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Short communication

Amino acid substitution in P3 of *Soybean mosaic virus* to convert avirulence to virulence on *Rsv4*-genotype soybean is influenced by the genetic composition of P3

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SUMMARY

The modification of avirulence factors of plant viruses by one or more amino acid substitutions converts avirulence to virulence on hosts containing resistance genes. Limited experimental studies have been conducted on avirulence/virulence factors of plant viruses, in particular those of potyviruses, to determine whether avirulence/virulence sites are conserved among strains. In this study, the *Soybean mosaic virus* (SMV)–*Rsv4* pathosystem was exploited to determine whether: (i) avirulence/virulence determinants of SMV reside exclusively on P3 regardless of virus strain; and (ii) the sites residing on P3 and crucial for avirulence/virulence of isolates belonging to strain G2 are also involved in virulence of avirulent isolates belonging to strain G7. The results confirm that avirulence/virulence determinants of SMV on *Rsv4*-genotype soybean reside exclusively on P3. Furthermore, the data show that sites involved in the virulence of SMV on *Rsv4*-genotype soybean vary among strains, with the genetic composition of P3 playing a crucial role.

Keywords: genetic background, *Glycine max*, potyvirus, resistance gene, virulence determinants.

Plants have evolved diverse strategies to resist pathogens including viruses (Hull, 2002). One strategy involves classical nucleotide-binding site leucine-rich repeat (NBS-LRR) resistance (*R*) genes (Maule *et al*., 2007). Resistance mediated by this group of *R* genes operates on the basis of a 'gene-for-gene' model, in which an *R*-gene-encoded protein in a host recognizes, directly or indirectly, a pathogen avirulence gene product, termed an avirulence factor (Flor, 1971; Jones and Dangl, 2006). In the majority of viral pathosystems, *R*-gene-mediated recognition activates host defence signalling pathways leading to the expression of a hypersensitive response (HR) (Dangl and Jones, 2001; Palukaitis and Carr, 2008). However, there are instances involving the NBS-LRR class of *R* genes in which the resistance phenotype is expressed as extreme resistance (ER) and HR expression is not required to limit infection (Bendahmane *et al*., 1999; Hajimorad and Hill, 2001).

Interestingly, unusual *R* genes against plant viruses have been reported that differ from the NBS-LRR class and the mechanism of resistance does not involve HR or ER. These include *RTM1*/*RTM2*/*RTM3* in Arabidopsis against *Tobacco etch virus* (TEV), *Scmv2* in maize against *Sugarcane mosaic virus* (SCMV) and *Rsv4* in soybean against *Soybean mosaic virus* (SMV) (Chisholm *et al*., 2000; Cosson *et al*., 2010; Ingvardsen *et al*., 2010; Kuntze *et al*., 1997; Saghai Maroof *et al*., 2010; Whitham *et al*., 2000). The *RTM* genes prevent systemic, but not cell-to-cell, movements of TEV within the inoculated leaves (Chisholm *et al*., 2001; Decroocq *et al*., 2009; Mahajan *et al*., 1998). Similarly, the mechanism of resistance against SCMV in *Scmv2*-genotype maize seems to operate at the level of long-distance movement (Ingvardsen *et al*., 2010; Kuntze *et al*., 1997). However, *Rsv4*, which belongs to a previously uncharacterized type or class of *R* gene, restricts SMV movement and accumulation in inoculated leaves and also prevents systemic movement (Gunduz *et al*., 2004; Khatabi *et al*., 2012; Saghai Maroof *et al*., 2010).

Regardless of the class or type of *R* gene, resistance phenotypes or the mechanism of resistance, experimental modification of a single virus-encoded protein by one or more mutations converts avirulent viruses to virulent or *R*-genotype plants (Decroocq *et al*., 2009; Hajimorad *et al*., 2011; Meshi *et al*., 1989; Wen *et al*., 2013). Avirulence/virulence function has been assigned to a number of viral-encoded proteins of non-potyviruses on *R*-genotype hosts; however, studies on potyviral-encoded proteins serving as avirulence factors are limited (Hull, 2002). Genome organization and gene expression strategy vary widely among plant viruses. In some, such as tobamoviruses and potexviruses, viral-encoded proteins are expressed from single open reading frames (ORFs) and avirulence/virulence function dissociated from

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virus infection has been assigned to diverse proteins of these groups of viruses (Bendahmane *et al*., 1999; Malcuit *et al*., 1999; Meshi *et al*., 1989; Padgett and Beachy, 1993; Pfitzner and Pfitzner, 1992). In contrast, a potyviral genome contains one single long ORF that is expressed as a precursor polypeptide, which is subsequently cleaved co-translationally and post-translationally by three virus-encoded proteases to produce 8–10 functional proteins (Urcuqui-Inchima *et al*., 2001). Thus, at any given time, a number of functional potyviral-encoded proteins, which are produced following processing *in trans*, are present in different polypeptide contexts within the infected cells. Although some *in trans* cleavage events occur rapidly and efficiently, others are slow events, which seem to have biological significance (Urcuqui-Inchima *et al*., 2001). Despite this fundamental difference in genome expression strategy, it has been demonstrated that a single virus-encoded protein serves as the avirulence/virulence factor in a number of potyviral pathosystems involving *R* genes. However, the majority of these studies were conducted in the context of virus infection and in the presence of other viral functional proteins (Chowda-Reddy *et al*., 2011a; Jenner *et al*., 2000; Khatabi *et al*., 2012; Kim *et al*., 2010; Seo *et al*., 2009a; Zhang *et al*., 2009). Interestingly, there are a few other potyviral pathosystems involving *R* genes in which avirulence/virulence function has been attributed to more than one virus protein (Chowda-Reddy *et al*., 2011b; Chu *et al*., 1997; Eggenberger *et al*., 2008; Hajimorad *et al*., 2008, 2011; Jenner *et al*., 2002a; Krause-Sakate *et al*., 2005). In two of these pathosystems, *Turnip mosaic virus–Brassica napus* line 165 and SMV–*Rsv1* pathosystems, subsequent studies have shown the presence of more than one *R* gene against each virus (Jenner *et al*., 2002a; Wen *et al*., 2013).

SMV is a species within the genus *Potyvirus*. A number of SMV strains, G1–G7, have been reported in North America (Cho and Goodman, 1979). Expression of the SMV genome results in the production of a number of multifunctional proteins, including helper-component proteinase (HC-Pro), P3 and cytoplasmic inclusion (CI) (Urcuqui-Inchima *et al*., 2001). In addition, a small ORF (i.e. *pipo*) embedded in the P3 cistron encodes a protein in the +2 frame that is involved in virus movement (Chung *et al*., 2008; Wen and Hajimorad, 2010). *Rsv4*, a single atypical *R* gene in V94-5152 and PI88788 soybean, confers resistance against SMV (Buss *et al*., 1997; Gunduz *et al*., 2004; Khatabi *et al*., 2012; Saghai Maroof *et al*., 2010). V94-5152 (*Rsv4*) confers resistance against a larger number of SMV strains relative to PI88788 (*Rsv4*) (Gunduz *et al*., 2004; Khatabi *et al*., 2012). The source of the difference in resistance effectiveness between these two genotypes is unknown. Regardless, the phenotype of resistance conferred by *Rsv4* is not associated with ER or HR (Gunduz *et al*., 2004; Khatabi *et al*., 2012).

It has been shown previously that P3 of SMV isolates belonging to the G2 strain serves as an avirulence/virulence factor on V94-5152 (Chowda-Reddy *et al*., 2011a; Khatabi *et al*., 2012). Experimentally, substitution at polyprotein position 1033 [glutamine (Gln) to lysine (Lys)] or 1054 [glycine (Gly) to arginine (Arg)] of SMV isolates and variants derived from the G2 strain converted avirulence to virulence on V94-5152 (Chowda-Reddy *et al*., 2011a; Khatabi *et al*., 2012). Recently, the virulence site of an SMV isolate originating from Iran, unassigned to a specific strain, was mapped experimentally to polypeptide position 1053 located on P3, where a serine (Ser) to asparagine (Asn) substitution converted avirulence to virulence (Ahangaran *et al*., 2013). In contrast, simultaneous mutations in multiple SMV proteins (HC-Pro, P3, CI) have been reported to be essential for the gain of virulence of an isolate belonging to the G7 strain (SMV-G7) on V94-5152 (Chowda-Reddy *et al*., 2011b).

Our main objective in this study was to evaluate the gain of virulence (i.e. ability to move systemically) by two isolates of the G7 strain, SMV-G7 and SMV-G7d, on V94-5152 and PI88788. Two hypotheses were tested: (i) avirulence/virulence determinants of SMV on *Rsv4*-genotype soybean reside exclusively on P3; and (ii) the site on P3 essential for gain of virulence on *Rsv4*-genotype soybean is influenced by the genetic composition of P3. In this article, we have defined virulence as the capability of an SMV strain, or an SMV variant, to move systemically in *Rsv4*-genotype soybean regardless of the symptom severity displayed.

When SMV-G7 and SMV-G7d tagged with β-glucuronidase (GUS) were inoculated biolistically on attached primary leaves of V94-5152 and PI88788, SMV-G7-GUS and SMV-G7d-GUS both established local infection (Fig. 1). However, unlike infection in inoculated leaves of Essex (*rsv4*), a universally susceptible cultivar to SMV, the infected foci in the inoculated leaves of both *Rsv4*-genotype soybeans did not extend to the veins (Fig. 1). Furthermore, in contrast with infection in Essex, in which both viruses moved systemically, none was detected in non-inoculated leaves of the two *Rsv4*-genotype soybeans regardless of mechanical or biolistic inoculation and the presence or absence of the *uidA* gene (Fig. 1; Table 1).

It should be noted that interactions of SMV-G7 and SMV-G7d with V94-5152 are no different from that of SMV-N, an isolate belonging to the G2 strain; however, SMV-N is virulent on PI88788, whereas both SMV-G7 and SMV-G7d are avirulent (Khatabi *et al*., 2012). A number of other field isolates of SMV are also virulent on PI88788, but not on V94-5152 (Khatabi *et al*., 2012; Fig. S1, see Supporting Information). Previously, we have shown experimentally that substitution of glutamine (Gln) to Lys or Gly to Arg at polyprotein positions 1033 and 1054 of SMV-N, respectively, results in the virulence of both SMV-N_{Q1033K} and SMV-N_{G1054R} on V94-5152 (Khatabi *et al*., 2012). Alignment of P3 of SMV-G7 and SMV-G7d with that of SMV-N shows that Gln is also present at polypeptide position 1034 of SMV-G7 and SMV-G7d, which corresponds to position 1033 on the SMV-N genome (Fig. S2, see Supporting Information). However, at polypeptide

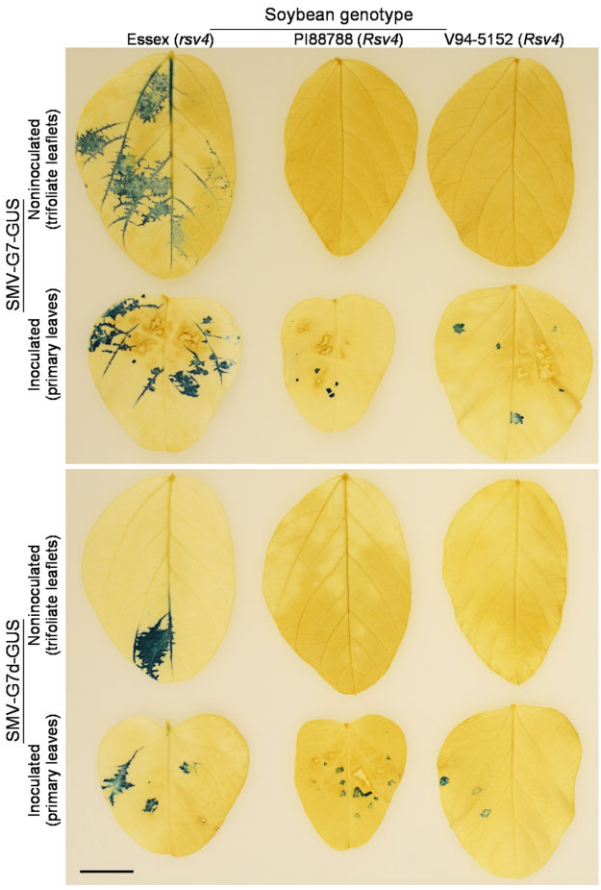

Fig. 1 β-Glucuronidase (GUS) expression in soybean leaf tissues following biolistic inoculation. Fully expanded attached primary leaves of soybean seedlings were inoculated with plasmids containing the full-length infectious cDNA clone of *Soybean mosaic virus* (SMV) strains G7 or G7d tagged with GUS (SMV-G7-GUS or SMV-G7d-GUS). The inoculated plants were analysed histochemically at 21 days post-inoculation. Scale bar, 2 cm.

position 1055 of SMV-G7 and SMV-G7d, corresponding to position 1054 of SMV-N, Ser instead of Gly is present (Fig. S2). To evaluate the impact of amino acid substitutions at polyprotein positions 1034 and 1055 on the virulence of SMV-G7, we synthesized SMV-$G7_{Q1034K}$ and SMV-$G7_{S1055R}$ (the details of the creation of point mutations, reverse transcription-polymerase chain reaction (RT-PCR) and sequencing are listed in Materials and Methods S1 and the primers used are listed in Table S1, see Supporting Information). Biolistic inoculation of SMV-$G7_{Q1034K}$ resulted in virulence on PI88788 and V94-5152, but SMV-$G7_{S1055R}$ remained avirulent on both *Rsv4*-genotype soybeans regardless of biolistic or mechanical inoculations (Table 1; Fig. 2). SMV-$G7_{S1055R}$ infected systemically Essex and the mutation was stable (Table 1). When Q1034K + S1055R were introduced simultaneously into P3 of SMV-G7, the rate of systemic movement of SMV-$G7_{Q1034K+S1055R}$ on *Rsv4*-genotype soybean was similar to that of SMV-$G7_{Q1034K}$ (Table 1; Fig. 2). In general, biolistic inoculation with plasmids was more

Table 1 Abilities of *Soybean mosaic virus* (SMV) strains G7 and G7d and derivative P3 mutants to infect systemically *rsv4*- and *Rsv4*-genotype soybeans following biolistic or mechanical inoculations†.

	Soybean genotypes		
Viruses/mutants/chimeras	Essex (*rsv4*)	PI88788 (*Rsv4*)	V94-5152 (*Rsv4*)
Viruses			
SMV-G7	(5/5)‡ 10/11§	(0/7) 0/22	(0/8) 0/11
SMV-G7d	(5/5) 9/9	(0/7) 0/12	(0/9) 0/12
SMV-G7-derived mutants			
SMV-$G7_{Q1034K}$	(10/10) 13/22	(4*¶/4) 3/17	(3*/4) 0/25
SMV-$G7_{S1055R}$	(6**/6) 18/18	(0/6) 0/11	(0/6) 0/12
SMV-$G7_{Q1034K+S1055R}$	(12/13) 13/16	(3/7) 5/15	(6*/7) 3/23
SMV-$G7_{H1054N}$	(9**/9) 5/5	(0/9) 0/9	(0/9) 0/18
SMV-G7d-derived mutants			
SMV-$G7d_{Q1034K}$	(4**/12) 8/13	(0/3) 0/12	(0/3) 0/10
SMV-$G7d_{S1055R}$	(8**/8) 13/13	(0/3) 0/21	(0/3) 0/24
SMV-$G7d_{Q1034K+S1055R}$	(11/11) 14/27	(2/6) 3/16	(1*/6) 3/20
SMV-$G7d_{H1054N}$	(6**/6) 9/9	(0/4) 0/32	(0/3) 0/23
SMV-G7d-derived chimeras			
SMV-G7d/G7P3	(4**/4) 10/10	(0/5) 0/10	(0/7) 0/10
SMV-G7d/$G7P3_{Q1034K}$	(6/6) 8/8	(2/3) 3/5	(1*/3) 0/14
SMV-G7d/$G7P3_{Q1034K+S1055R}$	(6/6) 5/5	(3/3) 5/5	(3*/3) 9/10

†For biolistic inoculation, plasmid containing full-length infectious cDNA clones was delivered into fully expanded attached primary leaves of soybean seedlings. For mechanical inoculation, sap derived from biolistically inoculated Essex was used as inoculum and rub inoculated onto fully expanded primary leaves of soybean seedlings. The inoculated plants were evaluated for infection based on symptom expression at 21 days post-inoculation. Absence of virus in asymptomatic plants was confirmed by enzyme-linked immunosorbent assay (ELISA) using antiserum to recombinant coat protein of SMV-G7d (see Materials and Methods S1).

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

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

¶The asterisks indicate that total RNA was extracted from one of the infected *Rsv4*-genotype plants and subjected to reverse transcription-polymerase chain reaction (RT-PCR). The stability of the introduced mutation(s) and the lack of any newly emerged mutation in helper-component proteinase (HC-Pro), P3 and cytoplasmic inclusion (CI) proteins of progeny viruses were confirmed by sequencing the entire cistron (*). For non-virulent mutants on *Rsv4*-genotype plants, total RNA was extracted from one of the systemically infected Essex plants and subjected to RT-PCR, but only the entire P3 cistron was sequenced (**). Infection of Essex with SMV-$G7d_{Q1034K}$ was associated with an additional mutation in the P3 cistron, which resulted in K1034N substitution. No newly emerged mutation was detected in P3, HC-Pro or CI derived from other progenies of molecularly cloned mutant viruses recovered from *Rsv4*-genotype soybean.

efficient than mechanical inoculation using progeny viruses derived from biolistically inoculated Essex (Table 1). To rule out the emergence of any new mutation *in planta*, the entire HC-Pro, P3 and CI cistrons of progeny viruses were RT-PCR amplified from one of the systemically infected *Rsv4*-genotype soybeans inoculated biolistically with each of the SMV-G7-derived P3 mutants. Sequence analysis showed the absence of any new mutation in

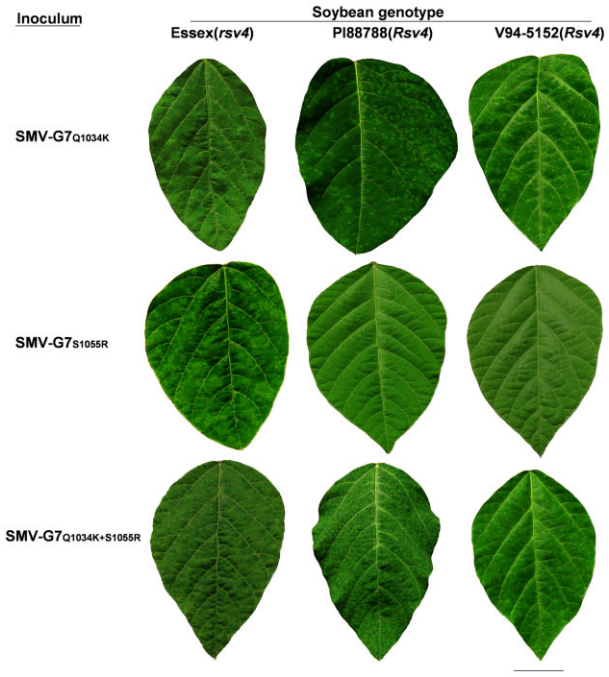

Fig. 2 Phenotypic differences in the systemic responses of soybean genotypes following biolistic inoculation of fully expanded attached primary leaves with plasmids containing the full-length infectious cDNA clone of *Soybean mosaic virus* (SMV)-G7-derived P3 mutants. Representative leaflets from non-inoculated trifoliate leaves were photographed at 21 days post-inoculation. Note that the phenotypic responses of *Rsv4*-genotype soybeans to biolistic inoculation with molecularly cloned SMV-G7 are similar to those expressed by SMV-$G7_{S1055R}$. Scale bar, 2 cm.

HC-Pro, P3 and CI cistrons, and verified the stability of the introduced mutations (Table 1). To determine whether any additional adaptive or compensatory mutation emerges following multiple passages of progeny viruses derived from SMV-$G7_{Q1034K+S1055R}$ in V94-5152, progeny viruses were passaged five times in V94-5152. All inoculated V94-5152 were systemically infected in the last passage (data not shown). The full-length HC-Pro, P3 and CI cistrons of progeny viruses derived from two systemically infected V94-5152 were RT-PCR amplified and sequenced. Analyses showed the absence of any newly emerged mutation in HC-Pro or CI cistrons; however, a single non-synonymous mutation was present in P3 of progeny viruses derived from both plants at polypeptide position 938 changing phenylalanine (Phe) with Ser. This mutation was synonymous in the *pipo* ORF.

Surprisingly, substitution at position 1034 or 1055 of SMV-G7d had no impact on virulence, as SMV-$G7d_{Q1034K}$ and SMV-$G7d_{S1055R}$ remained avirulent on *Rsv4*-genotype soybean regardless of biolistic or mechanical inoculations (Table 1). However, when both mutations were introduced simultaneously into P3, SMV-$G7d_{Q1034K+S1055R}$ gained the ability to move systemically in both *Rsv4*-genotype soybeans, but inefficiently (Table 1). No newly emerged mutation was present in HC-Pro, P3 and CI of progeny viruses derived from a single systemically infected V94-5152 (Table 1). Attempts to passage further the progeny viruses derived from this single infected V94-5152 on additional V94-5152 was unsuccessful (data not shown).

P3 of SMV-G7d differs from that of SMV-G7 by four amino acid substitutions (Hajimorad *et al*., 2003; Fig. S2). To determine whether the genetic composition of P3 as a whole has an influence on virulence on *Rsv4*-genotype plants, P3 of SMV-G7d was replaced precisely with that of SMV-G7 (Hajimorad *et al*., 2005). As expected, SMV-G7d/G7P3, similar to parental viruses, remained avirulent on both *Rsv4*-genotype soybeans (Table 1). When Q1034K was introduced at position 1034, SMV-G7d/$G7P3_{Q1034K}$ moved inefficiently systemically in both *Rsv4*-genotype soybeans (Table 1). However, when both Q1034K + S1055R mutations were introduced simultaneously into P3 of the chimera, SMV-G7d/$G7P3_{Q1034K+S1055R}$ was highly efficient in infecting systemically both *Rsv4*-genotype soybeans regardless of the method of inoculation (Table 1). Sequence analyses of HC-Pro, P3 and CI cistrons derived from one of the systemically infected V94-5152 with each of the SMV-G7d-derived chimeras showed the stability of the mutations and the absence of any newly emerged substitution.

Ahangaran *et al*. (2013) have shown experimentally that a Ser to Asn substitution at position 1053 of an avirulent SMV isolate converts avirulence to virulence on V94-5152. We introduced this mutation into P3 of SMV-N. SMV-N_{S1053N} remained avirulent on V94-5152 and, similar to parental SMV-N, was virulent on PI88788 (Table S2, see Supporting Information). Interestingly, both SMV-G7 and SMV-G7d encode histidine (His), instead of Ser, at position 1054, which corresponds to position 1053 of SMV-N (Fig. S2). Replacement of His with Asn did not confer virulence to SMV-$G7_{H1054N}$ and SMV-$G7d_{H1054N}$ as neither moved systemically in either V94-5152 or PI88788 (Table 1). SMV-$G7_{H1054N}$, SMV-$G7d_{H1054N}$ and SMV-N_{S1053N} infected systemically Essex and the mutations were stable in viral progenies (Tables 1 and S2). These observations further demonstrate the context dependence of the virulence site on P3 of SMV. It seems that the ability of SMV to move systemically in *Rsv4*-genotype soybean depends on the overall P3 structure. Hence, the three-dimensional context within which these residues reside is expected to be crucial for P3 gain of function. Unfortunately, the three-dimensional structure of P3 has not been resolved from any of the potyviruses.

In most viral pathosystems involving typical or atypical *R* genes, a single viral-encoded protein serves as the avirulence factor (Hull, 2002). However, it has been reported recently that gain of virulence of SMV on soybean genotypes containing *Rsv1*, *Rsv3* or *Rsv4* individually requires simultaneous mutations in HC-Pro, P3 and CI (Chowda-Reddy *et al*., 2011b). The *Rsv1* locus is multigenic, whereas *Rsv3* is a single *R* gene; nevertheless, the NBS-LRR class of *R* genes is associated with both *Rsv1*- and *Rsv3*-mediated resistance against SMV (Hayes *et al*., 2004; Suh *et al*., 2011; Wen

et al., 2013). Chowda-Reddy *et al*. (2011b) replaced P3 of SMV-G7 with that of a SMV-G2 virulent mutant that contained the G1054R mutation. In our study, we used the same SMV-G7 clone (Hajimorad *et al*., 2003) and demonstrated that avirulence/virulence determinants of SMV-G7 on *Rsv4*-genotype soybean, as well as those of SMV-G7d, reside exclusively on P3. However, P3 genetic composition plays a crucial role in virulence, as mutation at position 1034 (Gln to Lys), but not 1055 (Ser to Arg), converts avirulent SMV-G7 to virulent on both *Rsv4*-genotype soybeans. SMV-G7 is not the only isolate with a preferred virulence mutation at position 1034 instead of 1055 (Khatabi *et al*., 2012; Fig. S3, see Supporting Information). It should be noted that the CI cistrons of SMV-G7 and SMV-G7d are both identical and there is only a single amino acid difference in the HC-Pro cistron between these two viruses (Hajimorad *et al*., 2003). However, neither HC-Pro nor CI cistrons played a role in the virulence of these viruses on *Rsv4*-genotype soybean, because SMV-$G7_{Q1034K+S1055R}$ and SMV-G7d/$G7P3_{Q1034K+S1055R}$ were virulent on both *Rsv4*-genotype soybeans. This is further supported by the absence of any newly emerged mutation in HC-Pro or CI cistrons of progeny viruses derived from systemically infected V94-5152.

Wen *et al*. (2013) have also recently demonstrated that the involvement of both HC-Pro and P3 as avirulence/virulence factors of SMV on *Rsv1*-genotype soybean is caused by the multigenic nature of the *Rsv1* locus. Furthermore, no evidence for the involvement of CI in the virulence of SMV-N on *Rsv1*-genotype soybean has been reported (Eggenberger *et al*., 2008; Hajimorad *et al*., 2008, 2011; Wen *et al*., 2013). It should be noted that the selection of adaptive mutations in HC-Pro of SMV-N on *Rsv1*-genotype soybean is influenced by the P3 sequence content, as different sites on HC-Pro, clustered mostly at the N-terminus, convert avirulent SMV-N-derived P3 chimeras to virulent (Hajimorad *et al*., 2008; Wen *et al*., 2013). The impact of the genetic background surrounding the avirulence gene has been reported previously for gain of virulence of *Potato virus Y* (PVY) on pepper *pvr2* (Montarry *et al*., 2011). In the study by Chowda-Reddy *et al*. (2011b), position 682 corresponding to that of an isolate of the G2 strain was altered [Arg to methionine (Met)]. The R682M substitution in HC-Pro of SMV-N plays a role in avirulence/virulence of the virus on *Rsv1*-genotype soybean (Eggenberger *et al*., 2008; Hajimorad *et al*., 2008, 2011); however, this is not the preferred mutational pathway towards virulence (Hajimorad *et al*., 2011; Khatabi *et al*., 2013).

It also seems unlikely that the virulence of SMV on *Rsv3*-genotype soybean involves more than one SMV-encoded protein (Chowda-Reddy *et al*., 2011b). Studies on the SMV–*Rsv3* pathosystem by two groups identified CI as the avirulence/virulence factor; however, the avirulence/virulence site was not identical between the two isolates used (Seo *et al*., 2009a; Zhang *et al*., 2009). It is interesting to note that, unlike *Rsv1* and *Rsv4*, *Rsv3* has been defeated globally by SMV (Ahangaran *et al*., 2013; Choi *et al*., 2005; Khatabi *et al*., 2012; Seo *et al*., 2009b; Viel *et al*., 2009). If simultaneous mutations in multiple SMV genes are required for gain of virulence on *Rsv3*-genotype soybean (Chowda-Reddy *et al*., 2011b), it is expected that *Rsv3* will remain durable. Naturally occurring virulent isolates of SMV on *Rsv1* are not frequent (Ahangaran *et al*., 2013; Khatabi *et al*., 2013; Seo *et al*., 2009b; Viel *et al*., 2009); however, virulent isolates have been reported from South Korea (Choi *et al*., 2005). Unfortunately, the virulence determinants of the Korean isolates have not been characterized.

The deployment of *R* genes is the most effective, economically sound and environmentally friendly approach to control viral diseases of plants; however, concern about the durability of *R* genes remains. Avirulence determinants and their significance in the life cycle of pathogens are considered to be predictors of the durability of *R* genes (Lauge *et al*., 1998). The durability of the *Ry* gene in potato against PVY has been attributed to the targeting of nuclear inclusion protein a (NIa), which is a key factor in the processing of potyviral precursor polyproteins (Mestre *et al*., 2000). Other factors, such as the number of mutations required for gain of virulence, evolutionary constraints acting against amino acid substitutions and the high fitness cost associated with gain of virulence mutation(s), also contribute to *R*-gene durability (Bornemann and Varrelmann, 2013; Goulden *et al*., 1993; Harrison, 2002; Janzac *et al*., 2009, 2010; Jenner *et al*., 2002b; Khatabi *et al*., 2013). Durability can also be enhanced by careful selection and deployment of the most appropriate *R* genes individually or in combination using a gene stacking approach. Knowledge of the avirulence determinants and fitness costs associated with gain of virulence mutations may contribute to the appropriate implementation of this approach. Thus, the precise identification of avirulence determinants of viruses has practical implications in the durable management of *R* genes. Unfortunately, our knowledge of the avirulence/virulence sites residing on plant viral proteins is limited and it is unknown how widely sites are conserved amongst isolates/strains of the same virus. In some pathosystems, such as PVY–*Ry*, this question cannot even be addressed as there is not even a single virulent isolate available worldwide (Mestre *et al*., 2000). The context dependence of the required mutation(s) for gain of virulence by avirulent viruses asks for caution in assigning avirulence/virulence function to particular sites on viral proteins or genotyping of viral isolates for resistance-breaking capability.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1 Alignment of deduced primary amino acid sequences (residues 1020–1060) of P3 derived from *Soybean mosaic virus* (SMV) isolates recovered from systemically infected PI88788 (*Rsv4*).

Fig. S2 Alignment of the full-length deduced primary amino acid sequences of P3 of pSMV-N (N), pSMV-G7 (G7) and pSMV-G7d (G7d).

Fig. S3 Alignment of the deduced primary amino acid sequences (residues 1020–1060) of P3 from isolates of *Soybean mosaic virus* (SMV) virulent on V94-5152 (*Rsv4*).

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

Table S2 Ability of *Soybean mosaic virus* (SMV)-derived P3 mutant (SMV-N_{S1053N}) to infect systemically *rsv4*- and *Rsv4*-genotype soybeans following biolistic or mechanical inoculations.

Materials and Methods S1 The details of viruses, soybean genotypes, inoculation, detection, construction of point mutants, RT-PCR, sequencing and generation of polyclonal antibodies against bacterially expressed coat protein of SMV-G7d.