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Short communication

Gain of virulence by *Soybean mosaic virus* on *Rsv4*-genotype soybeans is associated with a relative fitness loss in a susceptible host

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SUMMARY

'Gene-for-gene' theory predicts that gain of virulence by an avirulent pathogen on plants expressing resistance (*R*) genes is associated with fitness loss in susceptible hosts. However, the validity of this prediction has been studied in only a few plant viral pathosystems. In this study, the *Soybean mosaic virus* (SMV)–*Rsv4* pathosystem was exploited to test this prediction. In *Rsv4*-genotype soybeans, P3 of avirulent SMV strains provokes an as yet uncharacterized resistance mechanism that restricts the invading virus to the inoculated leaves. A single amino acid substitution in P3 functionally converts an avirulent to a virulent strain, suggesting that the genetic composition of P3 plays a crucial role in virulence on *Rsv4*-genotype soybeans. In this study, we examined the impact of gain of virulence mutation(s) on the fitness of virulent variants derived from three avirulent SMV strains in a soybean genotype lacking the *Rsv4* gene. Our data demonstrate that gain of virulence mutation(s) by all avirulent viruses on *Rsv4*-genotype soybean is associated with a relative fitness loss in a susceptible host. The implications of this finding on the durable deployment of the *Rsv4* gene in soybean are discussed.

Keywords: avirulence, fitness penalty, *Glycine max*, P3, potyvirus, resistance durability.

Plants have evolved resistance (*R*) genes to counter attacks by pathogens, including viruses (Flor, 1971; Hull, 2014; Jones and Dangl, 2006). Structurally and mechanistically known dominant *R* genes against plant viruses fall into two broad classes. One class contains classical nucleotide-binding site leucine-rich repeat (NBS-LRR) genes with hypersensitive response (HR) or extreme resistance (ER) as the resistance mechanism(s) (Maule *et al.*, 2007). The other class contains genes that differ structurally from NBS-LRR genes and confer resistance by restricting avirulent viruses to inoculated leaves without the induction of ER or HR (Cosson *et al.*, 2010; Decroocq *et al.*, 2009; Gunduz *et al.*, 2004; Ingvardsen *et al.*, 2010; Khatabi *et al.*, 2012; Saghai Maroof *et al.*, 2010; Wang *et al.*, 2015). Regardless of the class of *R* gene, or the mechanism of resistance, it has been demonstrated empirically that mutation(s) in a single virus-encoded protein functionally converts avirulence to virulence on *R*-genotype plants (Decroocq *et al.*, 2009; Hajimorad *et al.*, 2011; Khatabi *et al.*, 2012; Wang *et al.*, 2015; Wen *et al.*, 2011, 2013).

'Gene-for-gene' theory predicts that the modification of a pathogen avirulence factor to gain virulence on an *R*-genotype plant results in a fitness loss on susceptible hosts (Flor, 1971). This is based on the theory that plant immune systems have evolved to target crucial pathogen-encoded proteins and that avirulence proteins are often pathogenicity factors (Dodds and Rathjen, 2010). For viruses, based on theoretical considerations, Sacristan and Garcia-Arenal (2008) have predicted an even higher fitness cost relative to other cellular pathogens for gain of virulence on *R*-genotype plants. This is presumably because of the multifunctional roles played by viral-encoded proteins in the life cycle of viruses (Hull, 2014). However, for plant–virus pathosystems, in particular potyviral pathosystems, limited direct experimental evidence in support of these assumptions has been published (Fraile *et al.*, 2011; Goulden *et al.*, 1993; Janzac *et al.*, 2010; Jenner *et al.*, 2002; Khatabi *et al.*, 2013; Kobayashi and Hohn, 2004; Rolland *et al.*, 2009). In this study, we utilized the *Soybean mosaic virus* (SMV)–*Rsv4* pathosystem to test whether gain of virulence by three avirulent SMV strains is associated with fitness loss in a susceptible soybean genotype lacking the *Rsv4* gene.

SMV is a species within the genus *Potyvirus* belonging to the *Potyviridae* family. Its genome is expressed as a single large polypeptide, which is cleaved post-translationally by three viral-encoded proteinases to yield a number of multifunctional proteins, including P3 (Urcuqui-Inchima *et al.*, 2001). In addition, a small open reading frame (ORF) embedded in the P3 cistron (i.e. *pipo*) encodes a protein in the +2 frame in relation to the polyprotein ORF that plays a role in virus movement (Chung *et al.*, 2008; Vijayapalani *et al.*, 2012; Wei *et al.*, 2010; Wen and Hajimorad, 2010). *Rsv4*, a single atypical *R* gene in soybean, belonging to a

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previously uncharacterized type or class of *R* gene, mediates the restriction of the systemic movement of avirulent SMV strains to the inoculated leaves of soybean lines V94-5152 and PI88788 without the expression of ER or HR (Buss *et al.*, 1997; Gunduz *et al.*, 2004; Khatabi *et al.*, 2012; Saghai Maroof *et al.*, 2010; Wang *et al.*, 2015). V94-5152 is an Essex isoline containing the *Rsv4* allele from PI486355 (Buss *et al.*, 1997), and PI88788 contains an allelic *R* gene to SMV at the *Rsv4* locus in V94-5152 (Gunduz *et al.*, 2004). Essex (*rsv4*) is a soybean cultivar that is universally susceptible to SMV (Buss *et al.*, 1997). For unknown reasons, the *Rsv4* allele in V94-5152 confers effective resistance against a larger number of SMV strains relative to the corresponding allele in PI88788 (Gunduz *et al.*, 2004; Khatabi *et al.*, 2012; Wang *et al.*, 2015). Regardless, the virulence/avirulence determinants of SMV on *Rsv4*-genotype soybeans reside solely on P3; however, the position of crucial virulence site(s) varies among strains (Ahangaran *et al.*, 2013; Chowda-Reddy *et al.*, 2011; Khatabi *et al.*, 2012; Wang *et al.*, 2015). It has been suggested previously that the three-dimensional context within which the virulence residue(s) resides is possibly crucial for gain of function (Wang *et al.*, 2015). However, this possibility cannot be explored because the three-dimensional structure of P3 has not been resolved for any potyvirus.

In this article, 'fitness' is defined as the ability of SMV to replicate and propagate in susceptible soybean cv. Essex (*rsv4*) measured on the basis of disease phenotype and accumulation level of virions (Holland *et al.* 1991). 'Virulence' is defined as the ability to overcome systemic movement restriction in PI88788 (*Rsv4*) or V94-5152 (*Rsv4*), whereas pathogenicity is defined as the capability to induce uniformly distributed mosaic symptoms in soybean cv. Essex (*rsv4*) coupled with a high level of virion accumulation (Shaner *et al.*, 1992).

Three molecularly cloned SMV strains [SMV-N (GenBank accession number D00507), SMV-G7 (AY216010) and SMV-G7d (AY216987)], as well as the P3 derivative mutants used in this study, have all been described previously (Hajimorad *et al.*, 2003, 2005; Khatabi *et al.*, 2012; Wang *et al.*, 2006, 2015).

SMV-N is avirulent on V94-5152 (*Rsv4*); however, it is virulent on PI88788 (*Rsv4*) (Fig. 1A; Khatabi *et al.*, 2012). Nevertheless, a mutation at polypeptide position 1033 [glutamine (Gln) to lysine (Lys)] or 1054 [glycine (Gly) to arginine (Arg)], both within P3, confers virulent to avirulent SMV-N on V94-5152 (*Rsv4*) with no impact on its virulence on PI88788 (*Rsv4*) (Fig. 1A; Khatabi *et al.*, 2012). However, infection of V94-5152 (*Rsv4*) with SMV-N_{G1054R} is associated with a sequence polymorphism at polypeptide position 1045 with the presence of both valine and alanine (Khatabi *et al.*, 2012). As expected, the simultaneous introduction of Q1033K and G1054R into P3 of SMV-N also results in virulence on V94-5152 (*Rsv4*) (Fig. 1A; Khatabi *et al.*, 2012). Ahangaran *et al.* (2013) have reported that a mutation within P3 at polypeptide position 1053 [serine (Ser) to asparagine (Asn)] also confers virulence on *Rsv4*-genotype soybeans to avirulent SMV strains from Iran, although SMV-N_{S1053N} is avirulent on V94-5152 (*Rsv4*) (Fig. 1A; Wang *et al.*, 2015).

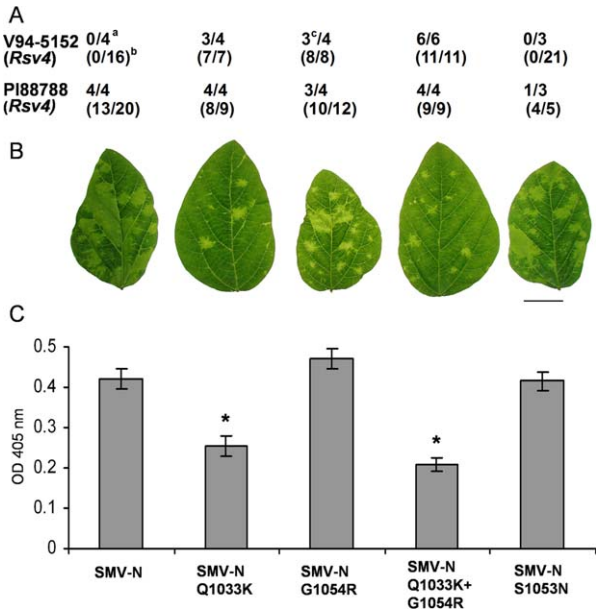

Fig. 1 (A) Virulence of SMV-N and its derivative P3 mutants on *Rsv4*-genotype soybeans inoculated biolistically with an infectious cDNA clone[a] or mechanically with infectious sap derived from biolistically inoculated Essex (*rsv4*)[b]; the number of plants systemically infected/number of plants inoculated is shown (Khatabi *et al.*, 2012; Wang *et al.*, 2015). [c]Infection of V94-5152 (*Rsv4*) with SMV-N_{G1054R} was associated with a sequence polymorphism in P3 of progeny viruses at position 3265, in which both GTA and GCA codons, encoding valine and alanine, respectively, were present at polyprotein position 1045 (Khatabi *et al.*, 2012). (B) Systemic symptoms on central leaflets from trifoliate leaves of soybean cv. Essex (*rsv4*) inoculated mechanically on unifoliate leaves with progeny viruses derived from cDNA clones. Plants were maintained in a growth chamber at 22°C until being photographed at 21 days post-inoculation (dpi). Scale bar, 2 cm. (C) Comparison of the accumulation level of viruses relative to SMV-N in systemically infected trifoliate leaves of soybean cv. Essex (*rsv4*) by enzyme-linked immunosorbent assay (ELISA). Plants were inoculated and maintained as in (B) until central leaflets from four fully developed trifoliate leaves of the inoculated plants were harvested at 21 dpi and analysed. Each bar represents the mean value of virion accumulation from two independent experiments, each with four replicate plants, with the standard errors indicated. Significant differences between the mean values for SMV-N and each of its derivative P3 mutants were determined using Kruskal–Wallis test [non-parametric statistical method from Data Processing System software (Tang and Zhang, 2013)] and are indicated by asterisks ($P < 0.05$). OD, optical density; SMV, *Soybean mosaic virus*.

To examine the impact of the virulence mutation(s) on the fitness of SMV-N-derived P3 mutants in the absence of the *Rsv4* gene, progenies derived from molecularly cloned viruses were inoculated mechanically onto unifoliate leaves of soybean cv. Essex (*rsv4*) in parallel with the parental SMV-N (the details of inoculum

source, inoculation procedure and plant maintenance are presented in Materials and Methods S1, see Supporting Information). As expected, all mutants were replication competent in soybean cv. Essex (*rsv4*) and moved systemically (Fig. 1B). Interestingly, SMV-N_{Q1033K} and SMV-$N_{Q1033k+G1054R}$, both virulent on V94-5152 (*Rsv4*), were less pathogenic in soybean cv. Essex (*rsv4*), evident by the failure to induce uniformly distributed mosaic symptoms on systemically infected leaves in comparison with avirulent SMV-N or SMV-N_{S1053N} (Fig. 1B). The level of accumulation of all mutants was compared by enzyme-linked immunosorbent assay (ELISA) relative to that of the parental SMV-N in systemically infected leaves of soybean cv. Essex (*rsv4*) (Fig. 1C) (the details of the ELISA procedures are presented in Materials and Methods S1). SMV-N_{Q1033K} and SMV-$N_{Q1033k+G1054R}$ accumulated to a significantly lesser extent than the other viruses. There was no significant difference in the accumulation level of virions from SMV-N_{G1054R} compared with that of avirulent SMV-N, despite being virulent on V94-5152 (*Rsv4*) (Fig. 1A,C). However, it should be noted that SMV-N_{G1054R}, unlike SMV-N_{Q1033K} and SMV-$N_{Q1033k+G1054R}$, is not stable in biolistically inoculated V94-5152 (*Rsv4*) (Fig. 1A; Khatabi *et al.*, 2012). We also evaluated the stability of all the mutant viruses in soybean cv. Essex (*rsv4*) after five successive passages by reverse transcription-polymerase chain reaction (RT-PCR) amplification of the full-length P3 cistron and sequencing (the details of RNA extraction, RT-PCR, sequencing and analysis are presented in Materials and Methods S1). Analysis showed that all mutant viruses, except SMV-N_{Q1033k}, in which Lys is substituted with Ser, were stable in the absence of the *Rsv4* gene (data not shown). To evaluate the stability of SMV-N_{Q1033k} during the second to fourth passages in Essex (*rsv4*), P3 of progeny viruses was also RT-PCR amplified and analysed. The analysis showed the absence of any newly emerged mutation during the second and third passages. However, a sequence polymorphism at polypeptide position 1043 was observed in P3 of progeny viruses derived from the fourth passage, resulting in the presence of both Asn and Ser at this position (data not shown).

To rule out the possibility that the fitness loss of SMV-N-derived virulent variants in the absence of the *Rsv4* gene was not strain specific, we used two additional avirulent SMV strains. SMV-G7 and SMV-G7d are biologically and genetically distinct from SMV-N, as well as from each other, and are both avirulent on the two *Rsv4*-genotype soybeans (Hajimorad *et al.*, 2003, 2005; Khatabi *et al.*, 2012; Wang *et al.*, 2006, 2015). SMV-G7 and SMV-G7d encode Gln at polypeptide position 1034, which corresponds to position 1033 on the SMV-N polypeptide; however, at polypeptide position 1055, corresponding to position 1054 of SMV-N, both SMV-G7 and SMV-G7d encode Ser (Wang *et al.*, 2015). Among SMV-G7-derived mutants with a single mutation in P3, only SMV-$G7_{Q1034K}$ is virulent on both PI88788 (*Rsv4*) and V94-5152 (*Rsv4*), whereas SMV-$G7_{S1055R}$ and SMV-$G7_{H1054N}$ are

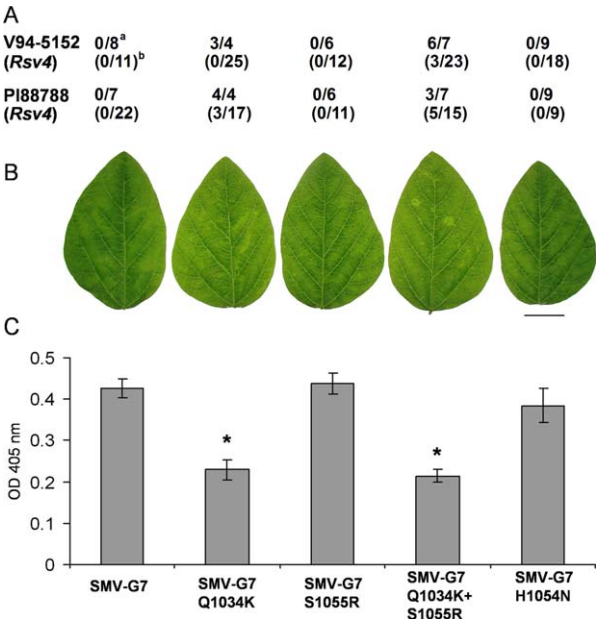

Fig. 2 (A) Virulence of SMV-G7 and its derivative P3 mutants on *Rsv4*-genotype soybeans inoculated biolistically with infectious cDNA clone[a] or mechanically with infectious sap derived from biolistically inoculated Essex (*Rsv4*)[b]; the number of plants systemically infected/number of plants inoculated is shown (Wang *et al.*, 2015). (B) Systemic symptoms on central leaflets from trifoliate leaves of soybean cv. Essex (*rsv4*) inoculated mechanically on unifoliate leaves with progeny viruses derived from cDNA clones. Plants were maintained in a growth chamber at 22°C until being photographed at 21 days post-inoculation (dpi). Scale bar, 2 cm. (C) Comparison of the accumulation level of viruses relative to SMV-G7 in systemically infected trifoliate leaves of soybean cv. Essex (*rsv4*) by enzyme-linked immunosorbent assay (ELISA). Plants were inoculated and maintained as in (B) until central leaflets from four fully developed trifoliate leaves of the inoculated plants were harvested at 21 dpi and analysed. Each bar represents the mean value of virion accumulation from two independent experiments, each with four replicate plants, with the standard errors indicated. Significant differences between the mean values for SMV-G7 and each of its derivative P3 mutants were determined using Kruskal–Wallis test [non-parametric statistical method from Data Processing System software (Tang and Zhang, 2013)] and are indicated by asterisks ($P < 0.05$). OD, optical density; SMV, *Soybean mosaic virus*.

avirulent (Fig. 2A; Wang *et al.*, 2015). SMV-$G7_{Q1034K+S1055R}$, harbouring two mutations simultaneously, is also virulent on both *Rsv4*-genotype soybeans (Fig. 2A; Wang *et al.*, 2015). When the pathogenicity of SMV-G7 was compared with that of its derivative P3 mutants in soybean cv. Essex (*rsv4*), only SMV-$G7_{Q1034K}$ and SMV-$G7_{Q1034K+S1055R}$ exhibited infection phenotypes that differed from those induced by the other SMV-G7-derived avirulent viruses (Fig. 2B). There was also a direct correlation between gain of virulence mutation on *Rsv4*-genotype soybeans and significant reduction of virion accumulation in systemically infected trifoliate leaves of soybean cv. Essex (*rsv4*) (Fig. 2A,C). Nevertheless, SMV-$G7_{Q1034K}$, SMV-$G7_{S1055R}$ and SMV-$G7_{Q1034K+S1055R}$ were all stable

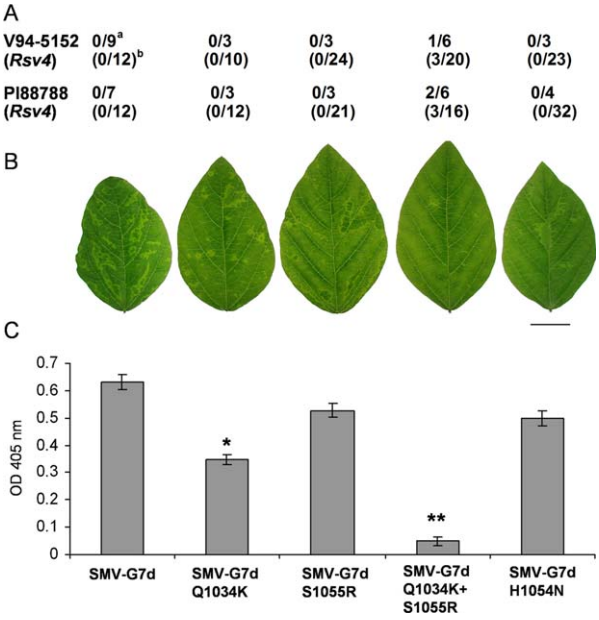

Fig. 3 (A) Virulence of SMV-G7d and its derivative P3 mutants on *Rsv4*-genotype soybeans inoculated biolistically with infectious cDNA clone[a] or mechanically with infectious sap derived from biolistically inoculated Essex (*rsv4*)[b]; the number of plants systemically infected/number of plants inoculated is shown (Wang *et al.*, 2015). (B) Systemic symptoms on central leaflets from trifoliate leaves of soybean cv. Essex (*rsv4*) inoculated mechanically on unifoliate leaves with progeny viruses derived from cDNA clones. Plants were maintained in a growth chamber at 22°C until being photographed at 21 dpi. Scale bar, 2 cm. (C) Comparison of accumulation level of viruses relative to SMV-G7d in systemically infected trifoliate leaves of soybean cv. Essex (*rsv4*) by enzyme-linked immunosorbent assay (ELISA). Plants were inoculated and maintained as in (B) until central leaflets from four fully developed trifoliate leaves of the inoculated plants were harvested at 21 days post-inoculation (dpi) and analysed. Each bar represents the mean value of virion accumulation from two independent experiments, each with four replicate plants, with the standard errors indicated. Significant differences between the mean values for SMV-G7d and each of its derivative P3 mutants were determined using Kruskal–Wallis test [non-parametric statistical method from Data Processing System software (Tang and Zhang, 2013)] and are indicated by single ($P < 0.05$) or double ($P < 0.01$) asterisks. OD, optical density; SMV, *Soybean mosaic virus*.

following five successive passages in soybean cv. Essex (*rsv4*) without the emergence of any compensatory mutation in their respective P3 cistrons.

Unlike SMV-G7, in which Gln to Lys substitution at polypeptide position 1034 results in a gain of virulence, SMV-G7d$_{Q1034K}$ and SMV-G7d$_{S1055R}$ are both avirulent on PI88788 (*Rsv4*) and V94-5152 (*Rsv4*) (Fig. 3A; Wang *et al.*, 2015). However, SMV-G7d$_{Q1034K+S1055R}$, which harbours two simultaneous mutations in P3, is virulent on both *Rsv4*-genotype soybeans, albeit to a limited extent (Fig. 3A; Wang *et al.*, 2015). When inoculated onto soybean cv. Essex (*rsv4*), SMV-G7d$_{Q1034K}$ and SMV-G7d$_{Q1034K+S1055R}$ differed phenotypically from the parental SMV-G7d (Fig. 3B). Furthermore, both mutants accumulated to a significantly lesser extent relative to the parental SMV-G7d (Fig. 3C). However, all the SMV-G7d-derived P3 mutants were stable on five successive passages in soybean cv. Essex (*rsv4*), except SMV-G7d$_{Q1034K}$, in which Lys was replaced with Asn. This substitution was accompanied by an additional compensatory mutation at polyprotein position 768 residing on P3 (E768K). This mutation (E768K) is located outside of *pipo*. It should be noted that the reversion of Q1034K to Q1034N in P3 of SMV-G7d$_{Q1034K}$ has also been reported previously in biolistically inoculated Essex (*rsv4*) (Wang *et al.*, 2015). Analyses of P3 from SMV-G7d$_{Q1034K}$-derived progeny viruses in the second to fourth passages in Essex (*rsv4*) also showed only the presence of Q1034N.

It is now well established that virulence determinant(s) of SMV on *Rsv4*-genotype soybean reside solely on P3, with the genetic composition of P3 playing a crucial role (Ahangaran *et al.*, 2013; Chowda-Reddy *et al.*, 2011; Khatabi *et al.*, 2012; Wang *et al.*, 2015). An SMV-G7d-derived recombinant having the precise P3 cistron from SMV-G7, SMV-G7d/G7P3, similar to the parental viruses, is avirulent on both *Rsv4*-genotype soybeans (Fig. 4A; Wang *et al.*, 2015). However, SMV-G7d/G7P3$_{Q1034K}$ and SMV-G7d/G7P3$_{Q1034K+S1055R}$ are both virulent (Fig. 4A; Wang *et al.*, 2015). Interestingly, the pathogenicity of these two virulent mutants on soybean cv. Essex (*rsv4*) also differed from those of avirulent SMV-G7d and SMV-G7d/G7P3, and both accumulated at significantly lower levels (Fig. 4B,C).

Our data demonstrate that gain of virulence on *Rsv4*-genotype soybean by avirulent SMV strains is associated with a loss of fitness in a susceptible host. In addition, our data provide experimental evidence that mutation at polypeptide position 1033 is not beneficial for the pathogenicity of SMV, regardless of its direct (SMV-N and SMV-G7) or indirect (SMV-G7d) impact on virulence on *Rsv4*-genotype soybeans (Figs 1–4). This conclusion is further supported by the absence of Lys at polypeptide position 1033 among field isolates of SMV originating from different geographical regions (Ahangaran *et al.*, 2013; Khatabi *et al.*, 2012). It should be noted that, although SMV-G7d$_{Q1034K}$ was avirulent on *Rsv4*-genotype soybean, SMV-G7d/G7P3$_{Q1034K}$ was virulent (compare Fig. 3 with Fig. 4).

The deployment of *R* genes is one of the most effective, environmentally friendly and economically sound approaches to control plant diseases caused by viruses. However, the main concern is the durability of *R* genes, in particular those against plant RNA viruses, because of their potential for a high mutation rate and rapid adaptation (Domingo and Holland, 1997). Nevertheless, the role and significance of avirulence determinants in the life cycle of viruses, the number of mutations required to gain virulence on *R*-genotype plants and the high fitness cost in susceptible hosts associated with gain of virulence mutation(s), individually or in combination, contribute to the durability of *R* genes (Bornemann

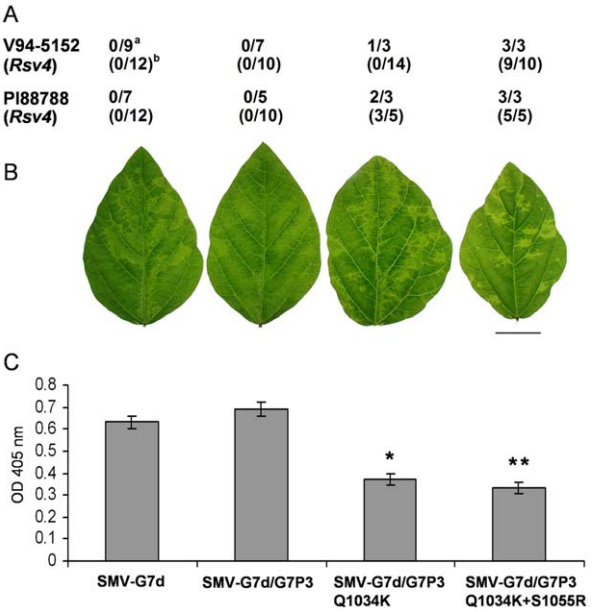

Fig. 4 (A) Virulence of SMV-G7d, recombinant SMV-G7d/G7P3 and its derivative P3 mutants on *Rsv4*-genotype soybeans inoculated biolistically with infectious cDNA clone[a] or mechanically with infectious sap derived from biolistically inoculated Essex (*rsv4*)[b]; number of plants systemically infected/number of plants inoculated is shown (Wang *et al.*, 2015). (B) Systemic infection on central leaflets from trifoliate leaves of soybean cv. Essex (*rsv4*) inoculated mechanically on unifoliate leaves with progeny viruses derived from cDNA clones. Plants were maintained in a growth chamber at 22°C until being photographed at 21 days post-inoculation (dpi). Scale bar, 2 cm. (C) Comparison of the accumulation level of viruses relative to SMV-G7d in systemically infected trifoliate leaves of Essex (*rsv4*) by enzyme-linked immunosorbent assay (ELISA). Plants were inoculated and maintained as in (B) until central leaflets from four fully developed trifoliate leaves of the inoculated plants were harvested at 21 dpi and analysed. Each bar represents the mean value of virion accumulation from two independent experiments, each with four replicate plants, with the standard errors indicated. Significant differences between the mean values for SMV-G7d and each of its derivative P3 mutants were determined using Kruskal–Wallis test [non-parametric statistical method from Data Processing System software (Tang and Zhang, 2013)] and are indicated by single ($P < 0.05$) or double ($P < 0.01$) asterisks. OD, optical density; SMV, *Soybean mosaic virus*.

and Varrelmann, 2013; Harrison, 2002; Janzac *et al.*, 2009, 2010; Jenner *et al.*, 2002; Khatabi *et al.*, 2013; Leach *et al.*, 2001). Durability could also be enhanced by careful selection and deployment of *R* genes targeting different functional proteins of a virus, as avirulence factors, via a gene stacking approach. In soybean, the complex *Rsv1* locus confers functional immunity against the majority of known SMV strains by recognizing two SMV-encoded proteins, HC-Pro and P3 (Ahangaran *et al.*, 2013; Eggenberger *et al.*, 2008; Hajimorad *et al.*, 2006, 2011, Khatabi *et al.*, 2012; Seo *et al.*, 2009; Viel *et al.*, 2009; Wen *et al.*, 2013). For gain of virulence of SMV on *Rsv1*-genotype soybeans, multiple mutations in HC-Pro and P3 are required (Eggenberger *et al.*, 2008; Hajimorad *et al.*, 2011; Wen *et al.*, 2013). In addition, a fitness penalty in susceptible hosts is also associated with gain of virulence by SMV on *Rsv1*-genotype soybean that is a consequence of mutation(s) in HC-Pro, but not P3 (Khatabi *et al.*, 2013). In this study, we also demonstrated a fitness penalty in a susceptible soybean genotype associated with gain of virulence by SMV on *Rsv4*-genotype soybeans as a consequence of mutation in P3. HC-Pro and P3 are both multifunctional proteins playing a number of essential roles in the life cycle of potyviruses (Revers and Garcia, 2015). Assuming that the fitness costs of overcoming *Rsv1* and *Rsv4* by SMV are soybean genotype independent, it is very likely that the stacking of *Rsv1* and *Rsv4* genes together will result in durable resistance against the virus.

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SUPPORTING INFORMATION

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

Materials and Methods S1 Details of viruses, soybean genotype, inoculation, maintenance, evaluation of virion accumulation by enzyme-linked immunosorbent assay (ELISA), RNA extraction, reverse transcription-polymerase chain reaction (RT-PCR), sequencing and analysis.

Fig. S1 Comparison of the accumulation level of mutant viruses relative to parental viruses in systemically infected trifoliate leaves of soybean cv. Essex (*rsv4*) in two independent experiments by enzyme-linked immunosorbent assay (ELISA).