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Rapid Communication

The charged residues in the surface-exposed C-terminus of the *Soybean mosaic virus* coat protein are critical for cell-to-cell movement

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ABSTRACT

The *Soybean mosaic virus* (SMV) coat protein (CP) is necessary for virion assembly and viral cell-to-cell and long-distance movements in plants. We previously showed that the C-terminal region of the SMV CP is required for CP self-interaction. In the present study, we generated SMV mutants containing CPs with single amino acid substitutions of the charged amino acids in the C-proximal region. Infectivity and cell-to-cell movement of the SMV mutants were examined in soybean plants. Through this genetic approach, we identified three charged amino acid residues (R245, H246, and D250) in the surface-exposed C-terminus of the SMV CP that are critical for virus cell-to-cell and long-distance movement. Our findings suggest that the identified charged amino acids in the surface-exposed C-terminus of SMV CP are critical for CP intersubunit interactions and thereby for cell-to-cell and long-distance movement and virion assembly.

Introduction

Soybean mosaic virus (SMV), which is a member of the genus *Potyvirus* in the family *Potyviridae*, has a single-stranded positive-sense RNA genome of approximately 9.6 kb. The major hosts of SMV are *Glycine max* and *Glycine soja*. Since SMV was first classified into seven strains (G1–G7) based on phenotypic reactions on different soybean cultivars (Cho and Goodman, 1979), emergence of variant SMV strains has been continuously reported (Cho et al., 1983; Choi et al., 2005; Hajimorad et al., 2003; Kim and Lee, 1991; Kim et al., 2003; Lim, 1985). Like the genomes of other members in the genus *Potyvirus*, the SMV genome encodes one large polyprotein, which is cleaved to yield at least 10 mature proteins, including P1, helper component-proteinase (HC-Pro), P3, 6K1, cylindrical inclusion (CI), 6K2, genome-linked viral protein (VPg), nuclear inclusion a proteinase (NIa-Pro), nuclear inclusion b (NIb), and coat protein (CP) (Riechmann et al., 1992). A small open reading frame embedded in the P3 cistron of potyviruses was recently discovered and named *pipo* (Pretty interesting *Potyviridae* ORF) (Chung et al., 2008). The PIPO protein is also encoded by SMV and is essential for SMV movement (Wen and Hajimorad, 2010).

Virus movement in plants is an active process mediated by virus-encoded proteins such as the movement proteins (MPs) and CPs. Viral MPs generally facilitate cell-to-cell movement of infectious entities by increasing the size-exclusion limits of plasmodesmata (Lucas, 2006; Ueki and Citovsky, 2011; Verchot-Lubicz et al., 2010). In potyvirus movement, no dedicated MP, like those for other viruses, has been identified. However, some potyviral proteins including HC-Pro, CI, VPg, CP, and P3N-PIPO have been demonstrated to be involved in viral cell-to-cell movement (Carrington et al., 1998; Cronin et al., 1995; Dolja et al., 1994, 1995; Dunoyer et al., 2004; Hofius et al., 2007; Rojas et al., 1997; Shen et al., 2010; Vijayapalani et al., 2012; Wei et al., 2010). The earlier studies demonstrated that CP is necessary for both cell-to-cell and long-distance movement of *Tobacco etch potyvirus* (TEV) (Dolja et al., 1994, 1995). The potyvirus CP has variable N- and C-terminal domains exposed on the virion surface and a conserved central core domain (Anindya and Savithri, 2003; Shukla and Ward, 1989). Mutational analyses of TEV CP have revealed that the central core domain is critical for virus cell-to-cell movement and that the variable N- and C-terminal domains function during long-distance movement (Dolja et al., 1994, 1995).

We previously found a strong interaction between subunits of SMV CP based on a yeast two-hybrid system (YTHS) and demonstrated that the C-terminal domain of SMV CP is required for the CP intersubunit interactions (Kang et al., 2004, 2006). In the current study, we further verified the *in planta* significance of

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the C-terminal domain of SMV CP in virus cell-to-cell and long-distance movements by generating SMV mutants containing CPs with single amino acid substitution of the charged amino acids in the C-terminal domain. Based on this approach, we demonstrate that some charged amino acid residues in the C-terminal domain of CP are critical not only for the intersubunit interactions (Kang et al., 2006) but also for virus movement (this study). Our findings also support the concept that potyviruses move as virions.

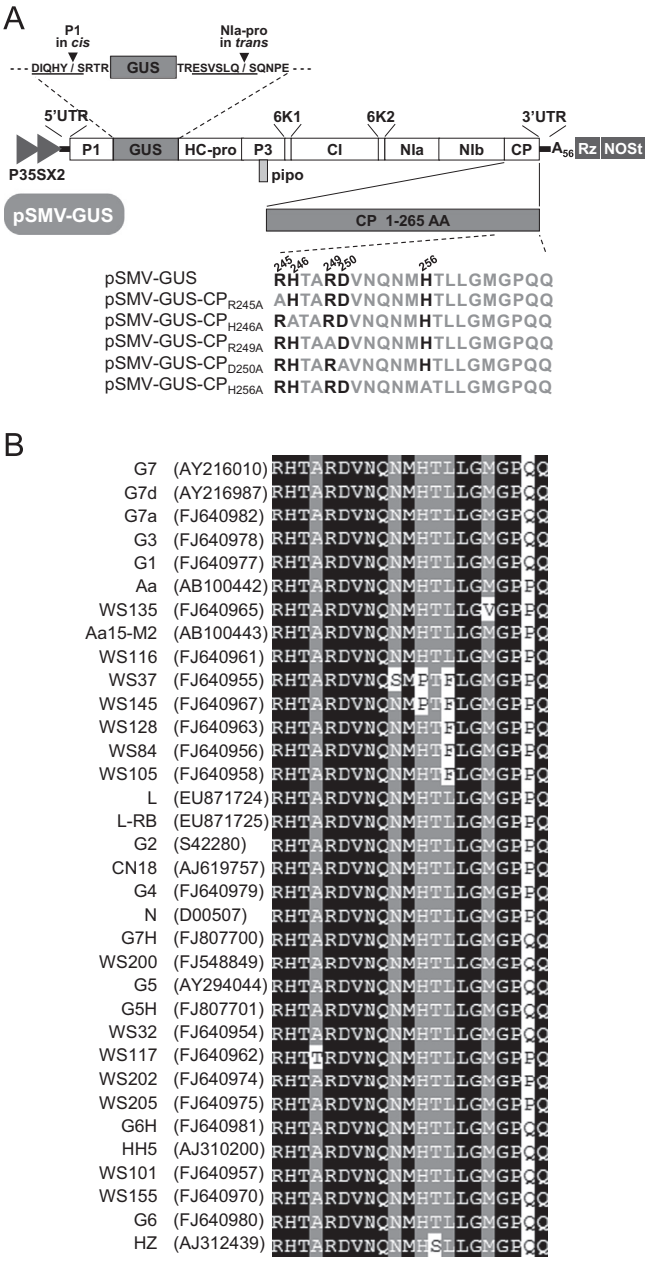

Fig. 1. Schematic representation of the construction of pSMV-GUS and the CP cistron showing the charged amino acid positions at the C-terminus. (A) SMV genome organization and insertion of the *gus* gene between P1 and HC-Pro. Amino acid sequences of the peptide cleavage sites recognized by either the P1 or Nla-Pro are underlined, and arrowheads indicate the location of the cleaved peptide bond. In vivo transcription of SMV-GUS is under control of a double 35S promoter (P35SX2), a cis-cleaving ribozyme sequence (Rz), and a NOS terminator (NOSt). The amino acid positions of alanine substitution mutations are shown in the schematic representation of SMV CP. (B) Alignment of the C-terminal amino acid sequences of CPs of various SMV strains and isolates. The names of SMV strains and isolates are indicated on the left side of the sequences together with their GenBank accession numbers. Sequences were aligned by ClustalX2.

Results and discussion

Introduction of single-substitution mutations into the C-terminal domain of CP of pSMV-GUS

We previously developed an SMV-based viral vector (pSMV-MCS) to express foreign genes in soybean by inserting the cloning sites into the polyprotein open reading frame (ORF) between the P1 and HC-Pro cistrons (Seo et al., 2009a). In the current study, the bacterial β-glucuronidase (GUS) gene was inserted into pSMV-MCS to visualize virus infection and movement in plants, and the resulting construct was designated as pSMV-GUS (Fig. 1A).

The amino acid sequences of the CP cistron are highly conserved among various SMV strains and isolates (Seo et al., 2009b). In particular, comparison of the amino acid sequence revealed that all of the charged amino acids (except H256) in the C-terminus of SMV CP are conserved among 34 representative SMV strains and isolates (Fig. 1B). Thus, to examine the significance of the C-terminal domain of SMV CP in virus cell-to-cell and long-distance movement, we introduced alanine substitutions into the charged amino acids in the C-terminal domain of the CP cistron of pSMV-GUS by site-directed mutagenesis. The resulting constructs containing the single-substitution mutations in the CP cistron were named pSMV-GUS–CP_{R245A}, –CP_{H246A}, –CP_{R249A}, –CP_{D250A}, and –CP_{H256A} (Fig. 1A).

Effect of the mutations in the C-terminus of SMV CP on virus infectivity

We first examined the infectivity of SMV-GUS and the derivative CP mutants. Plasmid DNAs of pSMV-GUS and the CP mutants were rub-inoculated onto the primary leaves of soybean seedlings (cv. Lee68). The results of the infectivity tests are summarized in Table 1. Typical systemic mild mosaic symptoms appeared in the upper uninoculated leaves of all the soybean plants inoculated with pSMV-GUS, pSMV-GUS–CP_{R249A}, or –CP_{H256A} at 9–14 days post inoculation (dpi). However, no symptoms were observed in the soybean plants inoculated with pSMV-GUS–CP_{R245A}, –CP_{H246A}, or –CP_{D250A} even at 45 dpi. To verify whether the inoculated soybeans were systemically infected with each SMV mutant, we extracted total RNAs from upper uninoculated leaves at 45 dpi and subjected the extracts to RT-PCR using SMV-specific primers. The RT-PCR results confirmed that none of the soybean plants inoculated with pSMV-GUS–CP_{R245A}, –CP_{H246A}, or –CP_{D250A} was infected with SMV while all soybean plants showing symptoms were systemically infected (data not shown). We also verified that the introduced mutations were maintained during systemic infection by analyzing (by RT-PCR) sequences of progeny viruses of pSMV-GUS–CP_{R249A} and –CP_{H256A}, which were recovered from upper uninoculated leaves (data not shown). Because CP modifications (i.e. deletions of the N- or C-terminal region and several substitution mutations in the central core domain) have no significant effect on potyvirus genome amplification (Dolja et al., 1994, 1995), we suspected that the residues R245, H246, and D250 of SMV CP may be critical for virus movement or assembly.

Effect of the mutations in the C-terminus of SMV CP on virus cell-to-cell movement

Because the loss of systemic infectivity of the CP mutants could be due to defects in virus movement, we next examined the cell-to-cell movement phenotypes of SMV-GUS and the CP mutants. To this end, the soybean leaves inoculated with pSMV-GUS or the CP mutants were subjected to a histochemical GUS assay at 3, 5 and 8 dpi. This assay allows quantitative measurement of the extent of virus cell-to-cell movement because only infected cells

express GUS activity. Results are summarized in Fig. 2 and Table 2. No infection foci were observed at 3 dpi in all leaves mechanically inoculated with the plasmid DNAs of pSMV-GUS and its derivative mutants. However, SMV-GUS spread radially to form infection foci with average diameters of approximately 573.2 ± 64.9 and 1463.4 ± 317.2 μm at 5 and 8 dpi, respectively. The mutants SMV-GUS–CP_{R249A} and –CP_{H256A}, which were previously determined to be capable of systemic infection, formed infection foci of similar sizes to those formed by SMV-GUS at 5 and 8 dpi (Fig. 2 and Table 2). In contrast, movement of the mutants SMV-GUS–CP_{R245A}, –CP_{H246A}, and –CP_{D250A}, which were previously determined to be incapable of systemic infection, was restricted to several epidermal cells surrounding the initially infected cells, and the foci were about 14-fold smaller than those produced by SMV-GUS at 5 dpi (Fig. 2 and Table 2). Furthermore, sizes of infection foci were marginally increased at 8 dpi when compared to those at 5 dpi in leaves inoculated with pSMV-GUS–CP_{R245A}, –CP_{H246A}, or –CP_{D250A}. These results demonstrated that the charged amino acids R245, H246, and D250 are critical for the functioning of SMV CP in cell-to-cell movement.

Table 1
Effect of SMV CP mutations on systemic infection of soybean plants.

Inoculum[a]	Infectivity[b]			
	1	2	3	Total
Mock	0/3	0/3	0/3	0/9
pSMV-GUS	3/3	3/3	3/3	9/9
pSMV-GUS–CP_{R245A}	0/5	0/5	0/5	0/15
pSMV-GUS–CP_{H246A}	0/5	0/5	0/5	0/15
pSMV-GUS–CP_{R249A}	5/5	5/5	5/5	15/15
pSMV-GUS–CP_{D250A}	0/5	0/5	0/5	0/15
pSMV-GUS–CP_{H256A}	5/5	5/5	5/5	15/15

[a] Soybean cultivar Lee68 (*rsv*) plants were inoculated with the corresponding plasmids as described in Materials and methods section.
[b] Number of systemically infected plants/number of plants inoculated. Virus infection of the upper non inoculated leaves was confirmed by RT-PCR using SMV specific primers at 45 dpi.

Table 2
Diameters of infection foci on soybean leaves inoculated with SMV-GUS and CP mutants.

Inoculum[a]	Infection foci diameter (μm)[b]		
	3 dpi	5 dpi	8 dpi
Mock	ND	ND	ND
pSMV-GUS	ND	573.2 ± 64.9	1463.4 ± 317.2
pSMV-GUS–CP_{R245A}	ND	36.5 ± 6.1	54.8 ± 11.3
pSMV-GUS–CP_{H246A}	ND	38.5 ± 6.1	62.1 ± 8.7
pSMV-GUS–CP_{R249A}	ND	596.7 ± 91.7	1341.5 ± 411.3
pSMV-GUS–CP_{D250A}	ND	41.8 ± 8.6	82.4 ± 21.6
pSMV-GUS–CP_{H256A}	ND	548.1 ± 95.4	1419.8 ± 271.6

ND = not detected.
[a] Soybean cultivar Lee68 (*rsv*) plants were inoculated with the corresponding plasmids as described in Materials and methods section.
[b] The diameters of foci were determined by GUS histochemical assays using light microscopy. Each value is the mean ± standard deviation of > 10 foci.

Effect of the mutations in the C-terminus of SMV CP on virion assembly

Because the C-terminal domain of SMV CP is required for interactions between CP subunits (Kang et al., 2006), we examined whether the charged amino acids in the C-terminus of SMV CP are

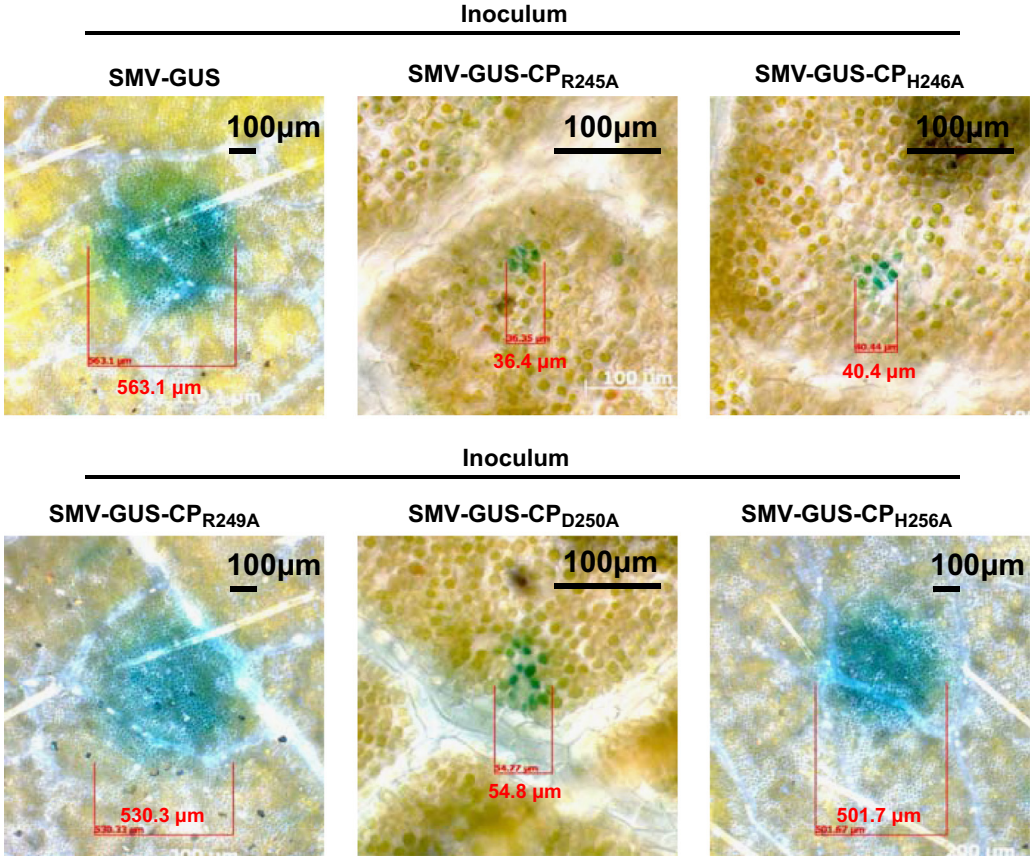

Fig. 2. Histochemical detection of GUS activity in soybean leaves inoculated with pSMV-GUS and its CP mutants. The inocula used to infect soybean leaves are indicated above each image. The GUS foci were photographed at 5 dpi.

required for virion assembly. Soybean leaves were inoculated with pSMV-GUS or the CP mutants, and leaf lysates were prepared from the inoculated leaves at 5 dpi and subjected to serologically specific electron microscopy (SSEM) in order to determine the presence of virions. Virions were easily detected in the leaf lysates inoculated with pSMV-GUS, $-CP_{R249A}$, or $-CP_{H256A}$ (Table 3). Despite extensive observations, however, no virions were detected in the leaf lysates inoculated with the movement-defective mutants SMV-GUS–CP_{R245A}, $-CP_{H246A}$, or $-CP_{D250A}$ (Table 3). This indicates that the charged amino acids R245, H246, and D250 in the C-terminus of SMV CP are required for successful virion assembly. In our previous study, based on a yeast two-hybrid system, we found that alanine substitution mutations at the charged amino acids R245, H246, and R249 of SMV CP disrupted the CP intersubunit interactions (Kang et al., 2006). In this regard, it is likely that the charged amino acids in the C-terminus of SMV CP mediate the intersubunit interactions that are critical for virion assembly. However, we cannot exclude the possibility that the lack of virions in leaf samples inoculated with the movement-defective CP mutants might be due to that the concentration of virions was below the level of detection by the method that we used. Future technical advances in analysis of SMV virion assembly at single-cell level may demonstrate clearly the significance of the C-terminal charged amino acids of SMV CP in virion assembly.

Structural prediction of SMV CP and functional significance of its C-terminal domain in virus assembly and movement

To better understand the involvement of the C-terminal domain of SMV CP in virus movement and virion assembly, we performed protein structure homology-modeling of SMV CP using the Phyre server (Kelley and Sternberg, 2009). The analysis predicted that the central region of SMV CP shares the highest structural similarity with the *Papaya mosaic virus* (PapMV) CP with 96.8% confidence (Fig. 3A). The predictive modeling showed that the central region of SMV CP contains four helices and a two-stranded antiparallel β sheet (Fig. 3B and C). Unfortunately, the protein structure homology-modeling by the Phyre server was unable to predict a three-dimensional structure of the C-terminal domain of SMV CP. Thus, we analyzed the secondary structure of SMV CP predicted by the Phyre server. Interestingly, the C-terminal region of SMV CP was predicted to contain a short α-helix near the charged amino acid residues (Fig. 3C). Thus, we examined whether the alanine substitutions of the charged amino acids in the C-terminus alter the α-helix secondary structure of the SMV CP C-terminus. The alanine substitutions at R245, H246, and D250 disrupted the original α-helix structure while the alanine substitution at R249 had no effect on the predicted secondary structure of SMV CP (Fig. 3D). On the other hand, the alanine substitution at H256 resulted in the formation of a β strand at the C-proximal region of SMV CP (Fig. 3D). Therefore, it is likely that the structural conformation of the C-terminal domain of SMV CP is important for virus movement as well as assembly because the SMV CP mutants with mutations in R245A, H246A, and D250A but not in R249A and H256A were defective in virus movement and assembly.

Table 3
Detection of virions in the leaves inoculated with SMV-GUS and CP mutants.

Inoculum[a]	Number of virions/microscope field[b]
pSMV-GUS	138.7 ± 77.5
pSMV-GUS–CP_{R245A}	ND
pSMV-GUS–CP_{H246A}	ND
pSMV-GUS–CP_{R249A}	98.7 ± 56.7
pSMV-GUS–CP_{D250A}	ND
pSMV-GUS–CP_{H256A}	128.2 ± 53.6

ND = not detected.

[a] Soybean cultivar Lee68 (*rsv*) plants were inoculated with the corresponding plasmids as described in Materials and methods section.

[b] Values are means ± standard deviation of number of virions observed per microscope field (40.96 μm^2/field); six fields were examined for each kind of inoculum.

The N- and C-terminal regions of potyviral CPs, which are predicted to be surface-exposed in virions, can be removed by limited trypsin treatment without disturbing virion assembly status (Jagadish et al., 1993). This suggested that the surface-exposed N- and C-terminal residues are dispensable for potyvirus virion assembly. However, Anindya and Savithri (2003) showed that the CP mutants lacking either N-terminal 53 or C-terminal 23 residues failed to form either virions or 16S ring-like assembly intermediates, suggesting that the surface-exposed N- and C-terminal residues are crucial for the CP intersubunit interactions that promote the initiation of virion assembly. Despite low amino acid sequence similarity among potyvirus CPs, the surface-exposed N- and C-terminal regions of potyviral CPs contain a relatively high ratio of charged amino acid residues. These charged residues in the surface-exposed regions are highly solvent accessible and can mediate intermolecular electrostatic interactions to promote the association of individual CP subunits. Indeed, Anindya and Savithri (2003) suggested that the charged amino acids in the N- and C-terminal regions of potyviral CPs might mediate head-to-tail interactions between CP subunits so that the subunits form ring-like intermediates and thereby virions. Consistent with Anindya and Savithri (2003), we previously reported that deletion of the C-terminal region of SMV CP resulted in loss of the CP intersubunit interaction and that the charged amino acids (i.e. R245, H246, and R249) in the C-terminal region of the CP are required for this interaction (Kang et al., 2006). In the current study, we found that the charged residues R245, H246, and D250 in the C-terminus of SMV CP are critical for virus cell-to-cell movement, further demonstrating the significance of the CP intersubunit interactions in virus movement.

In the prediction of protein structure, the alanine substitutions at R245, H246, and D250 appeared to modify the original C-terminal structure of SMV CP (Fig. 3C and D). These structural modifications might reduce the strength of intermolecular electrostatic interactions mediated by the C-terminal region of SMV CP, resulting in disruption of the CP intersubunit interactions. It is also possible that each charged residue itself is directly involved in the intermolecular electrostatic interactions and that mutation of a single charged reside is sufficient to abolish the CP subunit interactions. However, it is likely that the CP intersubunit interactions mediated by the C-terminal charged residues are only transiently required for promoting virion assembly because removal of the surface-exposed C-terminal region by limited trypsin treatment does not destroy virion assembly status (Anindya and Savithri, 2003; Jagadish et al., 1993). Unfortunately, a crystal structure of potyviral CPs has not been characterized yet. The functional and conformational significance of the C-terminal charged residues of SMV CP in the CP intersubunit interaction and virion assembly will become clearer once the three-dimensional structure of SMV CP is determined.

Researchers have recently suggested that potyvirus virions form movement complexes with CI proteins and that the virion-CI complex is transported to plasmodesmata for cell-to-cell movement by binding to P3N-PIPO (Vijayapalani et al., 2012; Wei et al., 2010). Research has also demonstrated that some host cellular proteins such as PcaP1 are involved in potyvirus cell-to-cell movement (Vijayapalani et al., 2012). Thus, the surface-exposed N- and/or C-terminal regions of potyvirus CP may interact with CI proteins to form the movement complex. Additional studies that examine this possibility will be required to increase our understanding of the significance of the surface-exposed regions of potyvirus CP in virus cell-to-cell movement.

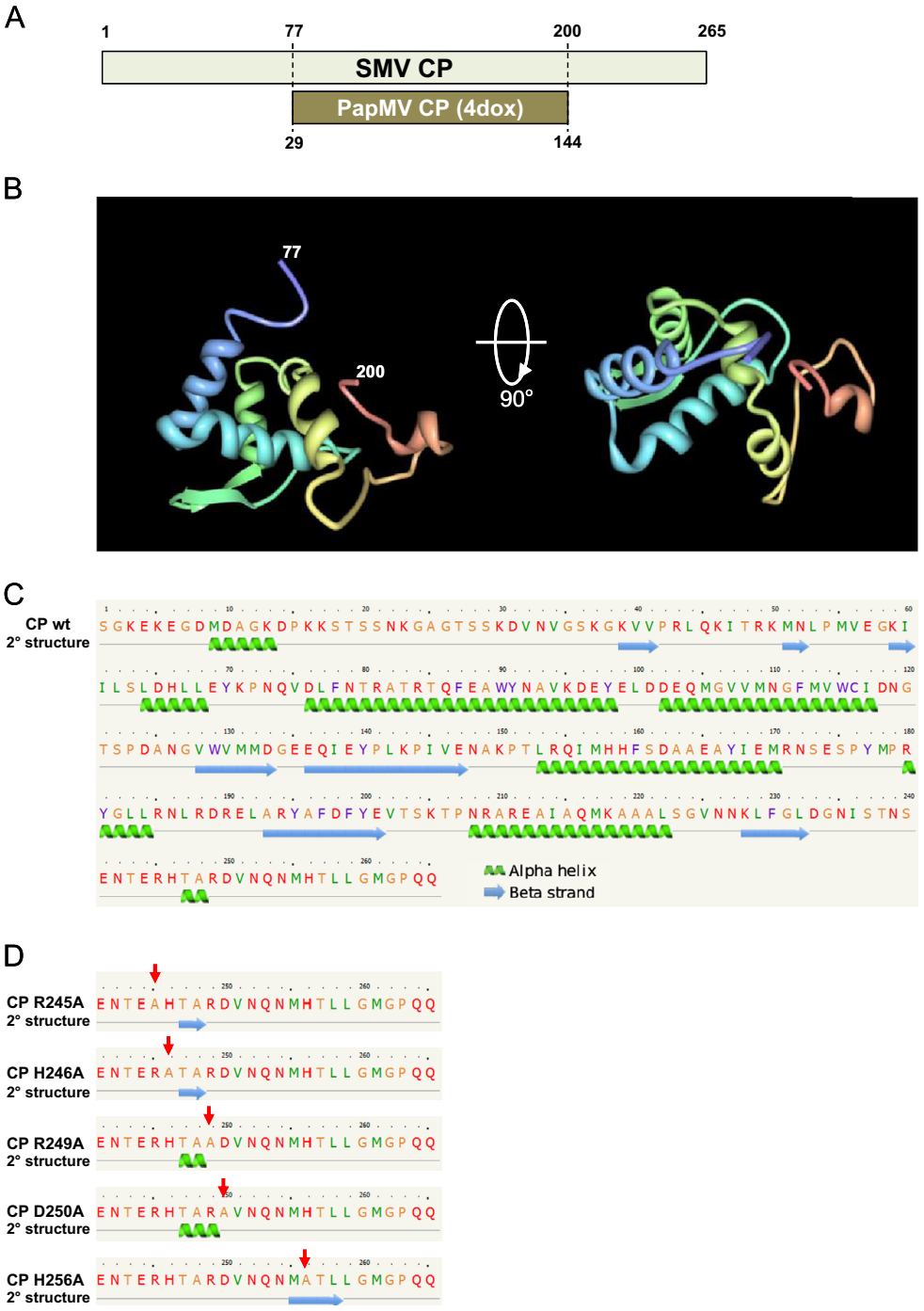

Fig. 3. Structural prediction of SMV CP. (A) Alignment coverage between SMV CP and PapMV CP. The central region (124 residues from the position 77 to 200) of SMV CP was aligned to the central region (116 residues from the position 29 to 144) of PapMV CP (4DOX) with 96.8% confidence. The homology alignment was performed using the Phyre server. (B) Three-dimensional structure model of the central region of SMV CP. The three-dimensional structure of the central region of SMV CP was determined by homology-modeling using the Phyre server. The three-dimensional images were generated using the Protein Workshop. (C) Secondary structure of wild-type (wt) SMV CP. The secondary structure elements predicted using the Phyre server are shown under the amino acid sequence of wt SMV CP. (D) Secondary structures of the C-terminal regions of the SMV CP mutants. The introduced substitution mutations are indicated with arrows.

Materials and methods

Insertion of the gus gene into the SMV viral genome

The coding region of the *gus* gene was amplified by PCR using a primer pair harboring *Xba*I sites (5′-GCTCTAGAATGTTACGTCCTG-TAGAAACCC-3′ and 5′-GCTCTAGATTGTTTGCCTCCCTGCTG-3′). The amplified fragments were digested with *Xba*I and cloned into pSMV-MCS (Seo et al., 2009a), which was opened with *Xba*I. The resulting construct with the GUS insert in the correct orientation was named pSMV-GUS.

Construction of SMV mutants

Individual mutations R245A, H246A, R249A, D250A, and H256A in the C-terminus of CP were introduced into pSMV-GUS by site-directed mutagenesis using appropriate primers (the list of primers used for the site-directed mutagenesis is available on

request) (Nassal and Rieger, 1990; Seo et al., 2009a). The resulting constructs were named pSMV-CP_{R245A}, $-CP_{H246A}$, $-CP_{R249A}$, $-CP_{D250A}$, and $-CP_{H256A}$, respectively.

Plant growth and inoculation

Soybean plants were grown in a growth chamber at 25 °C under a 16/8-h photoperiod. Seedlings were selected for inoculation when the cotyledons were fully expanded. Plasmid DNAs of pSMV-GUS and its derivative mutants were prepared using the Plasmid Maxi Kit (QIAGEN, Valencia, CA). Each cDNA plasmid was rub-inoculated as described previously (Seo et al., 2009a). To detect virus accumulation in the inoculated and upper uninoculated leaves, RT-PCR was performed using an SMV-specific primer pair designed to amplify the CP region (5′- TCAGGTAAGGAGAAGGAAGGA-3′ and 5′–CTGCTGTGGACCCATGCC-3′) and the resulting PCR products were sequenced to verify that the introduced mutations were maintained.

Histochemical GUS assays

GUS expression driven by virus infection was monitored by histochemical GUS assays as described previously (Dolja et al., 1992). Briefly, the inoculated soybean leaves were vacuum-infiltrated with the colorimetric GUS substrate 5-bromo-4-chloro-3-indoyl β-D-glucuronic acid, cyclohexylammonium salt (X-gluc) (1.2 mM) in 0.5 mM potassium ferricyanide/0.5 mM potassium ferrocyanide/10 mM EDTA. After overnight incubation at room temperature, the leaves were bleached in 70% ethanol and examined with a microscope to assess the diameters of GUS foci.

Serologically specific electron microscopy (SSEM)

To obtain crude virion preparations, the inoculated soybean leaves were ground in five volumes of a grinding buffer (10 mM Tris–HCl, 1 mM EDTA [pH 7.6]). Tissue debris was removed by centrifugation at 17,000 × *g* for 10 min. Anti-SMV serum-coated grids were prepared as described previously with minor modifications (Dolja et al., 1995). Formvar/carbon-coated EM grids were incubated with anti-SMV serum (1:500 dilution; Agdia, USA) for 1 h. The grids were rinsed with a washing buffer (50 mM Tris–HCl, 150 mM NaCl [pH 7.2]) and then incubated with the crude virion preparation for 2 h. The grids were rinsed with the washing buffer and incubated with 2% uranyl acetate for 5 min. The grid preparations were examined with a transmission electron microscope operated at 80 kV.

Structural prediction of SMV CP

Protein structure homology-modeling of SMV CP was performed using the Phyre server (Kelley and Sternberg, 2009) and was based on the crystal structure of PapMV CP (4DOX) (Yang et al., 2012), the closest related protein with available crystal structure. Prediction of secondary structures of SMV CP and mutants was also performed using the Phyre server. The three-dimensional images of the central region of SMV CP were generated using the Protein Workshop (Moreland et al., 2005).

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