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Cytoplasmic inclusion cistron of *Soybean mosaic virus* serves as a virulence determinant on *Rsv3*-genotype soybean and a symptom determinant

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ABSTRACT

Soybean mosaic virus (SMV; *Potyvirus*, *Potyviridae*) is one of the most widespread viruses of soybean globally. Three dominant resistance genes (*Rsv1*, *Rsv3* and *Rsv4*) differentially confer resistance against SMV. *Rsv1* confers extreme resistance and the resistance mechanism of *Rsv4* is associated with late susceptibility. Here, we show that *Rsv3* restricts the accumulation of SMV strain G7 to the inoculated leaves, whereas, SMV-N, an isolate of SMV strain G2, establishes systemic infection. This observation suggests that the resistance mechanism of *Rsv3* differs phenotypically from those of *Rsv1* and *Rsv4*. To identify virulence determinant(s) of SMV on an *Rsv3*-genotype soybean, chimeras were constructed by exchanging fragments between avirulent SMV-G7 and the virulent SMV-N. Analyses of the chimeras showed that both the N- and C-terminal regions of the cytoplasmic inclusion (CI) cistron are required for *Rsv3*-mediated resistance. Interestingly, the N-terminal region of CI is also involved in severe symptom induction in soybean.

Introduction

Soybean mosaic virus (SMV) is a member of the genus *Potyvirus* in the family *Potyviridae* (Shukla et al., 1994). The family *Potyviridae* comprises the largest number of known plant viruses many of which cause diseases in important plants (Shukla et al., 1994). SMV, the causal agent of soybean mosaic disease, is one of the most widespread viruses in soybean (*Glycine max* [L.] Merr.) globally (Hill, 1999). SMV infection in soybean results in significant yield loss as well as reduced seed quality. SMV isolates have been grouped into seven strains (G1–G7) based on phenotypic reactions on a set of differential soybean cultivars (Cho and Goodman, 1982). SMV has a single positive-stranded RNA genome approximately 9.6 kb that is encapsidated in filamentous virions that are 650–700 nm long and 15–18 nm wide (Brunt et al., 1996). The viral genome, which is expressed through synthesis and subsequent proteolytic processing of a polyprotein precursor, codes for at least nine mature proteins (Jayaram et al., 1992). The genome has a 3′ poly (A) tail and a virus protein genome-linked (VPg) that is covalently bound to the 5′ end.

There are three naturally occurring resistance (*R*) genes against SMV (*Rsv1*, *Rsv3* and *Rsv4*) in soybean. *Rsv1* was identified in the soybean line PI 96983, and it confers extreme resistance (ER) to SMV-N (an isolate of the SMV-G2 strain) but not to SMV-G7 (Chen et al., 1991; Hajimorad and Hill, 2001). *Rsv3* is a single dominant *R*-gene that was identified from the cultivar 'Harosoy' (Buss et al., 1999). In contrast to *Rsv1*, *Rsv3* confers resistance to SMV-G7 but not to SMV-N (this study). *Rsv1* and *Rsv3* have been mapped to different linkage groups (Hayes et al., 2004; Jeong et al., 2002). *Rsv4* is a single dominant *R*-gene from soybean line PI 486355 (Chen et al., 1993) that confers non-necrotic delayed susceptibility under greenhouse conditions to all SMV strains tested (Ma et al., 1995).

Most recent studies have focused on *Rsv1*-mediated resistance (Eggenberger et al., 2008; Hajimorad and Hill, 2001; Hajimorad et al., 2003, 2005, 2006, 2008). While the *Rsv1* resistance mechanism operates against all major SMV strains including SMV-N, strain SMV-G7 overcomes *Rsv1* and induces lethal systemic hypersensitive response (LSHR) in PI 96983 (*Rsv1*) (Cho and Goodman, 1979; Hajimorad and Hill, 2001). The mechanism of *Rsv1*-mediated resistance against SMV-N has been characterized as ER in which no virus can be detected in the inoculated leaf. However, the SMV-N–*Rsv1* interaction has the potential to produce a restricted systemic HR under certain conditions (Hajimorad and Hill, 2001). The elicitors of *Rsv1*-mediated resistance have been mapped to the helper-component proteinase (HC-Pro) and P3 (Eggenberger et al., 2008; Hajimorad et al., 2008). The features of the SMV-*Rsv1* interactions are phenotypically similar to those of *Potato virus X* resistance in potato conferred by the *Rx*-gene (Adams et al., 1986; Bendahmane et al., 1999).

In contrast, little is known concerning the interaction of SMV with *Rsv3*, which appears to be significantly different from that of *Rsv1*. We report here that resistance conferred by *Rsv3* to SMV-G7 is phenotypically different to that of *Rsv1* (ER) as it allows for a limited spread of avirulent SMV-G7 from the point of inoculation. *Rsv3* does not induce a typical HR phenotype either. By using the differential

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responses of SMV-N and SMV-G7 on *Rsv3*-soybean, we demonstrate that the cytoplasmic inclusion (CI) cistron of SMV is critical for induction of *Rsv3* resistance by SMV-G7. Furthermore, we demonstrate that the N-terminus of the viral CI gene is not only required for provoking *Rsv3*-mediated resistance, but it is also involved in elicitation of severe symptoms in SMV susceptible soybean.

Results

The Rsv3 mechanism of resistance differs phenotypically from those of HR or ER

Primary leaves of soybean line L29 (*Rsv3*) mechanically inoculated with SMV-G7 infectious sap consistently showed no symptoms and remained indistinguishable from mock-inoculated leaves. No visible HR was evident on leaves of inoculated L29 and the virus was not detected by double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) from the inoculated primary leaves or trifoliolate leaves of L29. In contrast, mild symptoms were induced on susceptible cultivar Williams (*rsv3*) inoculated with SMV-G7 infectious leaf sap and the infection was readily detected by DAS-ELISA (Fig. 4 and data not shown).

A more sensitive virus infection assay, using the GUS-tagged infectious SMV clones, SMV-G7-GUS and SMV-N-GUS (Wang et al., 2006; Zhang et al., 2009), showed extensive GUS infection foci on Williams (*rsv3*) inoculated with SMV-N-GUS or SMV-G7-GUS on the inoculated primary leaves (Fig. 1A) as well as the upper non-inoculated trifoliolate leaves (Fig. 1B) demonstrating systemic infection. Similar results were obtained with SMV-N-GUS inoculated L29 (*Rsv3*) (data not shown). In contrast, GUS infection foci indicative of limited GUS expression were observed on primary leaves of L29 inoculated biolistically with SMV-G7-GUS (Fig. 1A), but no GUS infection foci were evident on the upper trifoliolate leaves of the same plants at 3 weeks post-inoculation (Fig. 1B). As expected for ER, L78-379 (*Rsv1*) showed no GUS foci on inoculated primary or upper trifoliolate leaves when inoculated with SMV-N-GUS (Fig. 1). In addition, this treatment indicated that no residual GUS activity can be detected 3 weeks post-inoculation from the DNA inoculum used for biolistic inoculation. In another control experiment, we used non-GUS-tagged SMV infectious clones, SMV-G7 and SMV-N, and the infected plants showed no GUS staining (data not shown). The experiments were repeated three times and the results were consistent in plants maintained in either a growth chamber or in the greenhouse. The observation that limited infection by the avirulent SMV-G7 occurs in L29 suggests that the mechanism of resistance mediated by *Rsv3* is functionally different from ER that occurs in the SMV-N–*Rsv1* interaction for which no infection occurs.

The CI cistron is the Rsv3 virulence determinant

To identify the SMV cistron(s) that serves as the virulence determinant(s) for *Rsv3* resistance, a series of SMV chimeras was

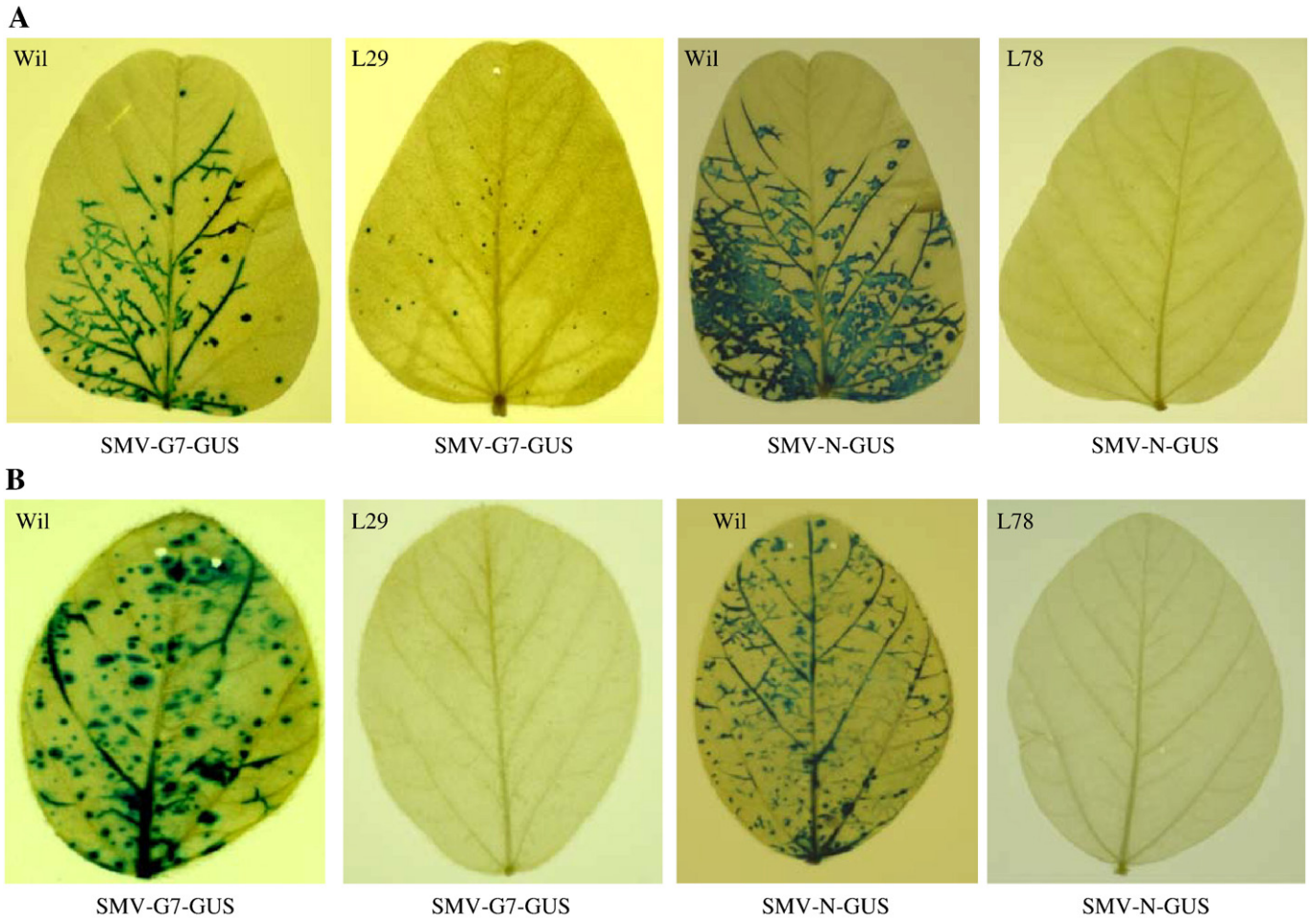


Fig. 1. GUS histochemical assay of *Soybean mosaic virus* (SMV) infection in soybean. (A) Primary leaves of Williams (*rsv3*), L29 (*Rsv3* isogenic line of Williams), and L78-379 (*Rsv1* isogenic line of Williams) were biolistically inoculated with SMV clones SMV-G7-GUS or SMV-N-GUS. After inoculation, plants were maintained in a growth chamber (20 °C) for 3 weeks before leaves were sampled for GUS histochemical assay. (B) Upper systemic second trifoliolate leaves after destaining. Note: Wil and L78 denote 'Williams' and L78-379 soybean genotypes, respectively.

constructed between virulent SMV-N and avirulent SMV-G7 (Fig. 2B). The chimeras were designed based on pairwise fragment exchanges to test the function of specific fragment(s) for their elicitor function on *Rsv3* soybean in either the SMV-N or SMV-G7 genomic background. All chimeras were infectious on Williams (*rsv3*) demonstrating that all are replication competent. However, on *Rsv3* soybean, chimeras SMV-N/G7$_{(1–3790)}$ and SMV-G7/N$_{(1–3787)}$ were avirulent showing that regions both upstream and downstream of the SalI site determine virulence independently. The two chimeras SMV-N/G7$_{(1608–3790)}$ and SMV-G7/N$_{(1605–3787)}$ containing the region upstream of the SalI site including the C-terminus of HC-Pro, P3 and N-terminus of CI were avirulent on *Rsv3*. Because HC-Pro and P3 were previously reported to be involved in inducing *Rsv1* resistance (Hajimorad et al., 2006; Eggenberger et al., 2008), we first tested the possibility that P3 may be important for inducing *Rsv3* resistance by two chimeras, SMV-N/G7P3 and SMV-G7/NP3, in which only the P3 cistrons were precisely switched between SMV-N and SMV-G7 (Hajimorad et al., 2006). The results showed that P3 is not involved in *Rsv3*-mediated resistance because the phenotypes of both chimeras were similar to those of the parental viruses (Fig. 2B). Data from four chimeras, SMV-N/G7$_{(1608–2023)}$, SMV-N/G7$_{(2017–2343)}$, SMV-G7/N$_{(1605–2020)}$, and SMV-G7/N$_{(2014–2340)}$ revealed that the C-terminus of HC-Pro is not a virulence determinant for *Rsv3* either (Fig. 2B). Replacement of the middle and C-terminal region of the SMV-N HC-Pro gene by SMV-G7 did not alter virulence of the chimeras on *Rsv3* (SMV-N/G7$_{(1608–2023)}$ and SMV-N/G7$_{(2017–2343)}$, Fig. 2B). As expected, the reciprocal replacement of the SMV-G7 HC-Pro cistron with fragments of SMV-N did not restore virulence of the corresponding chimeras (SMV-G7/N$_{(1605–2020)}$ and SMV-G7/N$_{(2014–2340)}$, Fig. 2B). These results showed that P3 and the C-terminus of HC-Pro, which are important SMV virulence determinants on *Rsv1*-genotype soybean, are not virulence determinants for *Rsv3*-mediated resistance. Nevertheless, there are at least two locations on the SMV genome that are involved in provoking *Rsv3* resistance.

The results above imply that the virulence determinants of *Rsv3*-mediated resistance reside on either the N-terminal region of CI or the

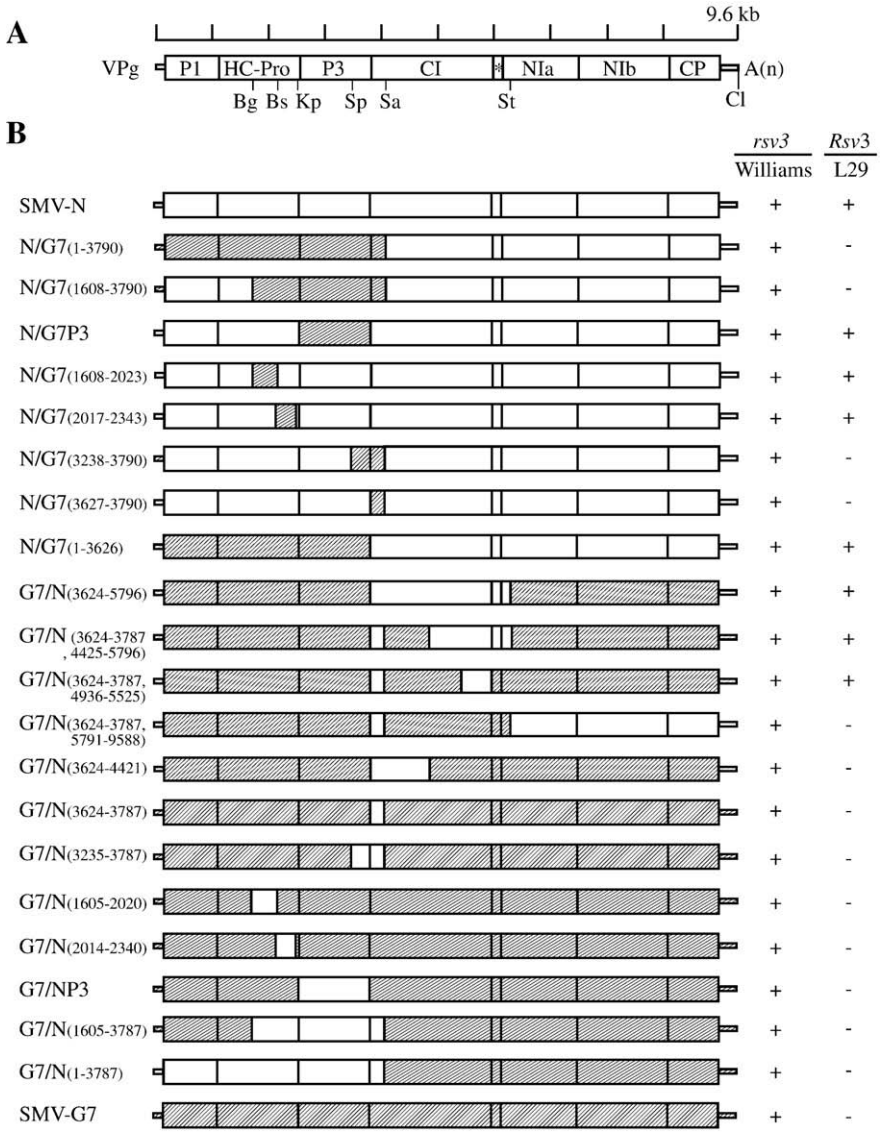


Fig. 2. Schematic representation of *Soybean mosaic virus* (SMV) parental clones or derivative chimeras and potential to infect Williams (*rsv3*) or L29 (*Rsv3*) soybean genotypes. (A) The genomic map of SMV and the position of the restriction sites BglII (Bg), BstEII (Bs), KpnI (Kp), SpeI (Sp), SalI (Sa), StuI (St) and ClaI (Cl) common between SMV-N and SMV-G7. (B) Schematic representation of infectious SMV clones N, G7, their derivative chimeras and response of Williams (*rsv3*) and L29 (*Rsv3*) to biolistic inoculation with plasmids. Following inoculations, plants were maintained in a growth chamber until evaluated 4 weeks post-inoculation for the presence (+) or absence (−) of infection by double-antibody sandwich enzyme-linked immunosorbent assay.

region upstream of BglII that contains the 5′ UTR, P1 and N-terminus of HC-Pro. To test the N-terminal region of CI, four chimeras were created (SMV-N/G7$_{(3238–3790)}$, SMV-G7/N$_{(3235–3787)}$, SMV-N/G7$_{(3627–3790)}$, SMV-G7/N$_{(3624–3787)}$, Fig. 2B). The SMV-N/G7$_{(3238–3790)}$, created by replacement of the region between SpeI and SalI of SMV-N with that of SMV-G7, failed to infect *Rsv3*-genotype soybean. As expected, due to the requirement for a downstream region, SMV-G7/N$_{(3235–3787)}$ did not gain virulence. Because previously P3 was shown not to be involved in induction of *Rsv3* resistance, the precise reciprocal replacement of the N-terminus of the CI rendered the chimeras SMV-N/G7$_{(3627–3790)}$ avirulent, but it did not make SMV-G7/N$_{(3624–3787)}$ virulent on *Rsv3*-genotype soybean. However, SMV-N/G7$_{(3627–3790)}$ and SMV-G7/N$_{(3624–3787)}$ were replication competent in Williams (*rsv3*) and induced mild symptoms (*rsv3*, Fig. 4C). We have shown that the N-terminal CI contains a critical virulence determinant for *Rsv3*-mediated resistance (SMV-N/G7$_{(3627–3790)}$, Fig. 2B). However, the possibility that the 5′ UTR, P1, and N-terminal portion of HC-Pro may also be involved had not been examined. To explore this possibility, the chimera SMV-N/G7$_{(1–3626)}$, containing the 5′ UTR, P1, HC-Pro, and P3 from SMV-G7, was generated and shown to be virulent on *Rsv3* soybean (Fig. 2B). Together, analyses of the chimeras SMV-N/G7$_{(3627–3790)}$ and SMV-N/G7$_{(1–3626)}$ on L29 showed that the N-terminus of CI, but not the 5′ UTR, P1, HC-Pro, or P3, is involved in eliciting *Rsv3* resistance.

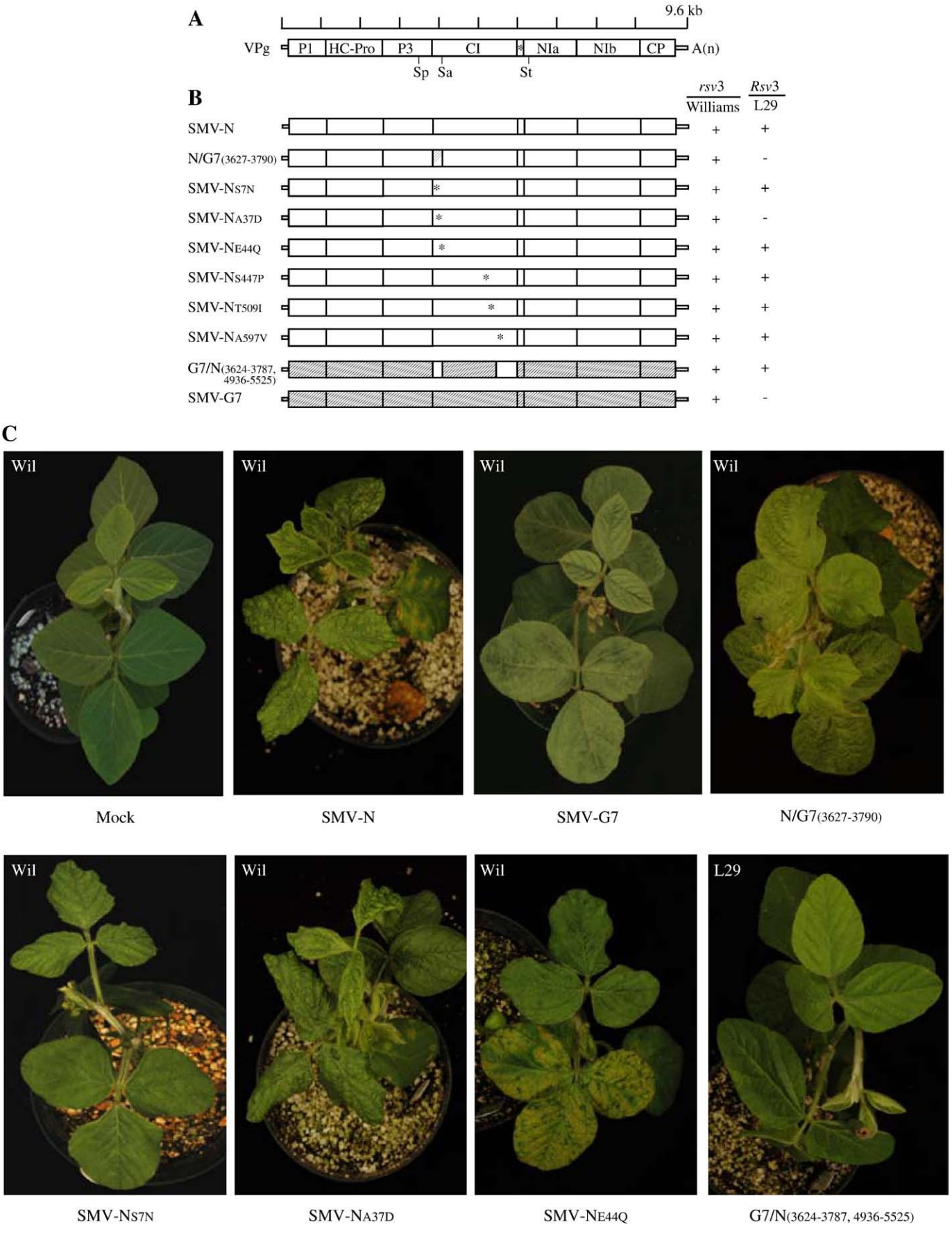
Subsequently, replacement of a region of SMV-G7 with the corresponding region derived from SMV-N containing the entire CI, virus protein genome-linked protein (VPg), and N-terminal nuclear inclusion protein a (NIa), resulted in a chimera (SMV-G7/N$_{(3624–5796)}$) that was virulent on *Rsv3* soybean (Fig. 2B). However, replacement of regions of SMV-G7 from nucleotides 3624 to 3787 and from 5791 to 9588 with corresponding regions of SMV-N (SMV-G7/N$_{(3624–3787,\ 5791–9588)}$) was not virulent on *Rsv3*-genotype soybean, but remained replication competent in Williams (*rsv3*). These data narrowed the second virulence determinant of *Rsv3* to the C-terminus of CI, VPg, or N-terminus of NIa. Two additional chimeras, SMV-G7/N$_{(3624–3787,\ 4425–5796)}$ and SMV-G7/N$_{(3624–3787,\ 4936–5525)}$ revealed that the C-terminal region of the CI encodes the second virulence determinant of SMV on *Rsv3*-genotype soybean, because they induced mild symptoms on L29 without visible necrotic lesions typical of HR (Fig. 2B).

Amino acid sequence alignment of CI between SMV-G7 and SMV-N showed that there are 3 differences at the N-terminus of CI (from SMV-N nt 3624 to the unique SalI site at nt 3783, Fig. 3). The three positions 7, 37, and 44 relative to the deduced amino acid sequence of the full-length CI, were selected for mutational analysis (Fig. 3). Each of the unique amino acids of SMV-N was replaced with the corresponding amino acid of SMV-G7 to generate SMV-N$_{S7N}$, SMV-N$_{A37D}$ and SMV-N$_{E44Q}$ (Fig. 4B). All were infectious on Williams (*rsv3*), but SMV-N$_{S7N}$ and SMV-N$_{E44Q}$ were infectious on L29 while SMV-N$_{A37D}$ was not. These results were confirmed by sequencing the RT-PCR products of the progeny viruses of SMV-N$_{S7N}$ and SMV-N$_{E44Q}$ from infected L29 plants and no mutations of the CI gene were detected. Leaf sap from SMV-N$_{A37D}$ infected 'Williams' was used to inoculate L29. No systemic infection was detected for L29 plants inoculated with SMV-N$_{A37D}$ leaf sap by either symptom expression or DAS-ELISA. Similarly, RT-PCR and CI cistron sequencing was performed to confirm the identity of SMV-N$_{A37D}$ using leaf tissue from infected 'Williams' plants. Among the 12 amino acid differences at the C-terminus between SMV-G7 and SMV-N (SMV-N nt 4936 to 5525, Fig. 3), three positions were chosen for mutational analysis (SMV-N$_{S447P}$, SMV-N$_{T509I}$ and SMV-N$_{A597V}$, Fig. 4B). Interestingly, these three point mutants were infectious on both Williams and L29. Similar to SMV-N$_{S7N}$ and SMV-N$_{E44Q}$, the results were confirmed by sequencing the RT-PCR products of the progeny viruses of SMV-N$_{S447P}$, SMV-N$_{T509I}$ and SMV-N$_{A597V}$ infected L29 plants and no mutations of the CI gene were detected. Other than biolistic inoculation, leaf sap inoculations were performed for the above 5 point mutants that are virulent on L29 and the results are consistent with the biolistic inoculation.

N-terminus of CI is involved in symptom phenotype

Surprisingly, the chimera SMV-N/G7$_{(3627–3790)}$ did not induce a severe symptom phenotype on Williams (*rsv3*) characteristic of the

Fig. 3. Cytoplasmic inclusion (CI) protein sequence alignment of SMV-N and SMV-G7. The amino acid differences are highlighted in black. Numbers in parenthesis are the positions of deduced amino acid sequences of SMV CI. Arrows indicate the nucleotide position based on SMV-N genome and the positions for chimeras exchanging parts of the CI coding sequence. GenBank accession numbers are SMV-N, D00507 and SMV-G7, AY216010.


A
9.6 kb
VPg
P1
HC-Pro
P3
CI
NIa
NIb
CP
A(n)
Sp
Sa
St
B
rsv3
Williams
Rsv3
L29
SMV-N
N/G7(3627-3790)
SMV-NS7N
SMV-NA37D
SMV-NE44Q
SMV-NS447P
SMV-NT509I
SMV-NA597V
G7/N(3624-3787, 4936-5525)
SMV-G7
C
Wil
Mock
SMV-N
SMV-G7
N/G7(3627-3790)
SMV-NS7N
SMV-NA37D
SMV-NE44Q
L29
G7/N(3624-3787, 4936-5525)

parental SMV-N (Fig. 4C). Since amino acid alignment revealed three amino acid differences between SMV-N and SMV-G7 in the N-terminal region of the CI at positions 7, 37, and 44, the plants infected with the CI mutants were observed for symptom phenotype (Fig. 4C). The SMV-N_{A37D} and SMV-N_{E44Q} displayed severe symptom phenotypes characteristic of the parental SMV-N on Williams (*rsv3*), but SMV-N_{S7N} displayed a mild symptom on both Williams (*rsv3*) and was infectious on L29 (*Rsv3*) (Fig. 4C). However, G7/$N_{(3624–3787)}$, a SMV-G7 derivative with the N-terminal CI replaced by that of SMV-N induced only mild symptoms (data not shown). This observation indicates that other regions of the SMV-N genome are required for the severe symptom induction. Nevertheless, the N-terminus of the SMV CI is critical for both pathogenicity and virulence on *Rsv3*-genotype soybean as two amino acid residues separated by only 30 amino acids in the N-terminal region of CI play roles in virulence by modulating disease symptoms or by specifying recognition by *Rsv3*.

Discussion

The results presented here show that L29 (*Rsv3*) plants inoculated mechanically with SMV-G7 expressed no symptoms and were ELISA negative. A more sensitive histochemical assay using SMV-G7-GUS showed limited infection foci in the primary leaves of biolistically inoculated L29 (*Rsv3*) plants. However, the virus failed to move systemically to the upper trifoliolate leaves of the same plants. No cell death lesions typical of HR were observed on L29 (*Rsv3*) inoculated with SMV-G7 or any of its derivatives. Although several SMV-G7 derived constructs induced severe symptoms on Williams (*rsv3*), the resistance phenotype remained unchanged compared with SMV-G7. As expected, L78-379 (*Rsv1*) plants inoculated with SMV-N showed no visible phenotype associated with resistance against SMV-N. The results demonstrate that the GUS-tagged virus can be useful for enhanced understanding of resistance and may be applicable to other pathosystems as exemplified by *TuRB01–Turnip mosaic virus* in canola (Walsh et al., 1999) and *RTM1/RTM2–Tobacco etch virus* in *Arabidopsis* (Chisholm et al., 2000; Whitham et al., 2000).

The mechanism of *Rsv3* resistance is different from that of ER characteristic of SMV-N–*Rsv1*, because avirulent SMV-G7 exhibits limited infection (this study) while no apparent infection is associated with ER (Hajimorad and Hill, 2001). Systemic viral infection involves both cell-to-cell and long distance movement in the phloem (Carrington et al., 1996). In addition, entry and exit from vascular tissue that is required for long distance movement are different from cell-to-cell movement (Nelson and Van Bel, 1998; Santa Cruz, 1999). At present, it is not known whether *Rsv3* resistance interferes with entry into or exit from vascular tissue or some other aspect of systemic virus movement. However, the observation that GUS foci induced by SMV-G7-GUS on L29 (*Rsv3*) is significantly smaller than those in the compatible interaction suggests that the cell-to-cell movement rather than the long distance systemic movement is impaired. Additional grafting experiments for the *Rsv3*–SMV-G7 pathosystem similar to those of *Rsv1*–SMV-N (Hajimorad and Hill, 2001) may reveal the aspect of viral transport that is inhibited by *Rsv3*-mediated resistance. Cloning of *Rsv3* and localization of its protein product could also reveal the point at which SMV movement is restricted.

Recent studies on *Rsv1*-mediated resistance have shown that SMV HC-Pro and P3 are elicitors of *Rsv1* (Eggenberger et al., 2008; Hajimorad et al., 2005, 2006, 2008). The data presented here demonstrate that by using the differential responses of SMV-N and SMV-G7 on *Rsv3* soybean, the N- and C-termini of the CI cistron are virulence determinants of *Rsv3* soybean. Point mutations at the N-terminus of SMV CI further showed that this region is also involved in severe symptom expression in SMV susceptible soybean.

The findings that SMV CI serves as a virulence determinant and influences symptom severity provide further evidence for its important role during virus infection of soybean. Cells infected with SMV accumulate pinwheel-shaped cytoplasmic inclusion bodies near the plasmodesmata that are unique and diagnostic for potyviral infection (Hunst and Tolin, 1983). Previously, it has been shown that the CI protein of potyviruses exhibit NTPase and RNA helicase activities, and functions in viral cell-to-cell movement (Carrington et al., 1998; Gómez de Cedrón et al., 2006; Rodríguez-Cerezo et al., 1997; Roberts et al., 1998) as well as virus replication (Carrington et al., 1998). In addition to the roles that both the N- and C-terminal regions of CI play in virulence of SMV on *Rsv3* soybean, the C-terminus of TuMV CI has also been shown to provoke *TuRB01*-mediated resistance to TuMV infection (Jenner et al., 2000, 2002). Similar to our finding that the C-terminus of SMV CI is required for *Rsv3*-mediated resistance, mutation of a single amino acid at the C-terminus of the TuMV CI cistron could overcome *TuRB01*-mediated resistance to TuMV (Jenner et al., 2000, 2002). The N-terminus of TEV CI was shown to be involved in TEV replication in the protoplast assay experiment and virus movement in plants (Carrington et al., 1998). Unique to the SMV CI, however, is the close proximity of two amino acids, located in the same N-terminal region, that are involved in *Rsv3*-mediated resistance and severe symptom phenotype.

The RNA helicase associated with the conserved central domain of the potyvirus CI has been classified as a member of superfamily 2 (SF2) along with the NS3 protein of flaviviruses (Eagles et al., 1994; Fernández et al., 1995; Fernández and García, 1996; Fernández et al., 1997; Kadaré and Haenni, 1997; Mackintosh et al., 2006; Sampath et al., 2006; Yamashita et al., 2008). Although accumulation of the CI protein in pinwheel inclusion bodies is well documented, structural analyses of the CI have not been performed. BLAST comparison (www.pdb.org) showed SMV CI helicase (aa 79–360) has 44% similarity to NS3 helicase of Yellow fever virus (YFV) and 43% similarity to NS3 of Hepatitis C virus (HCV) and Dengue virus (DEN; PDB ID: 1ymf, YFV; 2f55, HCV; 2bhr, DEN). Previous structural studies of the NS3 proteins of HCV, YFV, and DEN showed that these helicase domains all share a similar three-lobed structure that contains a central tunnel to accommodate RNA and the N-terminus is exposed on the surface of the three-lobed helicase structures (Mackintosh et al., 2006; Sampath et al., 2006; Yamashita et al., 2008). Because of the highly conserved amino acid sequences and the essential function of helicases in viral replication, it is possible that the central helicase domain (aa 79–360) of SMV CI assumes a similar structure and the N-terminus of SMV CI (aa 1–78) is also on the surface of the protein. By comparison with the NS3 helicase, the N-terminal SMV CI involved in virulence and pathogenicity as well as the C-terminal CI of TuMV (*TuRB01*) are proximal to the conserved sequences associated with helicase (Jenner et al., 2000; this study). It is likely that genetic alterations at the N- and C-terminal regions of SMV CI that affect virulence and pathogenicity do not compromise essential functional integrity of the helicase since all SMV chimeras and mutants were infectious on Williams (*rsv3*).

Fig. 4. Schematic representation of *Soybean mosaic virus* (SMV) parental clones, derivative chimeras, and point mutants and potential to infect Williams (*rsv3*) or L29 (*Rsv3*) soybean genotypes. (A) The genomic map of SMV and the positions of the restriction sites SalI (Sa), SpeI (Sp) and StuI (St) common between SMV-N and SMV-G7. (B) Schematic representation of infectious clones SMV-N, SMV-G7, derivative chimeras and point mutants. Response of Williams (*rsv3*) and L29 (*Rsv3*) to biolistic inoculation with plasmids and mechanical inoculation with progenies derived from the plasmids, respectively. Following inoculation, the plants were maintained and evaluated as described in Fig. 2. (C) Symptoms induced on Williams (*rsv3*) biolistically inoculated with SMV infectious clones. Plants were maintained in a growth chamber (20 °C) and photographed 3 weeks post-inoculation. Mock — plants mechanically inoculated with leaf sap from healthy soybean plants. Note: Wil denote 'Williams' soybean line.

The role of CI in virulence and pathogenicity remains uncertain and unclear due to lack of structural studies. We speculate that its function, particularly at the exposed N-terminus, may be in CI–viral and/or host protein interactions that influence RNA replication and/or movement. For example, using a yeast two-hybrid system, it was found that the N-terminus of *Plum pox virus* (PPV) CI is important in PPV CI self interaction (López et al., 2001). In another study, *Potato virus A* (PVA) CI was shown to interact with P1 and HC-Pro proteins encoded by PVA (Guo et al., 2001). Potyvirus CI has also been reported to interact with host factors such as tobacco protein P58IPK to TEV CI, *Nicotiana benthamiana* photosystem I protein PSI-K with (PPV) CI and an unknown protein from *N. benthamiana* with a HIT type zinc finger domain to PPV CI (Bilgin et al., 2003; Jiménez, 2004; Jiménez et al., 2006).

The putatively exposed N-terminal region of SMV CI, exclusive of the helicase domain, consists of about 78 amino acids and because of its size is also unlikely to impact the structure of the conserved central helicase domain. The A37D mutation in the N-terminus results in a change from a hydrophobic to a negatively charged hydrophilic amino acid which would be predicted to alter secondary structure, but the S7N and E44Q mutations are not predicted to alter secondary structure. While the A37D mutation did not significantly affect virus spread through Williams (*rsv3*), it did result in loss of virulence on *Rsv3* soybean. It is possible that the change alters interaction with a factor involved in *Rsv3* resistance.

Interestingly, the S7N mutation resulted in a change from severe to mild symptoms on Williams (*rsv3*). Although it is not unique that a single mutation results in an altered phenotype of potyviruses (Andersen and Johansen 1998; Atreya and Pirone 1993; Carrington et al., 1998; Jenner et al., 2000; Masuta et al., 1999), we are not aware of any report documenting both virulence and pathogenicity determinants within a 30 amino acid region in a single viral gene. While the three point mutants at the C-terminus failed to change the virulence of the mutants, further mutational analysis at this region may be instructive for understanding CI structure and its role in SMV virulence and pathogenicity. However, this is challenging because there are no convenient restriction sites at this region. Another possibility is that there might be multiple amino acids that are involved in *Rsv3* virulence at this region. It has been reported for *Rsv1*, that several amino acids of HC-Pro are required as SMV avirulence factors to *Rsv1* (Hajimorad et al., 2008). The C-terminus of SMV CI, which consists of 274 amino acids exclusive of the helicase domain, shares little homology with other potyvirus CI gene products. Additional knowledge of this region of SMV CI may enhance the understanding of potyvirus CI function during potyvirus infection. In addition, the understanding of divergent avirulence factors for *Rsv1* and *Rsv3* will assist in monitoring SMV virus population diversity to enable durable disease resistance management.

Materials and methods

Viruses, soybean genotypes, inoculation, and SMV detection

Plasmids containing infectious full-length cDNA clones of SMV-G7 (pSMV-G7) and SMV-N (pSMV-N), as well as the GUS-tagged infectious clones of SMV-G7-GUS and SMV-N-GUS, have been previously described (Hajimorad et al., 2003; Wang et al., 2006). The soybean (*G. max*) cultivar Williams (*rsv3*) (Bernard and Lindahl, 1972), susceptible to all strains of SMV, and line L29 (*Rsv3*), a near isogenic line of Williams (Buss et al., 1999) with the *Rsv3* allele derived from 'Hardee', and line L78-379 with the *Rsv1* allele derived from PI 96983 (Bernard et al., 1991) were used in this study. All seeds were harvested from greenhouse grown plants previously indexed for the absence of SMV. To establish infection, plasmid DNA was biolistically inoculated to primary leaves of soybean seedlings following the procedure described by Zhang et al. (2009). Leaf sap mechanical inoculation was done following the method described by Hajimorad et al. (2008). The inoculated plants were maintained in a growth chamber operating at 20 °C with a photoperiod of 16 h. The SMV detection was done by double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) (Hajimorad and Hill, 2001).

Construction of SMV chimeras and site-directed mutants

All plasmids were propagated in ElectroMax DH5α-E cells (Invitrogen, Carlsbad, CA, USA) and purified using the QiaPrep Spin MiniPrep kit (Qiagen, Valencia, CA, U.S.A.). Takara PrimeSTAR™ HS DNA Polymerase was used in all PCR reactions following the manufacturer's recommended protocol (TaKaRa Bio Inc., Otsu, Shiga, Japan). All primers used for PCR reaction and sequencing are listed in Table 1. Nucleotide sequencing was done using the Big Dye Terminator DNA Sequencing Kit (Applied Biosystems, Foster City, CA, U.S.A.) and the ABI Prism 310 genetic analyzer. Sequence analysis was performed using the Vector NTI program (Invitrogen).

SMV chimeras were generated by exchanging restriction fragments between cDNAs using standard molecular biological methods (Sambrook and Russell, 2001). SMV-N/G7P3 and SMV-G7/NP3 were reported by Hajimorad et al. (2006). The restriction sites used and their nucleotide positions in the SMV-N (GenBank accession number, D00507) genome were BglII (1605), BstEII (2013), KpnI (2335), SpeI (3234), SalI (3782) and StuI (5793) (Fig. 2A). The genomic positions for these restriction sites on SMV-G7 differ from those of SMV-N by three nucleotides, because SMV-N lacks a codon relative to SMV-G7 in the P1 region. In addition, SMV-G7 (GenBank accession number, AY216010) has a StuI (5797) site while SMV-N does not have StuI at a similar position. Mutation was introduced at the corresponding position on

Table 1
Primers for construction and sequencing of SMV chimeras and mutants.

Name	Sequence (5′→3′)
SMV-2914F	GATCGCTTGAAGCAGGAATG
SMV-3142F	CATCGGTGCGAGCCTTTG
SMV-3557F	CATGACAGGACAATCAGAAGATGT
SMV-3595R	AACATCCTCACCCATTGTGCTGA
SMV-3620F$_{S\rightarrow N}$	GCAGAGTCTTGATGAAATTCAAAATATTGAT
SMV-3642R$_{S\rightarrow N}$	TTGAATTTCATCAAGACTCTGC
SMV-3732F$_{A\rightarrow D}$	TGAGGATTGGTGGAATAGACAG
SMV-3732R$_{A\rightarrow D}$	CTGTCTATTCCACCAATCCTCA
SMV-3750F$_{E\rightarrow Q}$	TGCAGCAGAATAGAGTAATTCC
SMV-3750R$_{E\rightarrow Q}$	GGAATTACTCTATTCTGCTGCA
SMV-3873R	CCTGAGCCAACTGCACCTCT
SMV-4401F	GAAGTTGACCAATTGTCACGA
SMV-4421R	TCGTGACAATTGGTCAACTTC
SMV-4936R	TTGAGCAGTCTGTGGATTTCTGGGT
SMV-4960F	ACCCAGAAATCCACAGACTGCTCAA
SMV-4970F$_{S\rightarrow P}$	ACAGACTGCTCAAGCCATATAAA
SMV-4970R$_{S\rightarrow P}$	TTTATATGGCTTGAGCAGTCTGT
SMV-5012F	ACCAAGATAGCCATACCA
SMV-5148F$_{T\rightarrow I}$	ATGTTGTGGGACATCGTTTGTAAA
SMV-5148R$_{T\rightarrow I}$	TTTACAAACGATGTCCCACAACAT
SMV-5326F$_{A\rightarrow S}$	ACACAATTGGCTCTAGCGTCACTGGGTA
SMV-5326R$_{A\rightarrow S}$	TACCCAGTGACGCTAGAGCCAATTGTGT
SMV-5423F$_{A\rightarrow V}$	AGCATAATATAGTAGTTTTACAA
SMV-5423R$_{A\rightarrow V}$	TTGTAAAACTACTATATTATGCT
SMV-5503R	CTGTAGTTGGACTGCATTTAAAA
SMV-5526F	TTTTAAATGCAGTCCAACTACAG
SMV-N-StuF	TTGGTGAGGCCTATACCAAGA
SMV-N-StuR	TCTTGGTATAGGCCTCACCAA
SMV-6299F	GAGATTATAGTGGCATTTCC
SMV-6990F	ACAGTGACAGTACAAGGGA
SMV-8420F	GCACCATATATTGCAGAGACA
SMV-8526R	TCCTTGCCTGATTGTAAGGACA
SMV-9030F	TCTCAGATGCAGCAGAAG
SMV-9564F	CAACAAACATTGCCGCACCTC
Nos-Rev	AGACCGGCAACAGGATTCA

SMV-N to introduce StuI without changing the amino acid at that position. In the vector backbone of infectious SMV-N and SMV-G7, there is a unique ClaI site downstream of the poly(A) tail of the SMV genome. SMV chimeras SMV-N/G7$_{(1–3790)}$ and SMV-G7/N$_{(1–3787)}$ were generated by switching the fragment between SalI and ClaI. Because SMV-G7 contains two BglII sites, construct switching at the SMV-G7 BglII site uses partial digestion. The restriction sites used for generating the constructs are BglII and SalI for SMV-N/G7$_{(1608–3790)}$; BglII and BstEII for SMV-N/G7$_{(1608–2023)}$ and SMV-G7/N$_{(1605–2020)}$; BstEII and KpnI for SMV-N/G7$_{(2017–2343)}$ and SMV-G7/N$_{(2014–2340)}$; SpeI and SalI for SMV-N/G7$_{(3238–3790)}$ and SMV-G7/N$_{(3235–3787)}$.

A set of overlapping PCR reactions with overlapping primers SMV-3595R and SMV-3557F was used to switch the 5′ end of CI cistron between SMV-N and SMV-G7. PCR reaction A was performed with SMV-N as template and primers SMV-3142F (5′ end primer) and SMV-3595R (reverse overlapping primer). PCR reaction B was performed with SMV-G7 as template and primer pair SMV-3557F (forward overlapping primer) and SMV-3873R (3′ end primer). PCR reaction C was performed with PCR products of A and B as template and primer pair SMV-3142F and SMV-3873R. The products of PCR reaction C were digested with SpeI and SalI and ligated into similarly digested SMV-N to produce SMV-N/G7$_{(3627–3790)}$. SMV-G7/N$_{(3624–3787)}$ was generated similarly to SMV-N/G7$_{(3627–3790)}$. SMV chimera SMV-N/G7$_{(1–3626)}$ was generated by replacing the fragment between SalI and ClaI of SMV-G7/N$_{(3624–3787)}$ with that of SMV-N. SMV-N was used as a template with primer pair SMV-N-StuF and Nos-Rev for PCR reaction and the product was digested with StuI and ClaI, gel recovered and ligated to the 10.5 kb fragment of StuI and ClaI digestion product of SMV-G7/N$_{(3624–3787)}$ to generate SMV-G7/N$_{(3624–3787,\ 5791–9588)}$. SMV-N was used as template with primer pair SMV-3557F and SMV-N-StuR for PCR reaction and the PCR product was digested with SalI and StuI, gel recovered and ligated to the 12.1 kb fragment of SalI and StuI digestion product of SMV-G7/N$_{(3624–3787)}$ to generate SMV-G7/N$_{(3624–5796)}$. Overlapping primers SMV-SMV-4421R and 4401F were used in a set of overlapping PCR reactions with primers SMV-3557F (5′ end primer) and SMV-N-StuR (3′ end primer) to generate SMV-G7/N$_{(3624–4421)}$ and SMV-G7/N$_{(3624–3787,\ 4425–5796)}$. Similarly, 5′ and 3′ end primers SMV-3557F and SMV-N-StuR were used together with overlapping primers SMV-4936R and SMV-4960F, SMV-5503R and SMV-5526F, and templates SMV-G7 and SMV-N in a set of overlapping reactions to generate SMV-G7/N$_{(3624–3787,\ 4936–5525)}$ from SMV-G7/N$_{(3624–5796)}$. With primers SMV-3142F and SMV-3873R as 5′ and 3′ end primers, overlapping primers SMV-3642R and SMV-3620F$_{S\rightarrow N}$, SMV-3732R and SMV-3732F$_{A\rightarrow D}$ and SMV-3750R and SMV-3750F$_{E\rightarrow Q}$ were used with SMV-N as template to generate point mutants SMV-N$_{S7N}$, SMV-N$_{A37D}$ and SMV-N$_{E44Q}$ using restriction sites SpeI and SalI. With primers SMV-3557F and SMV-N-StuR as 5′ and 3′ end primers, overlapping primers SMV-4970R$_{S\rightarrow P}$ and SMV-4970F$_{S\rightarrow P}$, SMV-5148R$_{T\rightarrow I}$ and SMV-5148F$_{T\rightarrow I}$, and SMV-5423R$_{A\rightarrow V}$ and SMV-5423F$_{A\rightarrow V}$ were used with SMV-N as template to generate point mutants SMV-N$_{S447P}$, SMV-N$_{T509I}$ and SMV-N$_{A597V}$ using restriction sites SalI and StuI.

RNA extraction and RT-PCR

Total RNA from soybean was isolated from systemically infected fresh leaves or liquid nitrogen-frozen soybean tissues kept at −85 °C by using an RNeasy Plant Mini Kit (Qiagen). Reverse transcription was performed by using total RNA as template and random 9-mers (Takara Bio) following the standard protocol for SuperScript™ III RT reverse transcriptase (Invitrogen). The SMV genomic region including the CI gene was amplified using RT-PCR products as template with primer pair SMV-3557F and SMV-N-StuR following the Takara PrimeSTAR™ HS DNA Polymerase standard protocol (TaKaRa). Complete sequencing of the PCR products was done with primers SMV-3557F, SMV-4401F and SMV-N-StuR (Table 1).

Protein structure analyses

Predictions of protein secondary structures were performed using the Wisconsin Package Version 10.2 (Genetics Computer Group, Madison, WI, U.S.A.) and the PredictProtein server (Rost et al., 2004).

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