSMV-CPΔ85-100aa (Fig.
343 3A). Wheat seedlings inoculated with *in vitro* transcripts of this mutant developed mild chlorotic
344 streaks and chlorotic spots at 9 to 12 dpi compared to 6 to 8 dpi by the wild-type virus (Table 1;
345 Fig. 3B), suggesting that deletion of amino acid residues 85 to 100 in CP delayed the onset of
346 systemic symptoms, followed by moderate symptoms at 18 to 21 dpi. We next examined
347 possible cumulative effects of deletions comprising amino acid residues 48 to100 and 36 to 100
348 on systemic infection of wheat by creating pSP6-WSMV-CPΔ48-100aa and pSP6-WSMV-
349 CPΔ36-100aa, respectively (Fig. 3A). *In vitro* transcripts of these two mutants developed mild
350 systemic chlorotic streaks on wheat at 11 to 15 dpi compared to 9 to 12 and 6 to 9 dpi by
351 WSMV-CPΔ85-100aa and wild-type virus, respectively, albeit at slightly reduced infection rates
352 (Table 1; Fig. 3B). Systemic infection of wheat by WSMV with deletions encompassing amino
353 acids 85 to 100 was further confirmed by detecting smaller-sized RT-PCR amplicons from
354 deletion mutants-infected plants compared to that of the wild-type virus (Fig. 3C) and nucleotide
355 sequencing of PCR amplicons.
356 WSMV with overlapping deletions comprising amino acids 85 to 100 in the CP formed
357 virions in wheat that appeared to be morphologically similar to those of the wild-type virus (Fig.
358 3D). Western blot analysis of partially purified virions with an anti-WSMV serum revealed that
359 virions of WSMV-CPΔ85-100aa, WSMV-CPΔ48-100aa and WSMV-CPΔ36-100aa accumulated
360 2 to 5-fold less in wheat compared to those of the wild-type virus (Fig. 3E). Taken together,
361 these data suggested that WSMV with deletion of amino acid residues 36 to 100 in the CP is
362 capable of virion assembly and systemic infection of wheat, though at reduced efficiencies.

The N-proximal amino acids 6 to 27 and C-proximal 17 amino acids of CP are dispensable for long-distance transport in wheat. The requirement of the N- and C-proximal amino acids for systemic infection of wheat and virion assembly was examined by deleting codons encoding amino acid residues 6 to 27 at the N-terminus as well as both 14 and 17 amino acid residues from the C-terminus in WSMV-CPΔ6-27aa, WSMV-CPΔC14aa and WSMV-CPΔC17aa, respectively (Fig. 4A). *In vitro* transcripts of WSMV-CPΔN6-27aa infected 47 to 56% of wheat at 15 to 21 dpi with a few chlorotic streaks per leaf, and WSMV-CPΔC14aa infected 77 to 84% of wheat at 14 to 18 dpi with mild to moderate symptoms (Table 1; Fig. 4B). In contrast, deletion of 17 amino acids from the C-terminus had a deleterious effect on systemic infection with only 11 to 25% of plants infected at 15 to 21 dpi with few chlorotic streaks per leaf (Table 1; Fig. 4B). Infection of wheat by the N- and C-terminal deletion mutants was further confirmed by RT-PCR amplification of the CP cistron from systemically infected wheat (Fig. 4C), followed by nucleotide sequencing of PCR amplicons. These data demonstrated that amino acid residues 6 to 27 at the N-terminus and 17 amino acids from the C-terminus are dispensable for systemic infection of wheat.

The N- and C-proximal deletion mutants formed virions with no obvious morphological differences compared to wild-type virus (Fig. 4D), though these mutants accumulated virions at 2- and 10-fold less compared to wild-type virus (Fig. 4E). Taken together, these data revealed that the N-proximal amino acid residues 6 to 27 and C-proximal 14 and 17 amino acids are dispensable for virion formation and systemic infection, though presence of these amino acids enhanced the fitness of the virus.

386 **Amino acids 6 to 27, 85 to 100, and the C-terminal region of CP are required for**
387 **efficient cell-to-cell movement.** Deletion of 6 to 27, 85 to 100, or C-terminal 14 or 17 amino
388 acids in the CP delayed the onset of systemic infection and caused mild symptoms in wheat,
389 suggesting that these mutants are defective in movement. The movement of CP deletion mutants
390 in wheat was monitored by transferring deletions into pSP6-WSMV-S81-GFP-6K1/CI(7aa) (47).
391 The GFP-tagged variant of WSMV was successfully used as an excellent marker virus to
392 monitor virus movement and distribution in wheat and SDp2 maize (44, 45, 47).
393 *In vitro* transcripts of GFP-tagged variants of WSMV CP deletion mutants were
394 inoculated to two sets of wheat seedlings at the single-leaf stage. One set of inoculated plants
395 was observed for GFP fluorescent foci on inoculated leaves at 4 and 14 dpi and the other set of
396 plants was observed for systemic fluorescent foci at 12 or 21 dpi (Fig. 5). WSMV with deletion
397 of amino acids 36 to 84 in the CP formed foci of slightly larger in size than those of the wild-
398 type virus (Table 2; Fig. 5A1-B1), suggesting that amino acids 36 to 84 are not required for cell-
399 to-cell movement. In contrast, WSMV with deletions comprising amino acids 85 to 100, 48 to
400 100, or 36 to 100 formed smaller sized foci of 1.73, 1.45, 1.36 mm^2, respectively, compared to
401 2.31 mm^2 foci of the wild-type virus (Table 2; Fig. 5C1-E1). WSMV with deletion of 6 to 27, or
402 C-terminal 14 or 17 amino acids formed substantially smaller sized foci of 0.53, 0.64, and 0.40
403 mm^2 compared to 2.31 mm^2 of the wild-type virus (Table 2. Fig. 5F1-H1). These data revealed
404 that 85 to 100, 6 to 27 and C-terminal 17 amino acids are required for efficient cell-to-cell
405 movement in wheat. Furthermore, at 14 dpi, GFP fluorescence was spread throughout most of
406 the inoculated leaves of GFP-tagged wild-type virus or a deletion comprising amino acids 36 to
407 84 (Fig. 5A2-B2). In contrast, fluorescence was restricted to individual foci in leaves inoculated
408 with WSMV with deletions comprising 6 to 27, 85 to 100, or C-terminal 14 or 17 amino acids

409 (Fig. 5C2-H2), indicating that these amino acids are required for efficient cell-to-cell movement
410 in wheat.

411 At 12 dpi, GFP fluorescence covered most of the upper noninoculated leaves of wheat
412 infected with wild-type virus or WSMV-GFP-CPΔ36-84aa (Fig. 5A3-B3). In contrast, GFP
413 fluorescence was restricted to several foci without spreading throughout the entire leaf lamina of
414 plants inoculated with mutants comprising deletion of 85 to 100, 6 to 27, or C-terminal 14 or 17
415 amino acids at 12 to 21 dpi (Fig. 5C3-H3), which was consistent with the defective cell-to-cell
416 movement nature of these mutants.
417
418 **Amino acids 6 to 27 and 85 to 100 of CP are required for efficient virion assembly.**
419 The requirement of CP amino acids 6 to 27 and 85 to 100 for efficient virus translocation in
420 wheat prompted an examination of virion assembly competency of these mutants in an MIVA
421 assay. Deletion of codons encoding amino acid residues 6 to 27, 85 to 100 or 36 to 100 were
422 introduced into the CP cistron in pCAM-WSMV-S81-GFP-6K1/CI(7aa) (47). Wild-type virus
423 and a CP mutant with deletion of amino acids 36 to 84 were included as positive controls.
424 *Agrobacterium* harboring pCAM-WSMV-S81-GFP-6K1/CI(7aa) and deletion mutants were
425 infiltrated into *N. benthamiana* leaves.

426 Virions were partially purified from agroinfiltrated leaves at 7 dpa and analyzed by
427 Western blot using an anti-WSMV serum. WSMV with a deletion of amino acid residues 36 to
428 84 in the CP accumulated virions in similar amounts to that of the wild-type virus, while deletion
429 of amino acids 85 to 100 or 36 to 100 drastically reduced virion formation as CP accumulated at
430 much reduced levels in partially purified virions compared to those of the wild-type virus (Fig.
431 6A, B). Deletion of amino acids 6 to 27 also affected virion formation substantially compared to

wild-type virus, but accumulated virions at slightly higher levels than mutants with deletions comprising amino acids 85 to 100 (Fig. 6A, B). These results together with virion assembly in systemically infected wheat suggested that amino acids 6 to 27 and 85 to 100 are dispensable for virion formation, but presence of these amino acids enhances the fitness of virion assembly.

The C-terminus of CP is required for cell-to-cell movement, but is dispensable for virion assembly. WSMV with deletion of 14 amino acids from the C-terminus of CP delayed the onset of systemic symptoms and additional 3-amino acid deletion substantially affected cell-to-cell movement and the ability to infect wheat systemically (Table 1; Figs. 5 and 7A). We next introduced deletion of 22 and 27 amino acids from the C-terminus of CP in pSP6-WSMV-S81-GFP-6K1/CI(7aa) and *in vitro* transcripts of these mutants formed fluorescent foci confined to 3 to 10 cells at 14 dpi (Fig. 7A), but failed to establish systemic infection in wheat at 25 dpi. These results suggested that the C-terminal region of CP is involved in cell-to-cell movement of WSMV but it is not known whether these deletions affected virion encapsidation. To examine virion assembly of these mutants in an MIVA assay, deletion of 14, 17, 22 or 27 amino acids from the C-terminus of CP were introduced into pCAM-WSMV-S81-GFP-6K1/CI(7aa) and agroinfiltrated into *N. benthamiana* leaves. Western blot of partially purified virions from agroinfiltrated leaves indicated that CP in purified virions of deletion mutants accumulated in similar amounts to that of the wild-type virus (Fig. 7B). Moreover, mutants with deletion of 14, 17, 22, or 27 amino acids from the C-terminus formed virions similar to those of the wild-type virus (Fig. 7C; data not shown). These data demonstrated that 27 amino acids from the C-terminus of CP are dispensable for virion formation, but are crucial for cell-to-cell movement in wheat.

455 We next introduced deletion of 40, 49, 55, 65 or 73 amino acids from the C-terminus of
456 CP in pSP6-WSMV-S81-GFP-6K1/CI(7aa) and pCAM-WSMV-S81-GFP-6K1/CI(7aa). *In vitro*
457 transcripts of these mutants failed to form detectable fluorescent foci in wheat at 15 dpi (Fig.
458 7A), suggesting that these deletions debilitated the cell-to-cell movement function of WSMV.
459 Western blot of partially purified virions from agroinfiltrated *N. benthamiana* leaves revealed
460 that mutants with deletion of 40, 49, or 55 amino acids from the C-terminus accumulated CP in
461 partially purified virions in similar amounts to that of the wild-type virus (Fig. 7B). In contrast,
462 deletion of 65 amino acids at the C-terminal region assembled virions at ~15 to 20% of the wild-
463 type virus, while deletion of 73 amino acids completely abolished virion assembly (Fig. 7B).
464 Mutants with deletion of 40, 49, or 55 amino acids from the C-terminus formed flexuous
465 filamentous virus-like particles in an MIVA assay (Fig. 7C). These results indicated that the C-
466 terminal 65 amino acids of CP are dispensable for virion assembly, but required for cell-to-cell
467 movement of WSMV.
468
469 **DISCUSSION**
470
471 Viral CPs are involved in most aspects of the virus life cycle, with overlapping structural and
472 nonstructural functions in virion assembly and virus biology, respectively (1, 2). Thus, point
473 mutations or small deletions comprising a few amino acids in the CP can elicit negative effects
474 on CP functions (e.g., 4, 11-14, 58). Previously, we mapped differential infection of SDp2 maize
475 and wheat by WSMV to five amino acids at the N-terminus and two aspartic acid residues at the
476 C-terminus of CP (44, 45). In this study, in contrast to other viruses, we found that WSMV CP

477 is unusually tolerant of extensive deletions with continued systemic infection, which facilitated
478 identifying the CP determinants involved in cell-to-cell movement and virion assembly.

479 Three point mutations, $W_{165}A$, $R_{237}A$ and $D_{282}A$, targeting conserved amino acids in the
480 core domain of CPs of potyvirid species (56), restricted the virus mostly to a few cells and failed
481 to form virions at detectable levels, suggesting that these mutations might have affected protein-
482 protein or protein-RNA interactions required for virion assembly and/or cell-to-cell movement.
483 Though the CP cistron is not required for WSMV replication, the core domain mutants failed to
484 form virions at detectable levels, suggesting a functional role for these amino acids in virion
485 assembly and/or stability. Our predictive structural model of the core indicates that R237 and
486 D282 are likely involved in a salt bridge that confers structural stability to the CP. The analogous
487 mutations in potyviral CPs also abolished cell-to-cell transport and virion assembly (11, 13, 24).
488 Taken together, the core domains of poty- and tritimoviral CPs possess similar functions in
489 virion assembly and possibly in cell-to-cell movement.

490 WSMV CP is unusual in that 49 amino acid residues comprising amino acids 36 to 84 are
491 dispensable for normal virion assembly and systemic infection. What are the roles of these amino
492 acids in WSMV biology? Why is the virus keeping amino acids that are not required for virion
493 assembly and systemic infection? The ClustalW alignment of CP sequences from representative
494 species of each genera of the *Potyviridae* family revealed no conserved or similar amino acids
495 located at the N-terminal region. Additionally, the CPs of members of the *Tritimovirus* genus
496 contain fewer conserved amino acids at the N-terminal region than those in the central core
497 domain and C-terminal region. The variable nature of the N-terminal region of CPs of the
498 potyvirid species, together with amino acids 36 to 84 that are expendable for systemic infection,
499 suggests that these amino acids may possess virus-specific 'specialized functions' such as vector

500 transmission, host range, cross protection, etc. The N-terminal 'DAG' motif of potyviral CPs
501 was reported to be involved in aphid transmission (59); in contrast, WSMV CP does not contain
502 such a motif (37). However, mite transmission studies of CP deletion mutants may decipher
503 CP's role, if any, in vector transmission. Moreover, efficient infection of wheat by WSMV with
504 a 49 amino acid deletion (amino acids 36 to 84) in the CP could facilitate expression and display
505 of specialty epitopes/peptides embedded in virions.

506 Additionally, WSMV with overlapping deletions encompassing amino acids 85 to 100 in
507 the CP were capable of systemic infection, albeit with delayed and milder symptoms. Yet, it is
508 rare for a virus to systemically infect plants with a deletion of as large as 65 amino acid residues
509 (amino acids 36 to 100) in the CP. These mutants displayed restrictive cell-to-cell movement and
510 delayed the onset of systemic infection in wheat compared to wild-type virus. The delayed
511 systemic infection phenotype of these mutants could be due to the requirement of amino acids 85
512 to 100 in virion assembly and/or cell-to-cell transport. WSMV mutants with deletions
513 comprising amino acids 85 to 100 formed virions 2 to 5-fold less than the wild-type virus in
514 wheat, but at substantially reduced levels in an MIVA assay, suggesting that these amino acids
515 are required for efficient virion assembly. The differences in the levels of virion assembly by CP
516 deletion mutants in MDVA (in wheat) and MIVA (in *N. benthamiana*) assays might be due to
517 limited cell-to-cell and long-distance movement in wheat compared to virus restriction
518 exclusively to infiltrated mesophyll cells in *N. benthamiana*. Our data suggest that amino acids
519 85 to 100 are required for efficient virion assembly, but we cannot exclude the possible role of
520 these amino acids in cell-to-cell movement independent of virion assembly. It is also possible
521 that deletion of amino acids 85 to 100 might have affected virion stability. However, deletion

522 mutants in crude sap are highly infectious in wheat, suggesting that virions lacking amino acids
523 85 to 100 are stable and are capable of protecting viral genomic RNA.

524 In contrast to potyviral CPs, we found several differences with regard to the role of the
525 N- and C-proximal regions in virus biology. The potyviral N-proximal region (amino acids 5 to
526 29) was dispensable for virion assembly but is required for long-distance movement (13). In
527 contrast, we found that the N-terminal amino acids 6 to 27 of WSMV CP are required for
528 efficient virion assembly but dispensable for long-distance transport. It is possible that the
529 observed differences between TEV and WSMV might be due to the deletion of the N-proximal
530 region of TEV CP being slightly longer than the corresponding deletion in WSMV CP (amino
531 acids 5 to 29 vs 6 to 27). Nonetheless, the N-terminal regions of poty- and tritimoviral CPs have
532 clearly different requirements for virion assembly. Progressive deletions up to 65 amino acids
533 from the C-terminus of CP increasingly affected cell-to-cell movement of WSMV though virions
534 or virus-like particles were formed. Two plausible reasons can be deduced from these results: the
535 C-terminal 65 amino acids are involved in a dedicated cell-to-cell movement function and
536 virions with deletions from the C-terminus end are defective in virion stability/disassembly.
537 Because progressive deletions from the C-terminal region increasingly affected cell-to-cell
538 movement, it is likely that the C-terminus of CP functions as a determinant for cell-to-cell
539 movement. However, we cannot exclude a possible role for these amino acids in virion stability
540 and/or disassembly. In contrast to systemic infection of WSMV with a 17-amino acid deletion
541 from the C-terminus, the analogous mutant of TEV failed to infect tobacco systemically (11),
542 suggesting that either differential requirement of the C-terminal region of these two viruses or
543 differences in long-distance transport of viruses in eudicot and monocot plants could account for
544 these variations.

545 Systemic infection of wheat by cell-to-cell movement defective N- and C-terminal
546 deletion mutants suggest that these mutants entered the vascular system and successfully
547 egressed at distal regions. The delayed systemic infection by these mutants is most likely due to
548 defects in cell-to-cell movement, which might have caused delayed virus entry into the vascular
549 system. Since the CP of WSMV has not been demonstrated to have suppressor activity against
550 host RNA silencing (42), defects in combating host defense system are not likely causing
551 delayed systemic infection by CP deletion mutants. Though it is not known for WSMV, the N-
552 and C-termini of potyviral CPs are exposed outside of the virion (32, 33) and it is possible that
553 these amino acids are potential targets for interactions with host factors. Thus, deletion of these
554 amino acids might have prevented such interactions, consequently, affecting cell-to-cell
555 movement of virus. We observed a correlation between virion assembly and cell-to-cell
556 transport of WSMV: mutants that affected virion assembly also affected cell-to-cell transport,
557 and no mutant was found with cell-to-cell movement function without virion formation. These
558 results suggest that the cell-to-cell movement function of WSMV is facilitated as the virion form
559 as reported for como- and closteroviruses (60, 61). However, virion formation is not the only
560 requirement for cell-to-cell transport as deletions from the C-terminus of CP affected cell-to-cell
561 movement but not virion assembly. The CPs of several plant viruses were also implicated in
562 virus translocation (1, 4, 12, 62).

563 The availability of a structural model of CP would be helpful to understand the structural
564 basis for the results of this study. However, currently there are no high resolution structural
565 models for CPs of the *Potyviridae* family that can be used for homology modeling of the WSMV
566 CP. The recent crystal structure of the CP of the distantly related PMV (55), which is also a
567 flexuous filamentous plant virus, has been used in this study to identify the likely core domain

568 region of WSMV CP using the predictive modeling server, iTASSER. Although, this core
569 domain model does not aid in the interpretation of the N-terminal deletion data, it does provide
570 insight into the effects of single site mutations of R237 and D282, which are predicted to form a
571 salt-bridge as well as the C-terminal deletions. The salt-bridge is critical in maintaining a stably
572 folded domain and removal of salt-bridge via mutagenesis disrupts the stable fold. In the CP core
573 model there is the presence of a second conserved Arg (R307) on the opposite side of the D282
574 residue from R237 that likely helps to further stabilize the folded structure. Figure 1 shows that
575 the predicted core domain contains two salt-bridges with D282 in the middle. Swapping these
576 residues would keep one salt-bridge but disrupt the other by placing two positively charged Arg
577 residues in close proximity, which could explain why swapping of two salt-bridge residues in
578 TEV CP failed to restore virion assembly (13). As for the C-terminal deletions, it is observed that
579 deletions up to 65 amino acids from the C-terminus are predicted by the model to have very little
580 effect on the folding of the core. This fits well with the C-terminal deletion data that indicates
581 that deletions up to 65 amino acids from the C-terminus form virions but a 73-amino acid
582 deletion does not. Additionally, this model also predicts that the N-terminal region is likely a
583 separate domain, possibly connected to the core by a short loop and may have a domain
584 swapping interaction similar to that of PMV CP (55). Deletions that do not disrupt this
585 interaction would not be expected to have an effect on virion assembly.

586 Point and deletion mutations in the CP cistron of WSMV facilitated the identification of
587 determinants for virion assembly and/or cell-to-cell movement. The N-terminal amino acids 6 to
588 27 and 85 to 100 are required for cell-to-cell movement and efficient virion assembly and the C-
589 terminal 65 amino acids are required for cell-to-cell movement but are dispensable for virion
590 assembly. Additionally, amino acid residues 36 to 84 are not required for virus translocation and

591 virion assembly. The ability of WSMV to infect wheat systemically despite extensive deletions
592 in the CP may have serious consequences on the development of CP-based resistance against
593 WSMV. Our results indicate that resistance based on CP antagonism may be overcome by
594 WSMV. Additionally, the availability of viable CP deletion mutants will facilitate defining the
595 roles of CP in the transmission of WSMV by the wheat curl mite and disease development.
596
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598
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604

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768

769 **FIGURE LEGENDS**

770

771 **FIG 1** The predicted structural model of the WSMV CP core domain. (A) Predicted ribbon
772 structure of the core domain of WSMV CP showing a three amino acid salt bridge between
773 R237, D282 and R307. This structural model was generated with iTASSER using WSMV CP
774 sequence C-term-R155-Delta35aa together with the PMV CP structural model, 4DOX (A chain)
775 (55) as user supplied restrain. (B) Enlarged view of the predicted three amino acid salt bridge
776 between R237, D282 and R307 in the core domain.

777

778 **FIG 2** The core domain of WSMV CP is required for cell-to-cell movement and virion assembly.
779 (A) Genomic organization of WSMV-S81-GFP-6K1/CI(7aa) showing the locations of individual
780 proteins encoded by the polyprotein. The vertical lines between the encoded proteins are
781 sequences that code for proteolytic processing sites. The enlarged view of the CP cistron is
782 shown below the genomic organization with the locations of point mutations $W_{165}A$, $R_{237}A$ and
783 $D_{282}A$. (B) Infection foci of *in vitro* transcripts of GFP-tagged wild-type virus, point mutants,
784 ΔGDD, and WSMV-GFP-ΔCP on wheat at 9 dpi. The ΔGDD mutant was created by deleting the
785 GDD motif and five amino acids on either side of the GDD motif in the NIb cistron. WSMV-
786 GFP-ΔCP was created by deleting codons encoding CP amino acid residues except 5 amino acids
787 each at the N- and C-termini. Note that viruses with point mutations in the core domain or
788 WSMV-GFP-ΔCP were restricted to 1 to 3 cells and ΔGDD mutant failed to form fluorescent
789 foci on inoculated wheat leaves. The wild-type virus formed large foci. Bars represent 200 µm.
790 (C) Expression of GFP in agroinfiltrated leaves of *N. benthamiana* at 7 days post-agroinfiltration
791 (dpa). Bars represent 200 µm. (D) Western blot of partially purified virions from agroinfiltrated

792 *N. benthamiana* leaves at 7 dpa. The immunoblot was probed with an anti-WSMV serum. The
793 locations of 45, 32 and 29 kDa protein bands are indicated with an arrow, arrow head and
794 asterisk, respectively. Note that only the wild-type virus formed virions. The protein size markers
795 used in SDS-PAGE are indicated on the left.
796
797 **FIG 3** WSMV with deletion of amino acids 36 to 100 in the CP systemically infects wheat. (A)
798 Genomic organization of WSMV with proteins encoded by the polyprotein. The N-terminal 125
799 amino acids are enlarged at the bottom of schematic representation of the genome. The positions
800 of deleted amino acids in the CP are indicated with solid boxes and respective mutants are
801 indicated at right. (B) Systemic symptoms induced by *in vitro* transcripts of WSMV CP deletion
802 mutants on wheat. The numbers on top of the leaves are mutants as indicated in (A). Symptoms
803 induced by mutants 1 and 2 and 3 to 5 were at 9 and 16 days postinoculation, respectively. H:
804 buffer inoculated healthy wheat leaves. (C) RT-PCR amplification of CP cistron from deletion
805 mutants infected wheat. The RT-PCR products were gel electrophoresed through 1.0% agarose
806 gel and the numbers on top of the gel correspond to mutants as indicated in (A). The sizes of
807 DNA ladder (lanes M) are indicated on the left. (D) Electron micrographs of virions of the wild-
808 type virus and deletion mutants from systemically infected wheat. Bars represent 500 nm. (E)
809 Western blot analysis of partially purified virions from wheat leaves infected with the wild-type
810 virus and CP deletion mutants. The numbers indicated on top of the immunoblot are as indicated
811 in (A). The location of CP is indicated with an arrow and the corresponding CP of deletion
812 mutants is marked with dotted lines. The position of CP and truncated CP of 31 and 29 kDa
813 proteins are indicated with an arrow, arrow head and asterisk, respectively. The protein size

814 markers used in SDS-PAGE are indicated on the left. The dilution of partially purified virions
815 used for the immunoblot is indicated at the bottom of the blot. UD, undiluted.
816
817 **FIG 4** The N- and C-proximal regions of WSMV CP are dispensable for systemic infection in
818 wheat. (A) The schematic diagram of WSMV genome organization and the location of deletions
819 (bold and underlined) in the N and C-terminal regions of CP are indicated below the genomic
820 organization. (B) Symptoms elicited by *in vitro* transcripts of the wild-type virus and N- and C-
821 proximal deletion mutants on wheat at 18 days postinoculation. H: buffer inoculated healthy
822 wheat leaves. (C) RT-PCR analysis of CP cistron from wheat plants infected with the wild-type
823 virus and mutants with N or C-terminal deletions in the CP. The sizes of DNA marker (lanes M)
824 are indicated at left. (D) Electron micrographs of the wild-type virus and N- or C-terminal
825 deletion mutants of WSMV in wheat. Bars represent 500 nm. (E) Western blot analysis of
826 partially purified virions from wheat leaves infected with the wild-type virus and N- or C-
827 terminal deletion mutants. The position of CP and truncated CP of 31 and 29 kDa are indicated
828 with an arrow, arrow head and asterisk, respectively. The protein size markers used in SDS-
829 PAGE are indicated on the left. The dilution of partially purified virions used for the immunoblot
830 is indicated at the bottom of the blot. UD, undiluted.
831
832 **FIG 5** The N- and C-proximal regions and amino acids 85 to 100 of WSMV CP are required for
833 efficient cell-to-cell movement in wheat. *In vitro* transcripts of GFP-tagged wild-type virus and
834 CP deletion mutants were inoculated to wheat seedlings at the single-leaf stage. The fluorescent
835 foci developed by the wild-type virus and CP deletion mutants at 4 and 14 days postinoculation
836 (dpi) are presented in the top (A1 to I1) and middle (A2 to I2) rows, respectively, and GFP

837 fluorescence in the upper noninoculated leaves at 12 dpi (21 dpi for ΔC17aa and ΔN6-27aa
838 mutant viruses) are presented in the bottom rows (A3 to I3). Bars represent 500 μm. Note that
839 WSMV with deletion of amino acids 36 to 84 in the CP elicited local and systemic infection foci
840 similar to wild-type virus; in contrast, deletions comprising 6 to 27, 85 to 100 or C-terminal 14
841 or 17 amino acids affected the efficiency of cell-to-cell movement in wheat.
842
843 **FIG 6** WSMV CP amino acids 6 to 27 and 85 to 100 are required for efficient virion assembly.
844 (A) Movement-independent virion assembly assay of the wild-type virus and CP deletion
845 mutants in agroinfiltrated *Nicotiana benthamiana* leaves at 7 days post agroinfiltration (dpa).
846 Western blot analysis of partially purified virions from agroinfiltrated *N. benthamiana* leaves
847 was performed with an anti-WSMV serum. Note that deletion of amino acids 36 to 84
848 accumulated virions approximately similar to the wild-type virus; in contrast, WSMV with
849 deletion of amino acids 85 to 100, 36 to 100 or 6 to 27 in the CP significantly affected virion
850 assembly. (B) Longer exposure of immunoblot lanes consisting of partially purified virions of
851 WSMV CP deletion mutants comprising amino acids 85 to 100, 36 to 100 or 6 to 27. The
852 positions of CP and truncated CP of 31 and 29 kDa proteins are indicated with arrows, arrow
853 heads and asterisks, respectively. The protein size markers used in SDS-PAGE are indicated.
854
855 **FIG 7** The C-terminal 65 amino acids of WSMV CP are dispensable for virion assembly but are
856 required for cell-to-cell movement. (A) Progressive deletions at the C-terminus of CP
857 increasingly debilitated cell-to-cell movement of WSMV in wheat. GFP fluorescent foci induced
858 by GFP-tagged WSMV mutants with progressive deletions at the C-terminus of CP on
859 inoculated wheat leaves at 14 days postinoculation are indicated with arrows. Bars represent 500

μm. (B) Movement-independent virion assembly assay of WSMV mutants with progressive deletions from the C-terminus of CP in agroinfiltrated *N. benthamiana* leaves at 7 days post-agroinfiltration (dpa). Immunoblot analysis of partially purified virions from agroinfiltrated *N. benthamiana* leaves at 7 dpa with an anti-WSMV serum. Note that WSMV with progressive deletions of up to 65 amino acids at the C-terminus of CP is capable of virion assembly. The protein size markers used in SDS-PAGE are indicated on the left. (C) Electron micrographs of virions (WT, ΔC17aa) and virus-like particles (non-infectious, ΔC40aa, ΔC49aa and ΔC55aa) in partially purified virions of GFP-tagged WSMV (WT, wild-type) and the C-terminal deletion mutants from agroinfiltrated *N. benthamiana* leaves at 7 dpa. Bars represent 200 nm.

Table 1. Infectivity of *in vitro* RNA transcripts generated from cloned DNA copies of *Wheat streak mosaic virus* coat protein deletion mutants on wheat at 21 days post inoculation (dpi).

Mutant	Experiment 1	Experiment 2
WSMV-S81 (wild-type)[a]	13/15 (87%)[g]	17/19 (89%)
WSMV-CPΔ36-84aa[a]	17/18 (94%)	18/19 (95%)
WSMV-CPΔ85-100aa[b]	15/17 (88%)	19/20 (95%)
WSMV-CPΔ48-100aa[c]	12/16 (75%)	25/26 (96%)
WSMV-CPΔ36-100aa[c]	10/15 (67%)	16/20 (80%)
WSMV-CPΔC14aa[d]	10/13 (77%)	16/19 (84%)
WSMV-CPΔC17aa[e]	4/16 (25%)	2/18 (11%)
WSMV-CPΔN6-27aa[e]	7/15 (47%)	10/18 (56%)
WSMV-GFP-$W_{165}A$[f]	0/16 (0%)	0/18 (0%)
WSMV-GFP-$R_{237}A$[f]	0/14 (0%)	0/17 (0%)
WSMV-GFP-$D_{282}A$[f]	0/17 (0%)	0/16 (0%)
WSMV-GFP-ΔGDD[f]	0/13 (0%)	0/17 (0%)
WSMV-GFP-ΔCP[f]	0/16 (0%)	0/14 (0%)
Mock	0/15 (0%)	0/17 (0%)

[a-e]First visible symptoms developed at 6 to 9[a], 9 to 12[b], 11 to 15[c], 14 to 18[d], and 15 to 21[e] dpi; [f]failed to infect wheat systemically at 25 dpi; [g]number of plants infected/number of plants inoculated and the percentage of plants infected in parenthesis.

Table 2. Infection foci sizes on wheat seedlings produced by *in vitro* RNA transcripts generated from cloned cDNA copies of GFP-tagged *Wheat streak mosaic virus* coat protein deletion mutants[a].

Mutant	Foci sizes (mm^2)
WSMV-S81-GFP-6K1/CI(7aa) (wild-type)	2.31±0.44
WSMV-GFP-CPΔ36-84aa	2.68±0.38
WSMV-GFP-CPΔ85-100aa	1.73±0.36
WSMV-GFP-CPΔ48-100aa	1.45±0.37
WSMV-GFP-CPΔ36-100aa	1.36±0.30
WSMV-GFP-CPΔC14aa	0.64±0.17
WSMV-GFP-CPΔC17aa	0.40±0.21
WSMV-GFP-CPΔN6-27aa	0.53±0.19
Mock	0

[a]Wheat seedlings were incubated in a greenhouse at 20 to 27ºC. Foci sizes estimated from 20 to 30 individual foci at 4 days postinoculation.

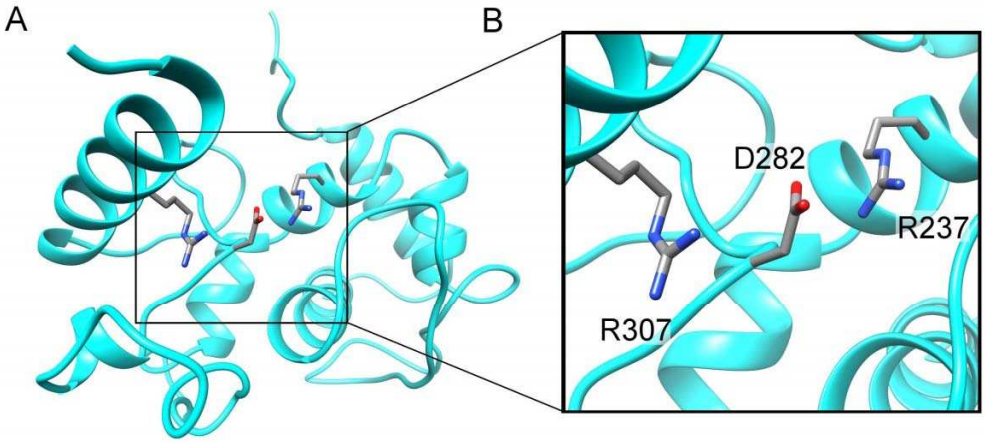


Figure 1

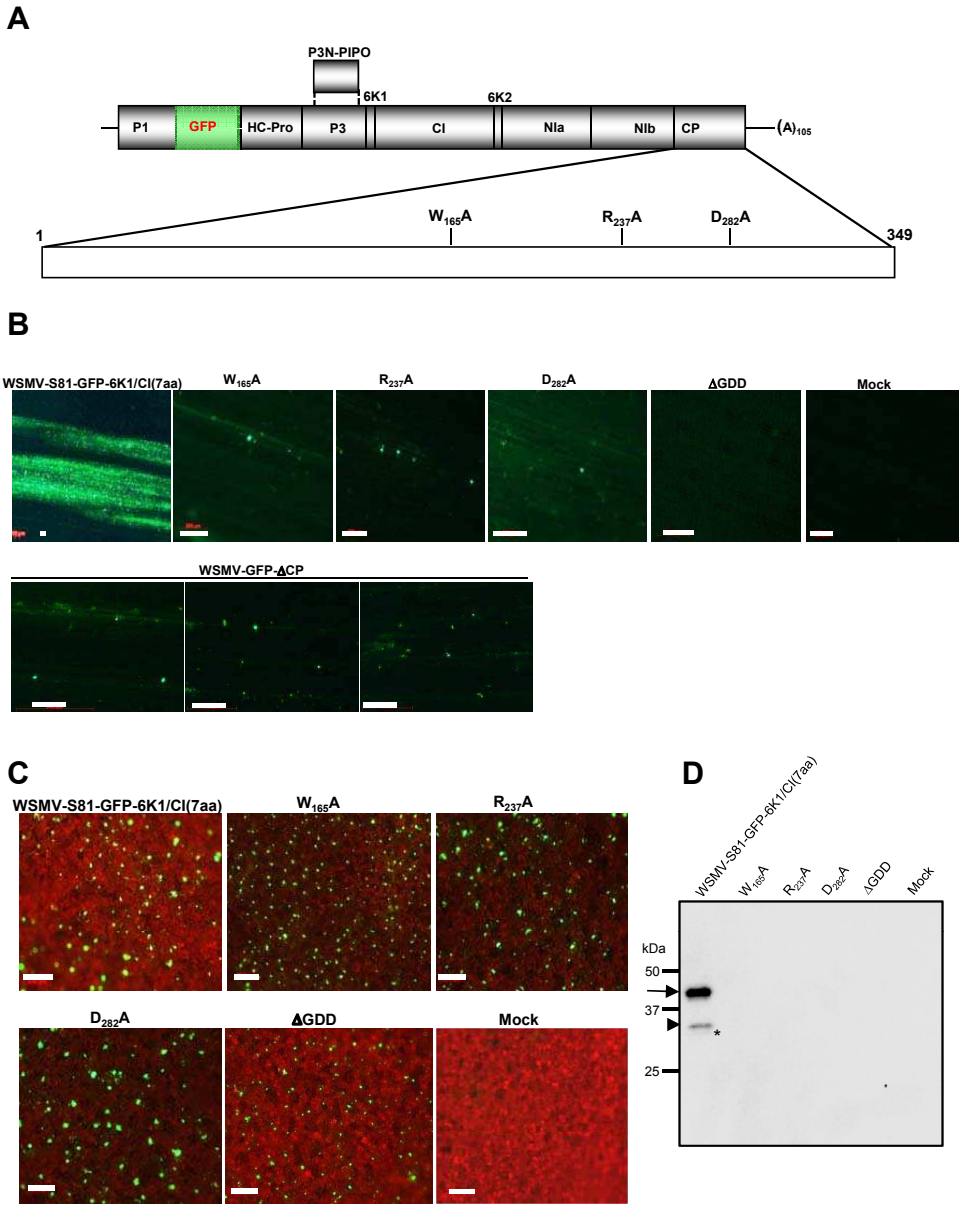
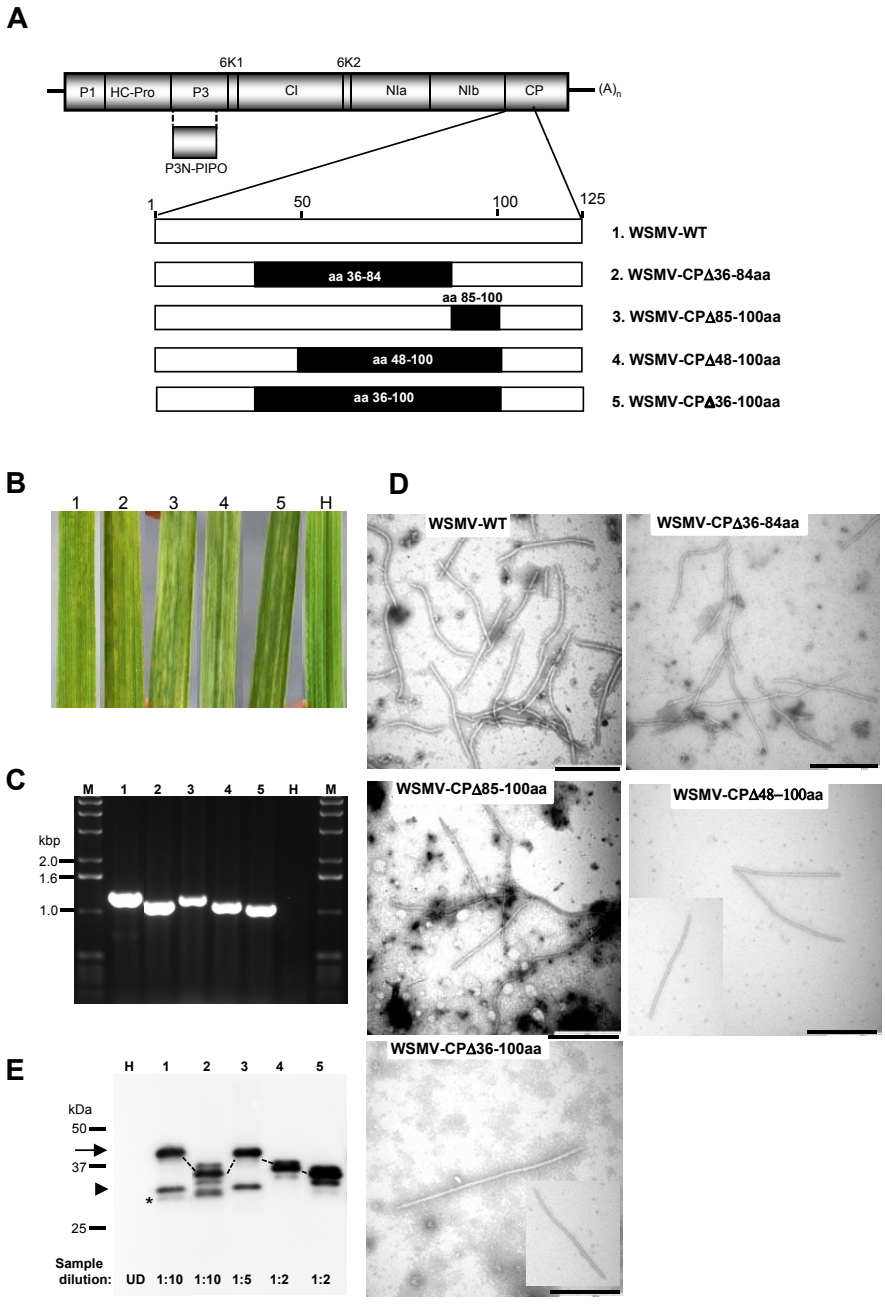


Figure 2



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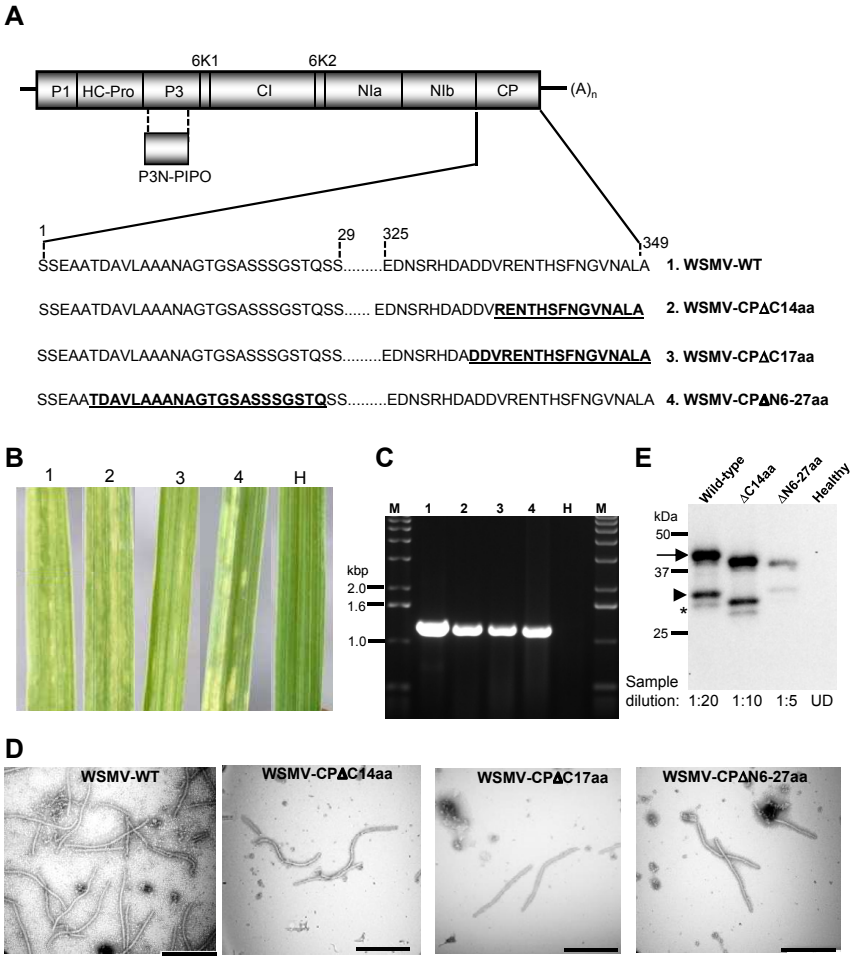

Figure 4

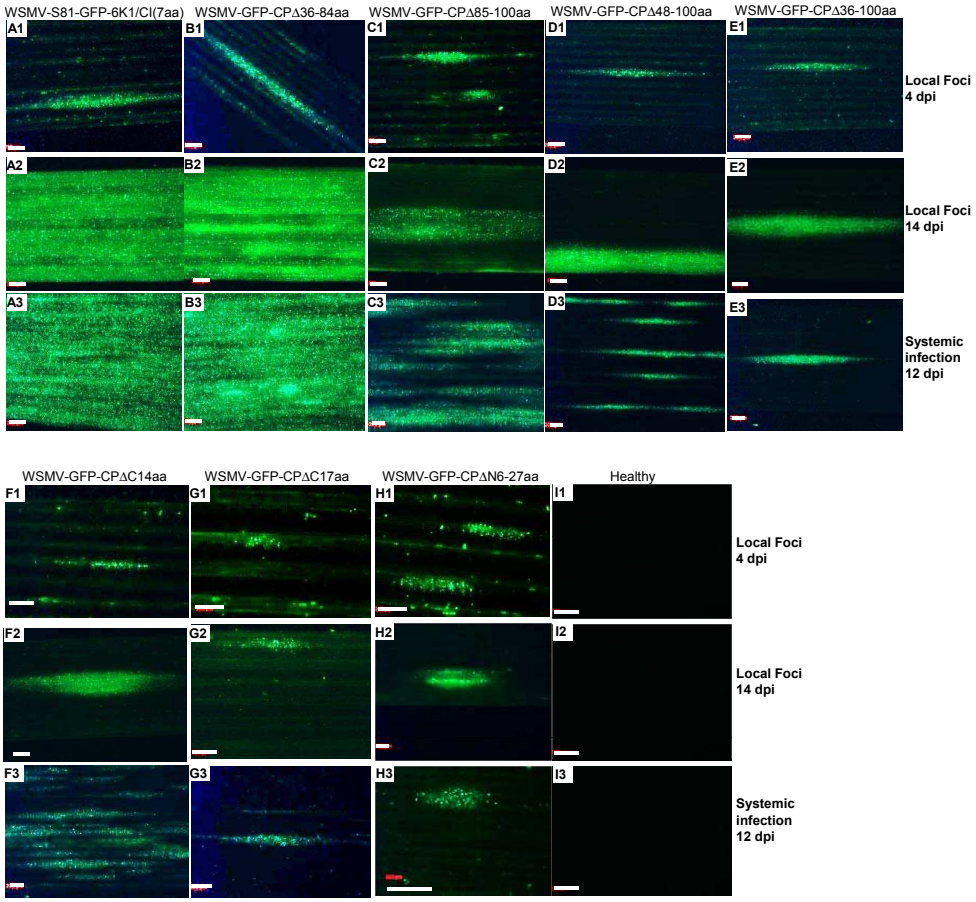

Figure 5

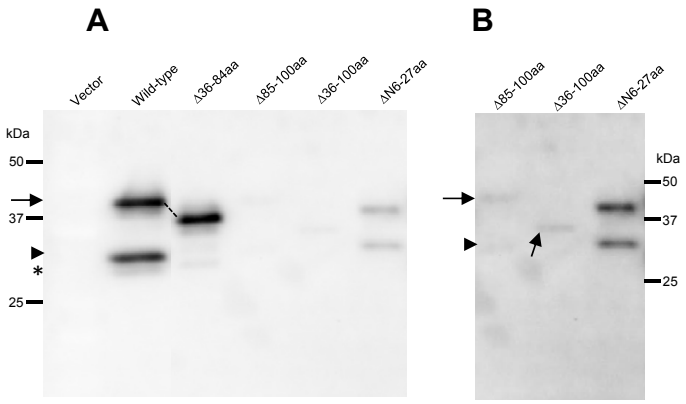

Figure 6

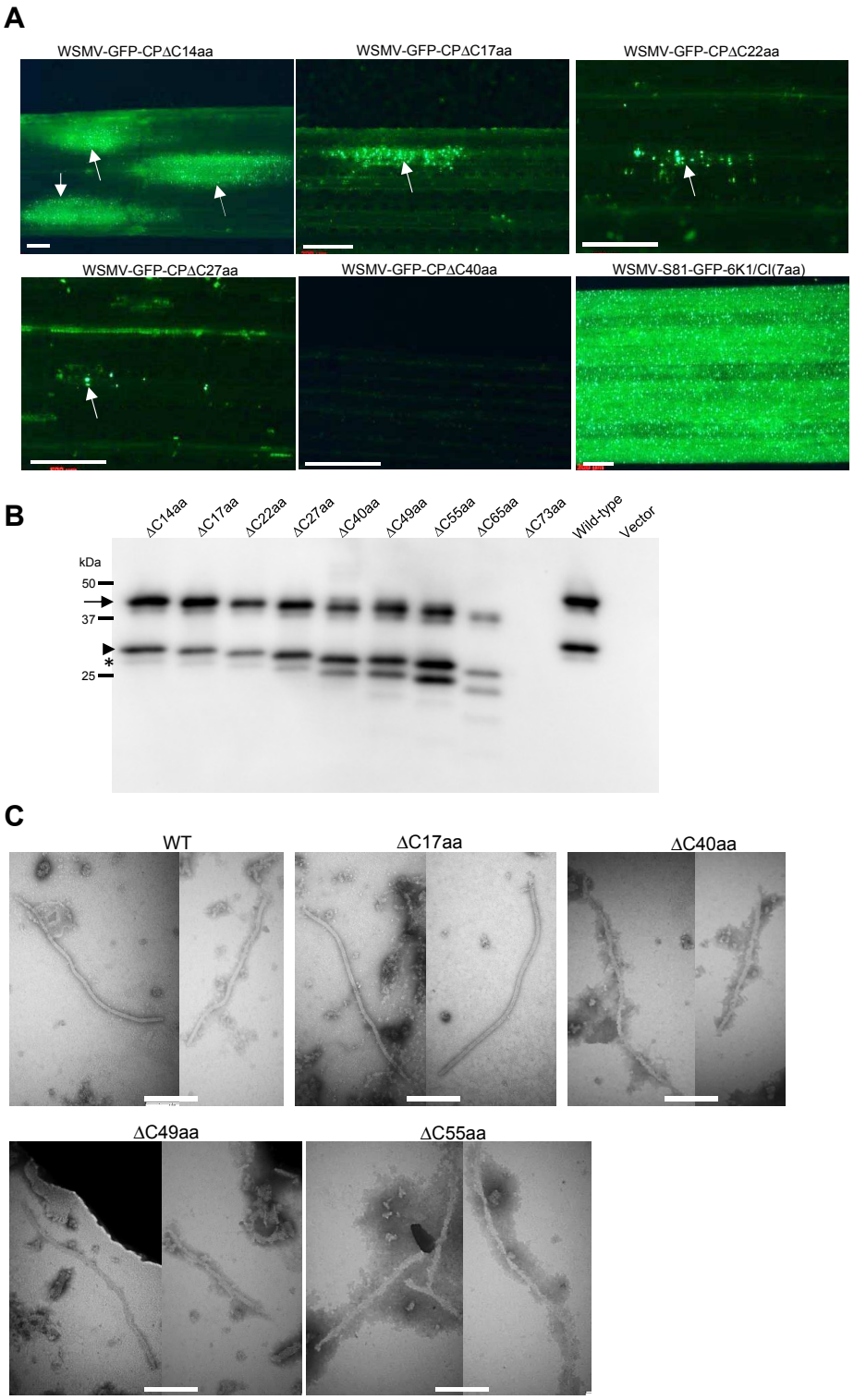

Figure 7