Amino acid changes in P3, and not the overlapping *pipo*-encoded protein, determine virulence of *Soybean mosaic virus* on functionally immune *Rsv1*-genotype soybean

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SUMMARY

A small open reading frame, termed '*pipo*', is embedded in the P3 cistron of potyviruses. Currently, knowledge on *pipo* and its role(s) in the life cycle of potyviruses is limited. The P3 and helper-component proteinase (HC-Pro) cistrons of *Soybean mosaic virus* (SMV) harbour determinants affecting virulence on functionally immune *Rsv1*-genotype soybeans. Interestingly, a key virulence determinant of SMV on *Rsv1*-genotype soybeans (i.e. soybeans containing the *Rsv1* resistance gene) that resides at polyprotein codon 947 overlaps both P3 and a *pipo*-encoded codon. This raises the question of whether PIPO or P3 is the virulence factor. To answer this question, the corresponding *pipo* of an avirulent and two virulent strains of SMV were studied by comparative genomics, followed by syntheses and analyses of site-directed mutants. Our data demonstrate that the virulence of SMV on *Rsv1*-genotype soybeans is affected by P3 and not the overlapping *pipo*-encoded protein.

INTRODUCTION

The genetic determinants of plant viruses mediating virulence on host genotypes containing resistance genes, dominant or recessive, often reside on viral encoded proteins and involve one or more amino acids (Abdul-Razzak *et al.*, 2009; Decroocq *et al.*, 2009; Diveki *et al.*, 2004; Eggenberger *et al.*, 2008; Hjulsager *et al.*, 2006; Janzac *et al.*, 2010; Malcuit *et al.*, 1999; Meshi *et al.*, 1988; Padgett *et al.*, 1997; Salvador *et al.*, 2008). In the case of viruses belonging to the family *Potyviridae*, such determinants have been mapped to various viral encoded proteins (Abdul-

Razzak *et al.*, 2009; Decroocq *et al.*, 2009; Eggenberger *et al.*, 2008; Fellers *et al.*, 2002; Hajimorad *et al.*, 2008; Hjulsager *et al.*, 2006; Janzac *et al.*, 2010; Jenner *et al.*, 2000, 2002, 2003; Johansen *et al.*, 2001; Krause-Sakate *et al.*, 2005; Mestre *et al.*, 2000; Salvador *et al.*, 2008).

The family *Potyviridae* represents one of the largest groups of plant RNA viruses affecting major crops worldwide (Adams *et al.*, 2005). The genome of the member species of the genus *Potyvirus*, including that of *Soybean mosaic virus* (SMV), is approximately 10 kb and contains a single long open reading frame (ORF) and a very small overlapping ORF, termed '*pipo*' (Chung *et al.*, 2008; Wen and Hajimorad, 2010). On expression, the long polyprotein is cleaved post-translationally by three viral encoded proteinases to yield a number of multifunctional mature proteins, including helper-component proteinase (HC-Pro) and P3 (Urcuqui-Inchima *et al.*, 2001). The small ORF *pipo* embedded in the P3 cistron of potyviruses has the potential to encode a protein in the +2 frame relative to the polyprotein ORF. It has been proposed that *pipo* is expressed *in planta* as a protein of approximately 25 kDa fused to the N-terminus P3 (P3N+PIPO) (Chung *et al.*, 2008); however, the presence of P3 amino acid sequences in the detected protein, serologically or by sequencing, remains to be demonstrated. The putative *pipo* of SMV is 225 nucleotides long, contains a GA_6 motif at its 5′ end and has the potential to encode 75 amino acids (Chung *et al.*, 2008; Wen and Hajimorad, 2010). Knowledge on *pipo* and its role(s) in the life cycle of potyviruses is limited (Choi *et al.*, 2005; Chung *et al.*, 2008; Wei *et al.*, 2010; Wen and Hajimorad, 2010).

The major host of SMV is soybean, and cultivars harbouring the *Rsv1* locus are functionally immune to strains G1–G6, but not to G7 or its experimentally evolved variant G7d (Bernard *et al.*, 1991; Hajimorad *et al.*, 2003). SMV-N, an isolate of strain G2, is avirulent on *Rsv1*-genotype soybeans (Hajimorad and Hill, 2001). Studies based on comparative genomics analyses between avirulent SMV-N (avrN) and virulent SMV-G7 (virG7), as well as experimental adaptation of avrN-derived P3 chimeras containing P3 sequences from virG7 or virulent G7d (virG7d),

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and direct adaptation of avrN to *Rsv1*-genotype soybeans, have all identified numerous virulence determinants of SMV residing on HC-Pro and P3 cistrons (Eggenberger *et al.*, 2008; Hajimorad *et al.*, 2008, 2011). Interestingly, a key virulence determinant within the P3 cistron at polyprotein codon 947 (Eggenberger *et al.*, 2008; Hajimorad *et al.*, 2011) also overlaps one of the *pipo*-encoded codons. This raises the possibility that PIPO, but not P3, is the virulence factor of SMV on *Rsv1*-genotype soybeans. In this article, virulence is defined as the genetic ability of a strain or a variant of SMV to overcome functional immunity mediated by the *Rsv1* locus (Shaner *et al.*, 1992). On the basis of this definition, with respect to *Rsv1*-genotype soybeans, SMV-G7 and SMV-G7d are both virulent, whereas SMV-N is avirulent (Hajimorad *et al.*, 2006).

To answer the question of whether *pipo* plays any role in the virulence of SMV, we have taken advantage of the differential interactions of avrN, virG7 and virG7d with *Rsv1*-genotype soybeans (Hajimorad *et al.*, 2003; Hajimorad and Hill, 2001). In this article, using a comparative genomics approach combined with site-directed mutagenesis, we present evidence that determinants of SMV effective for virulence on *Rsv1*-genotype soybeans residing on the P3 cistron are positioned on P3- and not the *pipo*-encoded protein.

RESULTS AND DISCUSSION

Genetic differences among *pipo* of avrN, virG7 and virG7d are limited

Alignment of the nucleotide sequence of the putative *pipo* of avrN with those of virG7 and virG7d showed that they are all 225 nucleotides in length (Fig. 1A). The position of *pipo* of avrN has been mapped to the genomic nucleotides 2882–3106 (Wen and Hajimorad, 2010). However, in the context of virG7 and virG7d genomes, *pipo* is located at nucleotides 2885–3109. This is the result of a lack of a single codon in the P1 cistron of avrN relative to those of virG7 and virG7d, which is positioned upstream of *pipo* (Hajimorad *et al.*, 2006). It has been proposed that AAA encoding lysine (Lys) located within the highly conserved GA$_6$ motif serves as the first codon for the putative SMV *pipo*-encoded protein, mainly because of the presence of an adjacent *pipo* in-frame stop codon (TGA) (Wen and Hajimorad, 2010) (Fig. 1A). Moreover, another *pipo* in-frame stop codon (TGA) located upstream of the GA$_6$ motif is conserved among all three SMV strains (Fig. 1A). Downstream of the GA$_6$ motif, the first *pipo* in-frame stop codon (TGA) in the genome of avrN is located at nucleotide positions 3107–3109, whereas those of

Fig. 1 (A) Partial nucleotide sequences of the P3 cistron of the infectious cDNA clones of *Soybean mosaic virus* (SMV) strains N (N), G7 and G7d containing the full-length sequences of *pipo*. The arrow indicates the starting nucleotide of *pipo*. The nucleotide sequences within the boxes are *pipo* in-frame stop codons upstream of the conserved GA$_6$ motif and the termination codon at the 3′-end of *pipo*. The sequences of the GA$_6$ motif at the 5′-terminus of *pipo* are shown in italic and boldface. The unique nucleotide of each strain not shared with the others is highlighted. (B) Alignment of the deduced primary amino acid sequences of the PIPO protein of N, G7 and G7d. The unique amino acid of each strain not shared with the others is highlighted. It should be noted that the genomic positions of *pipo* of G7 and G7d differ from that of N because of the presence of one additional codon upstream of the P3 cistron (GenBank accession numbers AY216010, AY216987 and D00507, respectively).

virG7 and virG7d are at positions 3110–3112 (Fig. 1A). This stop codon probably serves as the terminal signal for the expression of *pipo*-encoded protein.

There are a total of five and six nucleotide substitutions between the putative *pipo* of avrN and those of virG7 and virG7d, respectively (Fig. 1A). There is only one nucleotide substitution in the *pipo* of virG7 when compared with that of virG7d (Fig. 1A). These nucleotide differences result in a total of three amino acid substitutions in the *pipo*-encoded protein of avrN when compared with those of virG7 and virG7d (Fig. 1B). These unique amino acids of avrN not shared with virG7 and virG7d are located at positions 30, 35 and 65 of the PIPO protein. The PIPO protein of virG7 differs from that of virG7d by only a single amino acid substitution (Fig. 1B). This unique amino acid that is not shared with avrN as well as virG7 is located at position 35 of PIPO (Fig. 1B).

## PIPO of SMV lacks a determinant of virulence on *Rsv1*-genotype soybean

Recently, it has been shown that single mutations in P3 convert avrN to virN on L800 (*Rsv1*), but not PI96983 (*Rsv1*) (Hajimorad *et al*., 2011). For the virulence of avrN on PI96983 (*Rsv1*), simultaneous mutations in both HC-Pro and P3 are required (Hajimorad *et al*., 2008, 2011). L800 (*Rsv1*) is a recombinant soybean line selected from a population resulting from the crossing of SMV-susceptible Lee68 (*rsv1*) with PI96983 (*Rsv1*) (Hayes *et al*., 2004). The lower strength of resistance of L800 (*Rsv1*) to avrN, relative to PI96983 (*Rsv1*), is possibly a result of the possession of only one of the six nucleotide binding/leucine (Leu)-rich repeat-type candidate resistance genes at the *Rsv1* chromosomal region from PI96983 (*Rsv1*) (Hayes *et al*., 2004). However, the resultant phenotypes of L800 (*Rsv1*) against avrN, virG7 and virG7d resemble the phenotypic responses of PI96983 (*Rsv1*) (Fig. 2) (Hajimorad *et al*., 2003, 2006). As manipulation of the P3 cistron alone could convert avrN to virN on L800 (*Rsv1*) (Hajimorad *et al*., 2011), we utilized this *Rsv1*-genotype soybean to examine whether *pipo* carries virulence determinants of SMV.

Reciprocal exchanges of the unique nucleotide at genomic positions 2970 (G to A) of avrN and 2973 (A to G) of virG7 and virG7d resulted in nonsynonymous substitutions in *pipo* and the overlapping P3 codons of all the strains (Fig. 3). This was because the unique nucleotide at this position served as the first residue of the overlapping P3 codon, whereas it constituted the second residue of the *pipo*-encoded codon (Fig. 3). Thus, N_{C30Y}, $G7_{Y30C}$ and $G7d_{Y30C}$ all had an amino acid substitution in their respective P3. These were A947T in P3 of avrN and T948A in those of virG7 and virG7d (Figs 3 and 4; Table 1). All these mutants easily infected the *rsv1*-soybean genotypes (Table 1) (Hajimorad *et al*., 2011). N_{C30Y} gained virulence on L800 (*Rsv1*) (Fig. 4; Table 1); however, the virulence of $G7_{Y30C}$ and $G7d_{Y30C}$ was
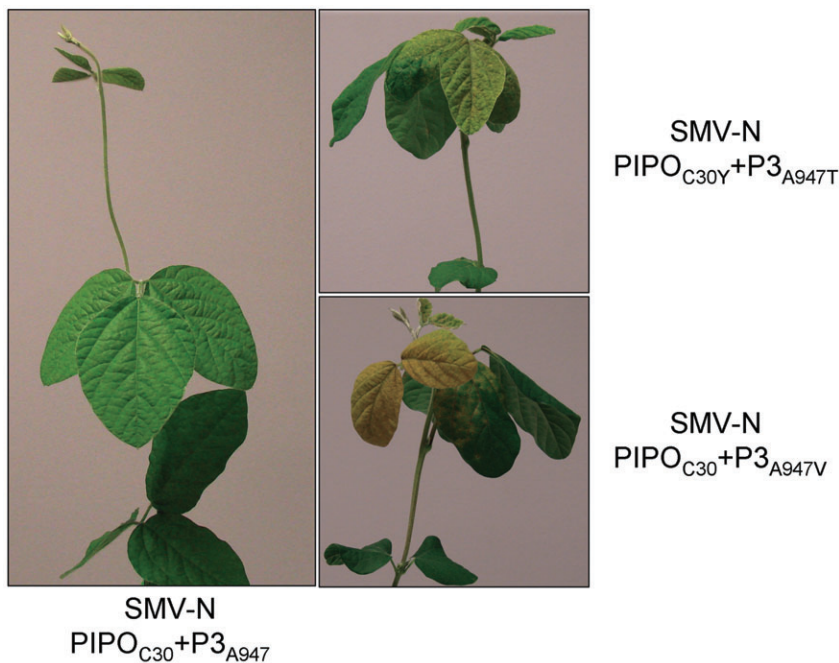

Fig. 2 Phenotypic responses of soybean L800 (*Rsv1*) to mechanical inoculation with progeny derived from the infectious cDNA clones of *Soybean mosaic virus* (SMV) strains N (SMV-N), G7 (SMV-G7) and G7d (SMV-G7d). Sap containing progeny of the viruses from biolistically inoculated Williams82 served as the inoculum. The inoculated plants were photographed 14 days post-inoculation. It should be noted that plants inoculated with SMV-N are functionally immune, do not exhibit any symptoms and the virus cannot be recovered from the inoculated plants. In contrast, L800 (*Rsv1*) inoculated with SMV-G7 and SMV-G7d is symptomatic. SMV-G7 induces stunting and systemic necrosis visible on the first trifoliate leaf. In contrast, SMV-G7d causes a systemic mosaic pattern.

severely compromised (Table 1). When Y30 in PIPO of virG7 was substituted with histidine (His) by changing *pipo*-encoded codon TAC to CAC (Fig. 3), $G7_{Y30H}$ remained virulent on L800 (*Rsv1*) (Table 1). This substitution [threonine (Thr) to His], unlike tyrosine (Tyr) to cysteine (Cys) replacement, did not alter the overlapping P3 codon (Table 1). Thus, the loss of virulence on L800 (*Rsv1*) by $G7_{Y30C}$ and $G7d_{Y30C}$ is probably a result of alteration in P3 and not PIPO. We also replaced Cys at position 30 of PIPO of avrN with arginine (Arg) and generated the N_{C30R} mutant. This mutation did not alter the overlapping P3 codon as it was synonymous (Table 1; Fig. 3). However, N_{C30R} was not stable and did not infect the *rsv1*-genotype soybeans efficiently, as two of the six biolistically inoculated Williams 82 (*rsv1*) and none of the Lee68 (*rsv1*) soybeans became infected (Table 1). Genotyping of the viral progeny from the infected Williams 82 (*rsv1*) soybean showed that those from one of the plants had a mutation in P3 at genomic position 2970 (G to U), which resulted in A947S and C30L replacements in P3 and PIPO, respectively. Analysis of the progeny from the second infected Williams 82 (*rsv1*) soybean showed reversion of the introduced substitution to the wild-type. As the C30R substitution did not alter the overlapping P3 codon, it is possible that this mutation affected a previously unrecognized *cis*-acting RNA sequence element. Regardless, N_{C30R} was not virulent on L800 (*Rsv1*) (Table 1). Hajimorad *et al*. (2011) have shown recently that N_{A947V}, similar to N_{A947T}, is virulent on L800 (*Rsv1*) (Fig. 4; Table 1). Unlike A947T substitution in the avrN genome, which changes the overlapping
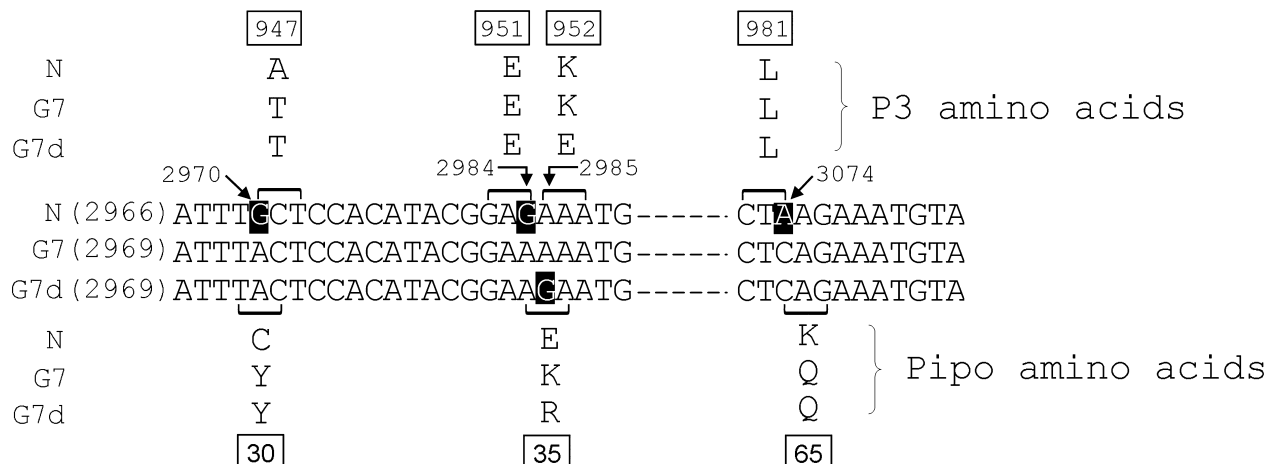
Fig. 3 Partial nucleotide sequences of the P3 cistron of the infectious cDNA clones of *Soybean mosaic virus* strains N, G7 and G7d harbouring codons expressing amino acids of P3 and the overlapping *pipo*-encoded protein. The square brackets above and below the nucleotide sequences show the codons encoding P3 and the unique PIPO amino acids, respectively. The unique nucleotide of each strain not shared with the others is highlighted. It should be noted that the genomic positions of *pipo* of G7 and G7d differ from that of N because of the presence of one additional codon upstream of the P3 cistron (GenBank accession numbers AY216010, AY216987 and D00507, respectively).

Fig. 4 Phenotypic responses of soybean L800 (*Rsv1*) to biolistic inoculation with the infectious cDNA clones of avirulent *Soybean mosaic virus* (SMV) strain N (SMV-N) and two SMV-N-derived virulent derivatives harbouring a parallel mutation in polyprotein codon 947 located in the P3 cistron. It should be noted that alanine (Ala) (A) to threonine (Thr) (T) substitution at position 947 leads to alteration in the overlapping PIPO codon (C30Y), whereas replacement with valine (Val) (V), instead of Thr, does not. This demonstrates that the determinant of virulence on L800 (*Rsv1*) resides on P3 and not the overlapping *pipo*-encoded protein. Plants were photographed at 14 days post-inoculation.

PIPO amino acid at position 30 from Cys to Tyr (Fig. 3), A947V substitution in P3 is synonymous for the *pipo*-encoded codon. This is because C to T substitution at genomic position 2971 of avrN alters the overlapping *pipo* codon TGC to TGT (Fig. 3). Both of these codons encode Cys. This clearly demonstrates that the determinant of virulence at this genomic position, involved in *Rsv1*-mediated resistance-breaking ability, resides on P3 and not PIPO.

Reciprocal substitutions between avrN and virG7 at position 35 of PIPO were synonymous for the overlapping P3 codons of these two viruses. This was because the unique nucleotide of *pipo*, corresponding to genomic position 2984 of avrN, constituted the third and first residues of the overlapping P3 and PIPO codons, respectively (Fig. 3). Thus, N_{E35K} and $G7_{K35E}$ were generated without alteration in P3 amino acids. Inoculation of N_{E35K} to L800 (*Rsv1*) resulted in one of five biolistically and one of 10

Table 1 Abilities of *Soybean mosaic virus* (SMV) strains N, G7 and G7d, as well as their derivative PIPO, P3 and PIPO+P3 mutants ,to infect systemically *rsv1*- and *Rsv1*-genotype soybeans following biolistic or mechanical inoculations.*

Viruses/mutants	Substitutions†			*rsv*1		*Rsv*1
	Nucleotide	P3 (aa)	PIPO (aa)	Lee68	Williams82	L800
SMV-N				(3/3)‡ 3/3§	(4/4) 4/4	(0/10) 0/4
SMV-G7				(3/3) 9/9	(3/3) 3/3	(3/3) 4/4
SMV-G7d				ND¶ 6/6	(3/3) 6/6	(3/3) 4/4
SMV-N$_{C30Y}$	G2970A	A947T**	C30Y	ND 5/5	(3/4) 5/5	(2/3) 6/6
SMV-N$_{C30R}$	T2969C	NA ††	C30R	(0/2) ND	(2ª‡‡/6) ND	(0/7) 0/3
SMV-N$_{E35K}$	G2984A	NA	E35K	(2/2) 3/3	(6/6) 4/4	(1ª/5) 1ª/10
SMV-N$_{K65Q}$	A3074C	NA	K65Q	(3/3) 3/3	(4/4) 2ª/2	(0/4) 0/4
SMV-N$_{E35K+K65Q}$	G2984A+A3074C	NA	E35K K65Q	(3/3) 6/6	(4/4) 3/3	(0/4) 1ª/10
SMV-N$_{E35R}$	G2984A+A2985G	K952E	E35R	ND 3/3	(6/6) 3ª/3	(0/4) 0/6
SMV-N$_{E35G}$	A2985G	K952E	E35G	ND 7/7	(4/4) 7/7	(0/3) 1ª/7
SMV-N$_{K816E+E35K}$	A2577G+G2984A	K816E	E35K	ND 3/3	(4/4) 4/4	(3/4ª) 6/6
SMV-G7$_{Y30C}$	A2973G	T948A	Y30C	ND 3/3	(3/3) 4ª/4	(0/4) 1ª/12
SMV-G7$_{Y30H}$	T2972C	NA	Y30H	(1/3) 5/5	(5/6) 4/5	(2ª/7) 6/6
SMV-G7$_{K35E}$	A2987G	NA	K35E	(3/3) 3/3	(4/4) 3/3	(4ª/4) 7/8
SMV-G7$_{Q65K}$	C3077A	NA	Q65K	(3/3) 3/3	(3/3) 3/3	(4ª/4) 6/8
SMV-G7$_{K35R}$	A2988G	K953E	K35R	ND 3/3	(4/4) 3/3	(3/4) 5/5
SMV-G7$_{K35E+Q65K}$	A2987G+C3077A	NA	K35E Q65K	(3/3) 6/6	(4/4) 3/3	(4ª/4) 9/9
SMV-G7d$_{Y30C}$	A2973G	T948A	Y30C	ND 4/5	(6/6) 2ª/4	(0/4) 1ª/4
SMV-G7d$_{R35E}$	A2987G+G2988A	E953K	R35E	ND 5/5	(6/6) 3ª/4	(3/4) 5/5
SMV-G7d$_{R35K}$	G2988A	E953K	R35K	ND 5/5	(7/7) 2ª/2	(3/4) 4/4
SMV-G7d$_{R35G}$	A2987G	NA	R35G	ND 6/6	(3/3) 7ª/7	(4/4) 4/4
SMV-G7d$_{Q65K}$	C3077A	NA	Q65K	ND 3/3	(4/4) 3/3	(4/4ª) 4/4
SMV-N$_{K816E}$	A2577G	K816E	NA	ND 3/3	(4/4) 4/4	(2ª/4) 6/6
SMV-N$_{V822M}$	G2595A	V822M	NA	ND 5/5	(3/3) 4/4	(2/3) 4ª/4
SMV-N$_{R945G}$**	A2964G	R945G	K28R	ND 5/5	(3/3) 2/3	(2/3) 4/5
SMV-N$_{A947V}$**	C2971T	A947V	NA	ND 6/6	(3/3) 7/7	(3/3) 8/9
SMV-N$_{P948L}$**	C2974T	P948L	NA	ND 7/7	(3/3) 7/7	(1/4) 4/5
SMV-N$_{V1045A}$**	T3265C	V1045A	NA	ND 6/6	(3/3) 3/3	(1/3) 4/5

*For biolistic inoculation, plasmid containing full-length infectious cDNA clones was delivered into fully expanded primary leaves of 2-week-old soybean seedlings. For mechanical inoculation, infectious sap from biolistically inoculated Williams82 (*rsv1*) was used as inoculum and rub-inoculated onto fully expanded primary leaves of 2-week-old soybean seedlings. The inoculated plants were maintained in a growth chamber (22 °C) until evaluation for infection based on symptom expression. Absence of virus in asymptomatic plants was confirmed by antigen-coated indirect enzyme-linked immunosorbent assay (ELISA).

†The positions of nucleotides or amino acid (aa) substitutions are based on genomic sequences of SMV strains N, G7 and G7d (GenBank accession nos. D00507, AY216010 and AY216987, respectively). For the position of PIPO amino acids, see Figs 1B and 3.

‡Number of plants systemically infected/number of plants inoculated biolistically.

§Number of plants systemically infected/number of plants sap inoculated mechanically.

¶Not done.

**Published data (Hajimorad *et al*., 2011).

††Not affected (substitution is either synonymous or located outside *pipo*).

‡‡The superscript letter 'a' indicates that, for each mutant, total RNA was extracted from one infected plant of the indicated genotype and subjected to reverse transcriptase-polymerase chain reaction (RT-PCR). The stability of the introduced mutation(s) and the lack of any newly emerged mutations in progeny viruses were confirmed by sequencing the entire P3 cistron of progeny viruses. Infection of one Williams82 (*Rsv1*) with SMV-N$_{C30R}$ was associated with an additional mutation in P3 at position C2970T, which resulted in A947S and C30L substitutions in P3 and PIPO, respectively. Progeny viruses from the second infected Williams82 with SMV-N$_{C30R}$ lacked the introduced mutation and the mutation had reverted to the wild-type. No mutation in P3 of the progenies derived from the single infected L800 (*Rsv1*) inoculated biolistically with SMV-N$_{E35K}$ was detected; however, these progenies failed to infect additional L800 (*Rsv1*) when inoculated mechanically. Progeny from L800 (*Rsv1*) mechanically inoculated with sap derived from biolistically inoculated Williams82 (*rsv1*) with SMV-N$_{E35K}$ contained a sequence polymorphism at position 2577. Thus, both codons AAG and GAG encoding lysine (Lys) and glutamic acid (Glu), respectively, were present at codon position 816 located in P3 (Fig. S1). Infection of L800 (*Rsv1*) with SMV-N$_{E35K+K65Q}$ was associated with C2671T substitution, which resulted in A847V mutation in P3. Infection of a single mechanically inoculated L800 (*Rsv1*) with SMV-G7$_{Y30C}$ was associated with only limited vein necrosis on the third trifoliate leaf at 21 days post-inoculation without any sign of necrosis on the leaf blade or any other visible symptoms; however, no additional mutation in P3 was detected. Infection of L800 (*Rsv1*) by SMV-N$_{E35G}$ was associated with a sequence polymorphism in P3 at position 2709, where both TCA and ACA codons encoding serine (Ser) and threonine (Thr), respectively, were present at polyprotein position 860. No newly emerged mutations in P3 cistrons of other progenies derived from any of the other molecularly cloned mutant viruses were detected.

mechanically inoculated plants (Table 1). However, sequences of progeny viruses from the mechanically inoculated L800 (*Rsv1*) showed polymorphism in the P3 amino acid at position 816 (Fig. S1, see Supporting Information). One additional passage of these progeny viruses in L800 (*Rsv1*) resulted in the fixation of Lys to glutamic acid (Glu) at the polyprotein position 816 (Fig. S1). This genomic position is located upstream of *pipo* of avrN (data not shown). When the K816E mutation was introduced into P3 of avrN and the mutant was inoculated to L800 (*Rsv1*), N_{K816E} was virulent (Table 1). We further reconstructed K816E+E35K mutations in avrN. Biolistic inoculation of $N_{K816E+E35K}$, as well as mechanical inoculation of its derivative progeny to L800 (*Rsv1*), showed that $N_{K816E+E35K}$, similar to N_{K816E}, was virulent (Table 1). Thus, E35K substitution in PIPO of avrN alone does not confer virulence on L800 (*Rsv1*). The observation that the virulence of $G7_{K35E}$ on L800 (*Rsv1*) and PI96983 (*Rsv1*) was not compromised (Table 1; data not shown) further allowed us to conclude that the PIPO residue at position 35 is not involved in the virulence of virG7 on *Rsv1*-genotype soybeans.

Reciprocal exchanges at position 35 of PIPO between virG7d and avrN, as well as virG7 and virG7d, were problematic. This was because the position of the unique nucleotide of *pipo* of virG7d constituted the first residue of the overlapping P3 codon (Fig. 3). The overlapping P3 codon in avrN and virG7 at this position, AAA, encodes Lys, whereas that of virG7d, GAA, codes for Glu (Fig. 3). Thus, the synthesis of N_{E35R} also resulted in K952E substitution in P3 (Fig. 3; Table 1). However, $N_{E35R+K952E}$, similar to avrN, remained avirulent on L800 (*Rsv1*) (Table 1). We also generated N_{E35G} that contained the K952E substitution in P3 as well; however, $N_{E35G+K952E}$, similar to $N_{E35R+K952E}$, remained avirulent on L800 (*Rsv1*) (Table 1). Synthesis of $G7_{K35R}$ also resulted in a substitution in P3 at position 953 (Lys to Glu) (Fig. 3). However, similar to virG7, $G7_{K35R}$, which contained the K953E substitution in P3, remained virulent on L800 (*Rsv1*) (Table 1). We also exchanged the amino acid at position 35 in PIPO of virG7d and generated $G7d_{R35E}$, $G7d_{R35K}$ and $G7d_{R35G}$. Both $G7d_{R35E}$ and $G7d_{R35K}$ contained a mutation in P3 at position 953 (Glu to Lys), whereas $G7d_{R35G}$ lacked this mutation (Table 1). However, inoculation of these three mutants on L800 (*Rsv1*) showed that all remained virulent on L800 (*Rsv1*) (Table 1). $G7d_{R35G}$ also remained virulent on PI96983 (*Rsv1*) (data not shown).

Reciprocal substitution at position 65 of PIPO was synonymous in relation to P3; hence, it did not alter the overlapping encoded P3 amino acid in any of the strains. This was because the unique nucleotide of avrN, corresponding to genomic position 3074, constituted the third residue with regard to the P3 codon (Fig. 3). However, the virulence of the resultant mutants did not vary from that of the parental viruses, as N_{K65Q} remained avirulent, whereas $G7_{Q65K}$ and $G7d_{Q65K}$ were both virulent on L800 (*Rsv1*) (Table 1). $G7_{Q65K}$ also remained virulent on PI96983 (*Rsv1*) (data not shown). We also introduced double mutations in

PIPO of avrN and virG7 without any alterations in the amino acid of their respective P3, and generated $N_{E35K+K65Q}$ and $G7_{K35E+Q65K}$. Inoculation of these two mutants, each with double substitutions in PIPO, to L800 (*Rsv1*) showed that $N_{E35K+K65Q}$ remained avirulent, whereas $G7_{K35E+Q65K}$ was virulent (Table 1). Furthermore, $G7_{K35E+Q65K}$ also remained virulent on PI96983 (*Rsv1*) (data not shown).

CONCLUSIONS

The main purposes of this study were to exploit the differential interactions of SMV strains avrN, virG7 and virG7d with *Rsv1*-genotype soybeans and to answer the question of whether the virulence determinants of SMV on functionally immune *Rsv1*-genotype soybeans reside on P3 or the overlapping *pipo*-encoded protein. Thus, this study did not aim to provide an understanding of the functional role(s) of mutations in virulence or to reveal the underlying mechanism(s) of functional immunity mediated by the *Rsv1* locus against SMV-N.

The reciprocal exchanges of the unique amino acid of PIPO, without alteration in P3 residues, among the three strains did not alter their phenotypes on L800 (*Rsv1*). Previously, via the experimental adaptation of avrN to *Rsv1*-genotype soybeans, we have identified several mutations in the P3 cistron that convert avrN to virN on L800 (*Rsv1*) (Hajimorad *et al.*, 2011). These include substitutions at polyprotein positions 945 [Arg to glycine (Gly)], 948 [proline (Pro) to Leu], 947 [alanine (Ala) to Thr], 947 [Ala to valine (Val)] and 1045 (Val to Ala) (Hajimorad *et al.*, 2011) (Table 1). In the present study, we also identified two additional mutations in the P3 cistron, V822M and K816E, which converted avrN to virN on L800 (*Rsv1*) (Table 1). Analyses of all of these mutations in relation to P3 and PIPO showed that substitution at polyprotein position 1045 is located downstream of *pipo*; hence, it affects only P3. However, substitutions at polyprotein positions 816 and 822 are upstream of *pipo*; thus none has a direct impact on *pipo*-encoded protein. However, R945G, A947T, A947V and P948L substitutions all overlap *pipo*-encoded codons. Among these mutations, R945G and A947T substitutions altered the overlapping PIPO codons as well. R945G substitution resulted in K28R substitution in PIPO of avrN. As avrN, virG7 and virG7d all encode a Lys at position 28 of PIPO, gain of virulence by N_{R945G} on L800 (*Rsv1*) is probably a result of substitution in P3 and not PIPO. However, A947T substitution resulted in C30Y replacement in PIPO. Interestingly, a parallel mutation at this site, A947V, which converted avrN to virN on L800 (*Rsv1*), altered only P3 but not the overlapping *pipo*-encoded amino acid (Fig. 3; Table 1). However, both $N_{C30Y+A947T}$ and N_{A947V} were virulent on L800 (*Rsv1*) and induced similar phenotypes (Hajimorad *et al.*, 2011) (Fig. 4). Hence, these data collectively demonstrate that virulence determinants of SMV on *Rsv1*-genotype soybean reside on P3 and not the overlapping *pipo*-encoded protein.

In a number of other potyviral pathosystems, the P3 cistron is involved in adaptations to new hosts or in conferring ability to overcome resistance or tolerance. We analysed the reported virulence determinants of these viruses to determine whether *pipo* is involved in any of these interactions. A mutation (A1047V) in the P3 cistron of *Tobacco etch virus* (TEV), which was associated with the adaptation of TEV to *Arabidopsis thaliana* (Agudelo-Romero *et al*., 2008), is located downstream of *pipo*. The determinants of virulence of *Turnip mosaic virus* (TuMV) to overcome two resistance genes in *Brassica napus* are mapped to the P3 cistron (Jenner *et al*., 2002, 2003), but are positioned outside *pipo*. An important determinant of TuMV to infect *Brassica* spp. and/or *Raphanus sativus*, which is positioned in the P3 cistron (Suehiro *et al*., 2004), does not overlap *pipo*. The N-terminal half of the P3 cistron of TuMV, which induces hypersensitive response-like cell death in *A. thaliana* (Kim *et al*., 2010), is positioned upstream of *pipo*. Most of the mutations in the P3 cistron of TuMV essential for adaptation to *R. sativus* (Tan *et al*., 2005) do not alter PIPO, except the C3172T substitution that changes the overlapping *pipo*-encoded amino acid. Multiple determinants in P3 of *Pea seed-borne mosaic virus*, involved in virulence against sbm-2 resistance in *Pisum sativum* (Hjulsager *et al*., 2006), are positioned upstream of *pip*o. Tolerance breaking of *Zucchini yellow mosaic virus* in tolerant zucchini cultivars is related to a point mutation in the P3 cistron (Desbiez *et al*., 2003; Glasa *et al*., 2007); however, the substitution at position 917 is located adjacent to the conserved *pipo* motif that affects only P3. Thus, in all of these pathosystems, except TuMV–*R. sativus*, the determinants are mapped to the P3 protein and *pipo* is not involved. However, the role of *pipo* of TuMV in the adaptation of the virus to *R. sativus* remains to be determined.

EXPERIMENTAL PROCEDURES

Viruses, soybean genotypes, inoculation and SMV detection

The plasmids pSMV-N (GenBank Accession No. D00507), pSMV-G7 (GenBank Accession No. AY216010), pSMV-G7d (GenBank Accession No. AY216987), pSMV-N$_{V822M}$, pSMV-N$_{A947T}$ and pSMV-N$_{A947V}$ have been described previously (Hajimorad *et al*., 2003, 2006, 2011; Wang *et al*., 2006). All plasmids were propagated in ElectroMax DH5α-E (Invitrogen, Carlsbad, CA, USA) and were purified using a QiaPrep Spin MiniPrep Kit (Qiagen, Valencia, CA, USA). To establish infection with plasmid DNA, fully expanded primary leaves of soybean seedlings were biolistically inoculated as described previously (Hajimorad *et al*., 2003, 2008). Sap containing viral progenies in 50 mM phosphate buffer, pH 7.0, derived from the infected tissues of biolistically inoculated soybeans, was used as inoculum to mechanically inoculate carborundum (600-mesh)-dusted primary leaves of

soybeans (Hajimorad and Hill, 2001). Soybean (*Glycine max*) genotypes Williams82 (*rsv1*) and Lee68 (*rsv1*), both universally susceptible to all SMV strains, and *Rsv1*-soybeans PI96983 and L800, both functionally immune to avrN, were used in this study (Bernard *et al*., 1991; Hayes *et al*., 2004). PI96983 (*Rsv1*) is a soybean plant introduction (PI) containing the *Rsv1* resistance locus, and was originally collected in Hwanghae Puk, South Korea, and introduced to the USA in 1932. Soybean L800 (*Rsv1*) is a recombinant line containing only one of the six nucleotide binding/Leu-rich repeat-type candidate resistance genes at the *Rsv1* chromosomal region from PI96983 (*Rsv1*) (Hayes *et al*., 2004). L800 (*Rsv1*) was selected from a population resulting from the crossing of Lee68 (*rsv1*) with PI96983 (*Rsv1*) (Hayes *et al*., 2004). All seeds were obtained from glasshouse- or field-grown plants shown to be free of SMV by indexing. The inoculated plants were maintained in a growth chamber operating at 22 °C with a photoperiod of 16 h. The detection of SMV in the inoculated plants was performed by symptomatology, enzyme-linked immunosorbent assay (ELISA) (Malapi-Nelson *et al*., 2009) or reverse transcriptase-polymerase chain reaction (RT-PCR) (Hajimorad *et al*., 2008).

Construction of point mutants by site-directed mutagenesis

The megaprimer PCR-based mutagenesis method (Sambrook and Russell, 2001) was used for the synthesis of all site-directed mutants employing PrimeStar HS DNA polymerase (Takara Bio, Madison, WI, USA). All the oligonucleotide primers used are listed in Table S1 (see Supporting Information).

To generate SMV-N$_{C30R}$, SMV-N$_{E35K}$, SMV-N$_{E35R}$, SMV-N$_{E35G}$, SMV-N$_{K65Q}$ and SMV-N$_{K816E}$, the mutagenic primers N-2969s, N-2984s, N-ERs, N-2985s, N-3074s and N-2577a (Table S1), respectively, were used in PCRs in the presence of N-3264a and pSMV-N, which served as the template. The resultant PCR products with each primer set served as megaprimers in the presence of SMV-2289s and pSMV-N to generate genomic fragments spanning nucleotides 2289–3264 and containing the desired mutations. The resultant amplified fragments were digested with *Kpn*I and *Spe*I and ligated into pSMV-N. To generate SMV-N$_{K816E+E35K}$, the mutagenic primer N-2577a (Table S1) was used in the presence of N-2289s and pSMV-N$_{E35K}$ as the template in the first PCR. The resultant amplified fragment served as a megaprimer in the presence of SMV-3264a and pSMV-N in the second PCR. The mutagenized fragment was digested with *Kpn*I and *Spe*I and ligated into pSMV-N. To generate SMV-N$_{E35K+K65Q}$, the mutagenic primer N-3074s was used in the presence of N-3264a primer and pSMV-N$_{E35K}$ as the template in the first PCR. The resultant amplified fragment served as a megaprimer in the presence of SMV-2289s and pSMV-N in the second PCR. The mutagenized fragment was digested with *Kpn*I and *Spe*I and ligated into pSMV-N.

To generate SMV-G7$_{Y30H}$, SMV-G7$_{Y30C}$, SMV-G7$_{K35E}$, SMV-G7$_{K35R}$ and SMV-G7$_{Q65K}$, the mutagenic primers G7-2972s, G7-2973s, G7-2987s, G7-KRs and G7-3077s (Table S1), respectively, were initially used in PCRs in the presence of primer G-3263a and pSMV-G7 as the template. The resultant PCR products with each of the primer sets served as megaprimers in the presence of SMV-2289s and pSMV-G7 to generate genomic fragments spanning nucleotides 2289–3263 containing the desired mutations. Subsequently, the fragments were digested with *Kpn*I and *Spe*I and ligated into pSMV-G7. To generate SMV-G7$_{K35E+Q65K}$, the mutagenic primer G7-3077s was used in the presence of primer G7-3263a and pSMV-G7$_{K35E}$, which served as the template. The resultant PCR products served as megaprimers in the presence of SMV-2289s and pSMV-G7 to generate the mutagenized fragment containing two mutations; this was subsequently digested with *Kpn*I and *Spe*I and ligated into pSMV-G7.

To generate SMV-G7d$_{Y30C}$, SMV-G7d$_{R35G}$, SMV-G7d$_{R35E}$, SMV-G7d$_{R35K}$ and SMV-G7d$_{Q65K}$, the mutagenic primers d-2973s, d-2987s, d-2988s, d-RKs and d-3077s (Table S1), respectively, were used in PCRs in the presence of primer G-3263a and pSMV-G7d as the template. The resultant PCR product with each of the primer sets served as a megaprimer in the presence of SMV-2289s and pSMV-G7d to generate mutagenized genomic fragments spanning nucleotides 2289–3264 containing the desired mutations. The resultant amplified fragments were digested with *Kpn*I and *Spe*I and ligated into pSMV-G7d.

To ensure the absence of any undesired PCR-generated mutation, the entire amplified regions of each of the mutant viruses were sequenced and analysed. The infectivity of all the mutants was tested on Williams82 by biolistic inoculation.

RT-PCR and sequencing

To verify the stability of the introduced mutations *in planta*, the genomic region of all the progeny viruses containing the entire P3 cistron was reverse transcribed in the presence of primer SMV-3910a (Table S1) with SuperScript reverse transcriptase (Invitrogen). PCR amplification of the entire P3 citrons was performed using primers SMV-2289s and SMV-3840a (Table S1) in the presence of Ex *Taq* polymerase (Takara Bio). The PCR products were purified with a QIAquick-PCR Purification Kit (Qiagen) and sequenced using the same primers. Sequencing was performed at the University of Tennessee DNA Sequencing Facility, Knoxville, TN, USA. The sequences were edited and analysed using Vector NTI (Invitrogen).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Electropherograms showing the sequences of reverse transcriptase-polymerase chain reaction (RT-PCR) products from progenies derived from SMV-N$_{E35K}$. Primary leaves of Williams 82 (*rsv1*) were biolistically inoculated with pSMV-N$_{E35K}$, and systemically infected trifoliate leaves harvested 21 days post-inoculation were either subjected to RT-PCR analysis or used as the source of inoculum to inoculate L800 (*Rsv1*). (A) Partial P3 sequences generated by a forward primer are shown. SMV-N nucleotides, the encoded amino acids and their positions within the genome and the viral polyprotein, located in the P3 cistron, are shown above the sequences, and soybean genotypes are given on the left side of the electropherograms. The polymorphism at nucleotide position 2577 (indicated by an arrow) in the sequences derived from the first passage of progenies in L800 (*Rsv1*), but not in those from Williams82 (*rsv1*), and fixation of the mutation in those derived from the second and fifth passages of progenies should be noted. (B) Electropherogram showing the sequences of an RT-PCR product from the first passage in L800 (*Rsv1*) generated with a reverse primer to demonstrate the polymorphism shown in (A), and generated with a forward primer; this is not a sequencing artefact.

Table S1 Sequences of oligonucleotide primers used for site-directed mutagenesis, polymerase chain reaction (PCR), reverse transcriptase-polymerase chain reaction (RT-PCR) and sequencing.

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