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Evolution of *Soybean mosaic virus*-G7 molecularly cloned genome in *Rsv1*-genotype soybean results in emergence of a mutant capable of evading *Rsv1*-mediated recognition

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Abstract

Plant resistance (*R*) genes direct recognition of pathogens harboring matching avirluent signals leading to activation of defense responses. It has long been hypothesized that under selection pressure the infidelity of RNA virus replication together with large population size and short generation times results in emergence of mutants capable of evading *R*-mediated recognition. In this study, the *Rsv1*/*Soybean mosaic virus* (SMV) pathosystem was used to investigate this hypothesis. In soybean line PI 96983 (*Rsv1*), the progeny of molecularly cloned SMV strain G7 (pSMV-G7) provokes a lethal systemic hypersensitive response (LSHR) with up regulation of a defense-associated gene transcript (PR-1). Serial passages of a large population of the progeny in PI 96983 resulted in emergence of a mutant population (vSMV-G7d), incapable of provoking either *Rsv1*-mediated LSHR or PR-1 protein gene transcript up regulation. An infectious clone of the mutant (pSMV-G7d) was synthesized whose sequences were very similar but not identical to the vSMV-G7d population; however, it displayed a similar phenotype. The genome of pSMV-G7d differs from parental pSMV-G7 by 17 substitutions, of which 10 are translationally silent. The seven amino acid substitutions in deduced sequences of pSMV-G7d differ from that of pSMV-G7 by one each in P1 proteinase, helper component-proteinase, and coat protein, respectively, and by four in P3. To the best of our knowledge, this is the first demonstration in which experimental evolution of a molecularly cloned plant RNA virus resulted in emergence of a mutant capable of evading an *R*-mediated recognition.

Keywords: *Soybean mosaic virus*; Virus quasispecies; Plant RNA virus; Host passage; RNA virus evolution; Soybean; Experimental evolution

Introduction

One of the common features of RNA viruses is a high mutation rate, which is attributed mostly to the lack of proofreading ability of RNA-dependent RNA polymerase (RdRp) (Drake and Holland, 1999; Steinhauer et al., 1992). The infidelity of RNA genome replication, together with large population size, and short generation times, results in a highly diverse replicating population known as viral quasispecies (Domingo et al., 1985, 2001; Eigen, 1996). It has been postulated that the genetic heterogeneity within a virus quasispecies population is advantageous to virus survival, since the simultaneous presence of multiple variant genomes allows rapid selection of mutants better suited to new environments (Domingo and Holland, 1997; Domingo et al., 2001). This implies that due to the quasispecies nature of RNA viruses they are capable of overcoming selective pressures that limit their replication. Indeed, increasing evidence suggests that the quasispecies nature of RNA viruses leads to selection and emergence of drug resistant (Lech et al., 1996; Najera et al., 1995; Pawlotsky, 2000; Pawlotsky et al., 1998), and antibody escape mutants (Chackerian et al., 1997; Feuer et al., 1999; Mortara et al., 1998), that create potential obstacles for control of human viral diseases either by drug therapy or vaccination.

Plants have developed surveillance systems mediated by resistance (*R*) genes to recognize and counterattack invading pathogens, including viruses (Takken and Joosten,

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2000). The recognition is conditioned through matched specificity between an *R* gene and a pathogen avirulence (*Avr*) gene, which is achieved by either direct or indirect interactions (Bonas and Lahaye, 2002; Dangl and Jones, 2001; Flor, 1971). Phenotypically, *R*-mediated recognition of a virus harboring an *Avr* gene results in expression of either extreme resistance (ER) or a hypersensitive response (HR) (Hull, 2002). ER and HR are components of the same defense response, which are activated following *R/Avr* recognition (Bendahmane et al., 1999). The arrest of an *Avr* virus in ER expressing tissues is associated with the lack of any visible symptoms, or virus accumulation (Bendahmane et al., 1999; Hajimorad and Hill, 2001). However, in HR expressing tissues, the arrest is associated with rapid, localized death of infected and neighboring cells, induction of a variety of defense-related genes including pathogenesis-related (PR) proteins, and a wide range of physiological changes (Hammond-Kosack and Jones, 1996). Some of the features of HR are similar to those involved in innate immunity in mammalian and insect systems (Cohn et al., 2001; Nurnberger and Scheel, 2001). Perturbation of *Avr*, or *R* genes, occasionally results in virus escape to distant tissues provoking systemic HR (SHR) (Culver et al., 1991; Dinesh-Kumar et al., 2000; Kim and Palukaitis, 1997). It has been postulated that SHR is a consequence of delayed occurrence of biochemical and physiological events that are associated with localized HR (Dinesh-Kumar et al., 2000). Thus, SHR represents a condition in which the resistance is incomplete.

It has long been proposed that in *R*-genotype plants, the error-prone replication of plant RNA viruses together with large population size and short generation times could result in emergence of mutants capable of evading *R*-mediated recognition. In fact, in several viral pathosystems, passages of *Avr* viruses in *R*-genotype plants under laboratory conditions resulted in emergence of variants capable of overcoming HR (Macnell and Boxall, 1974; de Jager and Wesseling, 1981; Jones, 1982; Khan and Jones, 1989; Paguio et al., 1988). Wild-type populations of viruses were used in these experiments and in a few of these studies passaged through a number of successive local-lesion transfers to obtain a biologically pure virus preparation. However, experimental evidence suggests that such attempts do not yield a genetically homogeneous virus population (Garcia-Arenal et al., 1984). Thus, the presence of the variants in the original viral inoculum and their subsequent selection cannot be excluded. In fact, in two of the pathosystems, when the initial inocula were replaced with clonal populations derived from RNA transcripts of cDNA copies of the viruses, mutants capable of overcoming HR failed to emerge (Khan and Jones, 1989; Santa Cruz and Baulcombe, 1993). Hence, these experiments do not convincingly demonstrate that infidelity of RNA genome replication in *R*-genotype plants results in emergence of mutants capable of evading *R*-mediated recognition. We used the *Rsv1*/SMV pathosystem to further investigate this.

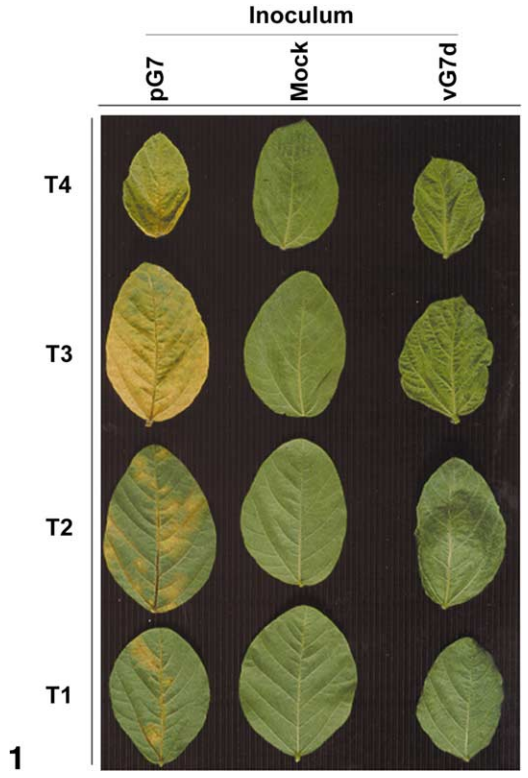
Soybean mosaic virus (SMV) is a single-stranded positive-sense RNA virus belonging to the virus family *Potyviridae*. Its genome encodes a single polypeptide, which is cleaved by three virus-encoded proteases to produce eight to nine mature proteins (Jayaram et al., 1992). *Rsv1*, a genetically mapped single dominant gene in soybean line PI 96983 (Yu et al., 1994), confers resistance against all SMV strains except G7 and G7a (Buzzell and Tu, 1984; Lim, 1985). SMV-G7 induces a lethal systemic hypersensitive response (LSHR) in PI 96983, which is associated with up regulation of the PR-1 protein gene transcript (Hajimorad and Hill, 2001). According to previous reports (Buzzell and Tu, 1984; Chen et al., 1994), SMV-G7a causes systemic mosaic and stunting in *Rsv1*-genotype soybean; however, results reported in this communication do not confirm those observations. Although the phenotype of *Rsv1*-mediated resistance against *Avr* strains of SMV is ER, restricted HR is induced if the virus is introduced continuously into an *Rsv1*-bearing scion from an infected rootstock (Hajimorad and Hill, 2001). Thus, in a manner that is phenotypically similar to the potato *Rx* gene (Bendahmane et al., 1999), *Rsv1* confers a two-tiered resistance mechanism against SMV.

In this article we demonstrate that serial passages of large population progeny of molecularly cloned SMV-G7 (pSMV-G7) in PI 96983 (*Rsv1*) results in emergence of a mutant population (vSMV-G7d) capable of evading *Rsv1* recognition. For the purpose of this communication, *Rsv1* recognition of SMV will be defined as expression of SHR and up regulation of PR-1 protein gene transcript. The molecular characteristics of an infectious clone of an individual mutant genome (pSMV-G7d) are presented. The emergence of SMV-G7d provides experimental support for the notion that the infidelity of RNA virus genome replication together with large population size and short generation times under selection pressure generates plant viral mutants capable of evading *R*-mediated recognition.

Results

Emergence of an SMV mutant population (vSMV-G7d) incapable of inducing LSHR in PI 96983 (Rsv1)

Early progeny of pSMV-G7 induced mosaic on noninoculated trifoliolate leaves of PI 96983 about 2-weeks postinoculation (wpi); however, at later stages of infection, 3–4 wpi, the systemically infected plants were severely stunted (not shown) and developed stem, petiole, leaf vein, and leaf blade necrosis (Fig. 1). Eventually, the necrotic trifoliolate leaves became desiccated and the stem apex developed necrosis, leading to partial death of the infected plants (not shown). These symptoms are consistent with the reported phenotype of the wild-type population of SMV-G7 in PI



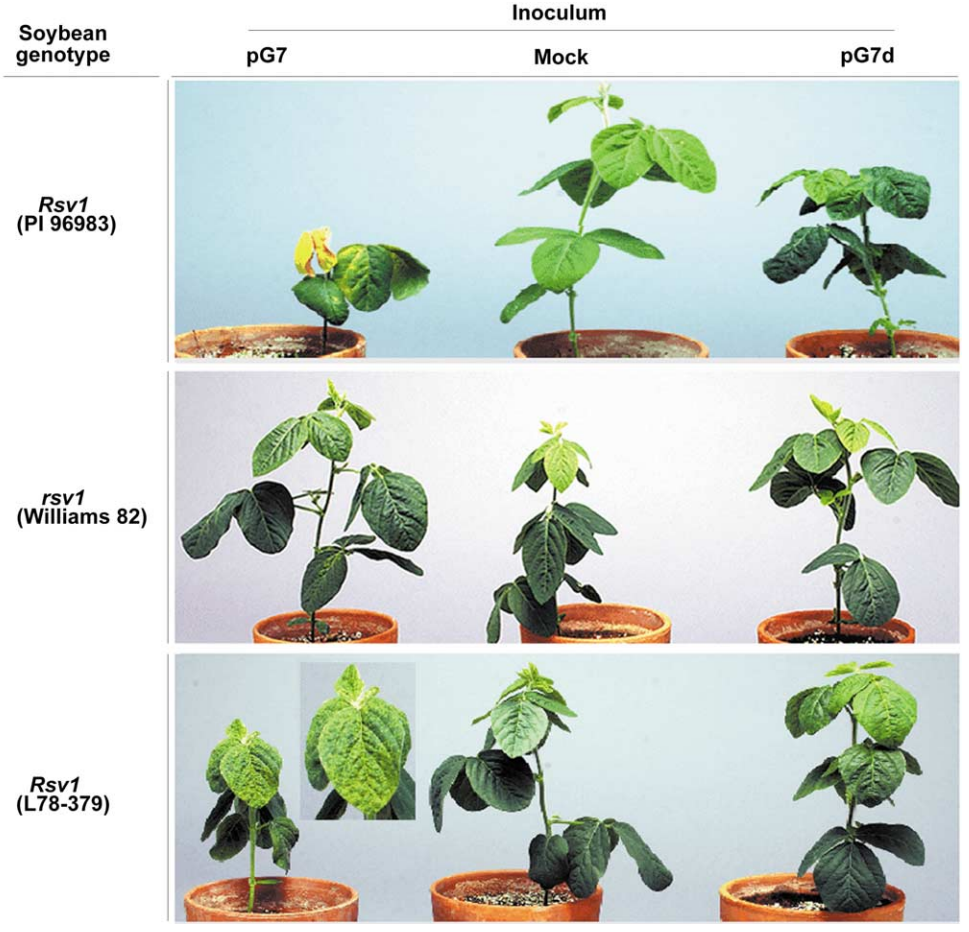


Fig. 1. Phenotypic differences in response of soybean line PI 96983 (*Rsv1*) to *Soybean mosaic virus* (SMV) infection. Adaxial (upper leaf) surface of primary leaves were inoculated with either buffer (mock), 6.25 μg/leaf purified virions of the progeny of pSMV-G7 (pG7), or the newly emerged mutant population (vG7d). Following inoculation, plants were maintained in a growth chamber (20°C) until a representative leaflet from trifoliolates 1–4 (T1–T4) was photographed 4 weeks postinoculation.

Fig. 4. Phenotypic responses of PI 96983 (*Rsv1*), Williams 82 (*rsv1*), and L78–379 (*Rsv1*) to inoculation with either buffer (mock), or infectious sap containing progenies of pSMV-G7 (pG7) and pSMV-G7d (pG7d). Following inoculation, the plants were maintained in a growth chamber (20°C) until photographed 4 weeks postinoculation. The insert presents an enlarged trifoliolate leaflet of a pG7 systemically infected L78–379 (*Rsv1*) plant displaying systemic hypersensitive response (SHR).

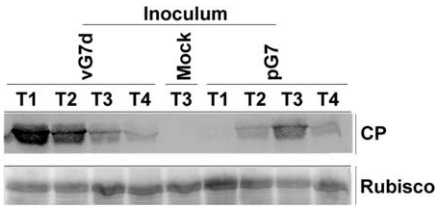


Fig. 2. Western immunoblotting analysis for the accumulation of *Soybean mosaic virus* (SMV) coat protein (CP) in PI 96983 (*Rsv1*) leaf tissues. Primary leaves were mechanically inoculated with either buffer (mock), 6.25 μg/leaf of purified virions of the newly emerged mutant population (vG7d), or the progeny of pSMV-G7 (pG7). Following inoculation, plants were maintained in a growth chamber (20°C) until a half of a leaflet from trifoliolate 1–4 (T1–T4) of the virus infected plants or trifoliolate leaflets 3 (T3) of mock-inoculated plants were collected 3 weeks postinoculation. Samples from corresponding trifoliolate leaflets of four independent replicate plants were combined; total protein was extracted in 5 volumes (w/v) of extraction buffer, and 15 μl of each sample was subjected to 12% SDS–PAGE. Separated proteins were either transferred to nitrocellulose for immunodetection using antiserum to SMV-CP or stained for the presence of Rubisco protein by Coomassie brilliant blue.

96983 (Cho and Goodman, 1979; Jayaram et al., 1992; Lim, 1985). The expression of a phenotype characteristic of SMV-G7 in PI 96983 by early progenies of pSMV-G7 suggests that the cloned SMV-G7 genome was stable. This was further supported by the observation that the consensus sequences of the P3 region, a hypervariable region among SMV strains and other potyviruses (Jayaram et al., 1992; Urcuqui-Inchima et al., 2001) in progenies from the fifth passage, were identical to the corresponding region of their parental sequences (pSMV-G7) (not shown). However, continuous passage of large population of the progeny of pSMV-G7 over a long period (about 18–20 months) in PI 96983 resulted in evolution of a mutant population (vSMV-G7d), which induced no visible stunting or necrosis in this soybean genotype (not shown). The first systemically infected trifoliolate leaves, however, occasionally developed a few nonrestricted chlorotic lesions 3–4 wpi, in particular, when plants were maintained at 20°C. All of the other systemically infected trifoliolate leaves developed mosaic, vein banding, and mild leaf distortion (Fig. 1). The systemic symptoms induced by vSMV-G7d appeared at least 1 week earlier than those of its progenitor (pSMV-G7) and spread more rapidly among the trifoliolate leaves of the infected plants (data not shown). The phenotype of vSMV-G7d in PI 96983 remained consistent irrespective of the nature of inoculum (i.e., infectious sap or purified virions). Furthermore, PI 96983 scions grafted onto vSMV-G7d-infected Williams 82 (*rsv1*) rootstocks showed a phenotype similar to those inoculated mechanically (data not shown). Under similar conditions, graft inoculation of PI 96983 with its progenitor (pSMV-G7) resulted in expression of apical necrosis and the death of all the infected PI 96983 scions (Hajimorad and Hill, 2001).

The vSMV-G7d accumulates efficiently in PI 96983 (Rsv1) leaf tissues, but fails to up regulate PR1 protein gene transcript

The possibility that inability of vSMV-G7d to provoke *Rsv1*-mediated LSHR in PI 96983-infected leaf tissues was due to its poor accumulation was investigated by both Western immunoblotting and slot-blot hybridization analyses. The accumulation of vSMV-G7d coat protein (CP), however, was higher than that of its progenitor (pSMV-G7) in trifoliolate leaves 1 and 2 and at a comparable level in all the other trifoliolate leaves examined (Fig. 2). The accumulation of its genomic RNA was also consistent with its CP accumulation (compare Figs. 2 and 3). pSMV-G7 accumulated to a very low level in trifoliolate leaves 1 as compared to the other trifoliolate leaves from the same infected plants (Figs. 2 and 3). However, irrespective of the level of virus accumulation, all pSMV-G7-infected trifoliolate leaflets displayed necrosis (Fig. 1), which suggests that the induction of *Rsv1*-mediated necrosis by pSMV-G7 is concentration independent. Despite the efficient accumulation of vSMV-G7d in all of the infected trifoliolate leaves examined, and unlike its progenitor (pSMV-G7), it failed to up regulate the expression of the soybean PR-1 protein gene transcript in the infected trifoliolate leaves 2–4 (Fig. 3). Furthermore, none of the infected vSMV-G7d-infected tri-

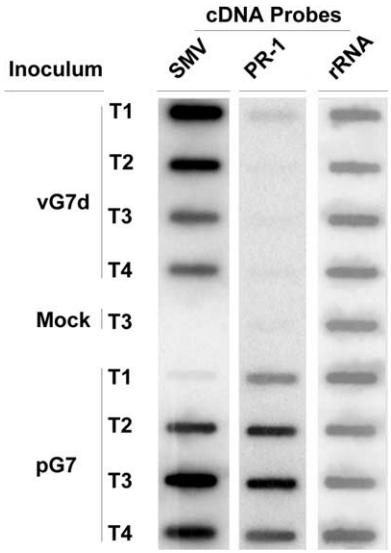


Fig. 3. Slot-blot hybridization analysis for accumulation of *Soybean mosaic virus* (SMV) RNA and soybean PR-1 (PR-1) protein gene transcript up regulation in PI 96983 (*Rsv1*) leaf tissues. Primary leaves were mechanically inoculated with buffer (mock), 6.25 μg/leaf of purified virions of the newly emerged mutant population (vG7d), or the progeny of pSMV-G7 (pG7). Following inoculation, plants were maintained in a growth chamber (20°C) until a half of a leaflet from trifoliolate 1–4 (T1–T4) of the virus infected or trifoliolate 3 (T3) of the mock inoculated plants was collected 3 weeks postinoculation. Samples from corresponding trifoliolates of four independent replicate plants were combined; total RNA was isolated and denatured, and 10 μg was slot-blotted onto a membrane and hybridized with ^{32}P-labeled cDNA probes.

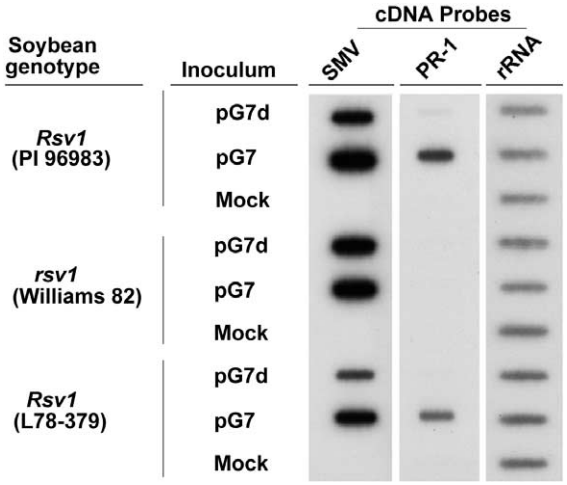


Fig. 5. Slot-blot hybridization analysis of accumulation of *Soybean mosaic virus* (SMV) RNA and soybean PR-1 protein gene transcript up regulation (PR-1) in soybean trifoliolate leaves. Primary leaves of PI 96983 (*Rsv1*), Williams 82 (*rsv1*), and L78–379 (*Rsv1*) were mechanically inoculated with buffer (mock) or infectious sap containing progenies of pSMV-G7d (pG7d) and pSMV-G7 (pG7). Following inoculation, plants were maintained in a growth chamber (20°C) until a leaflet from the third trifoliolate of infected plants was collected 4 weeks postinoculation. Samples from corresponding trifoliolate leaflets of four independent replicate plants were combined; total RNA was isolated and denatured, and 10 μg was slot-blotted onto a membrane and hybridized with ^{32}P-labeled cDNA probes.

foliolate leaflets displayed necrosis (Fig. 1). However, a slight up regulation of the PR-1 protein gene transcript was detected in vSMV-G7d-infected trifoliolate 1, which was associated with the presence of a few chlorotic lesions on the leaflet blades.

The infectious clone of SMV-G7d (pSMV-G7d) displays a similar phenotype to that of the virus population (vSMV-G7d)

Seven full-length clones of SMV-G7d fused downstream of the 35S promotor (pSMV-G7d) were tested for infectivity by biolistic inoculation of PI 96983. However, only one of the clones caused infection in the transfected plants and it displayed a phenotype similar to that of the vSMV-G7d (not shown). Infectious sap containing progeny of pSMV-G7d was used to inoculate PI 96983 (*Rsv1*), Williams 82 (*rsv1*), and L78-379 (*Rsv1*) and plant phenotypes were compared with those inoculated with the progeny of its progenitor (pSMV-G7) (Fig. 4). The progenies of both clones induced indistinguishable symptoms in Williams 82, consisting of very mild mosaic, mild leaf distortion with no visible necrosis, and without up regulation of the soybean PR-1 protein gene transcript (Figs. 4 and 5, respectively). Progeny of pSMV-G7 induced severe LSHR in PI 96983 (*Rsv1*) and severe SHR in L78–379 lines (Fig. 4, inset). Lack of LSHR expression in L78–379 line is possibly due to genetic differences with PI 96983 as L78-379 is a near isoline of Williams (*rsv1*) containing the *Rsv1* allele from PI 96983 (Bernard et al., 1991). It has been shown in other pathosystems that, in addition to *R* and *Avr* gene products, additional host factors are also required for *R*-mediated signaling responses downstream of the recognition event leading to HR-mediated cell death (Banerjee et al., 2001). This may be reflected in the differential responses of the *Rsv1*-containing PI 96983 and L78-379 soybean lines. As expected, *Rsv1*-mediated recognition of the progeny of pSMV-G7 in both PI 96983 and L78-379 was associated with strong up regulation of the PR-1 protein gene transcript (Fig. 5). However, the progeny of pSMV-G7d, similar to the vSMV-G7d population, did not induce necrosis in these two genotypes and caused only moderate mosaic, mild leaf distortion (Fig. 4), and no up regulation of the PR-1 protein gene transcript (Fig. 5). Similar to infection with vSMV-G7d, the progeny of pSMV-G7d caused a few chlorotic lesions on the first systemically infected trifoliolate leaf of PI 96983 (not shown).

Nucleotide and predicted amino acid sequences of pSMV-G7d differ from its progenitor (pSMV-G7) by a very limited number of substitutions

Comparative sequence analysis of the full-length infectious clone of pSMV-G7d with that of its progenitor (pSMV-G7) showed that both genomes consist of 9588 nucleotides excluding the 3′ poly(A) tail. However, pSMV-G7d differed from its progenitor (pSMV-G7) by 17 nucleotide substitutions of which 10 were translationally silent (Table 1). No nucleotide substitutions were observed in the cylindrical inclusion (CI), 6K, NIa-Vpg, and 3′ untranslated regions (UTR) of pSMV-G7d. The 3′-end sequence of the vSMV-G7d identified by 3′ RACE also was found to be identical to those of pSMV-G7. In contrast, one nucleotide substitution was found in the 5′ UTR of pSMV-G7d (Table 1), which was originally detected by 5′ RACE of the vSMV-G7d and subsequently incorporated into the primer G75′d (Table 2). NIb and CP regions each differed by one; helper component-proteinase (HC-Pro) and NIa-Proteinase (NIa-Pro) each differed by two; P1 proteinase (P1) differed by four and P3 differed by six nucleotides (Table 1). However, nucleotide substitutions resulted in only seven amino acid exchanges in the entire deduced polyprotein of pSMV-G7d; one each in P1, HC-Pro, and CP, and four in P3 (Table 1).

The sequences of pSMV-G7d are very similar but not identical to the consensus sequences of the vSMV-G7d population

To determine the sequence similarity between pSMV-G7d and the consensus sequences of the mutant population (vSMV-G7d), the sequences of the 5′ and 3′ portions of the genome were targeted (Table 3). P1, P3, the N-termini of HC-Pro and C-termini of NIb, and the entire CP regions of

Table 1
Nucleotide and amino acid differences between pSMV-G7d and pSMV-G7

Region[a]	Nucleotide			Amino acid		
	Position[b]	pSMV-G7	pSMV-G7d	Position	pSMV-G7	pSMV-G7d
5′ UTR (1–131)	12	C	A			
P1 (132–1058)	322 (2)	A	G	64	Lys	Arg
	512 (3)	T	C			
	650 (3)	A	G			
	872 (3)	T	C			
HC-Pro (1059–2429)	1142 (3)	C	T			
	1546 (2)	A	G	472	Gln	Arg
P3 (2430–3626)	2501 (3)	T	C			
	2598 (1)	G	A	823	Val	Met
	2615 (3)	T	C			
	2874 (1)	A	G	915	Met	Val
	2988 (1)	A	G	953	Lys	Glu
	3466 (2)	C	T	1112	Ala	Val
CI (3627–5528)	—[c]		—			
6K (5529–5687)	—		—			
NIa-VPg (5688–6257)	—		—			
NIa-Pro (6258–6986)	6281 (3)	T	G			
	6713 (3)	G	A			
NIb (6987–8537)	8363 (3)	A	G			
CP (8538–9335)	8655 (1)	G	A	2842	Val	Met
3′ UTR (9336–9588)	—		—			

[a] The order of SMV genomic regions and their predicted lengths are presented as proposed by Jayaram et al. (1992).
[b] The positions of mutated nucleotides and amino acids on the SMV genome are based on sequences of SMV strains G7 and G7d (GenBank Accession Nos. AY216010 and AY216987, respectively). Numbers in parentheses show position within the codon.
[c] No difference.

the SMV-G7d population were RT-PCR amplified, directly sequenced, and compared with the corresponding regions of its progenitor (pSMV-G7) and pSMV-G7d (Table 3). Sequence alignment showed that the P1 region of pSMV-G7d differed from the homologous region of vSMV-G7d population by two nucleotide substitutions, both of which remained translationally silent (Table 3). Sequences of the N-terminus of HC-Pro and the C-terminus regions of NIb and CP of pSMV-G7d were identical to those of the vSMV-G7d population (Table 3). However, the P3 region of pSMV-G7d differed by one nucleotide substitution from the consensus sequences of vSMV-G7d, which resulted in one amino acid substitution at this position (Table 3). The consensus nucleotide sequences of the vSMV-G7d population at positions 512, 650, and 2874 were all identical to its pSMV-G7 progenitor sequences (Table 3). The nucleotide at position 2874 in pSMV-G7d was stable and did not revert to its parental sequence (pSMV-G7) even after five passages in PI 96983 (not shown).

To further study the heterogeneity at position 2874 within the vSMV-G7d population, a portion of the HC-Pro and P3 genes of the vSMV-G7d population (nucleotides 1988–3206) was RT-PCR amplified using as a template the isolated virion RNA (from the same stock which pSMV-G7d was derived from), cloned into pGEM-T, and two independent clones were sequenced. The sequences of both the clones were identical to each other and to the consensus sequences of the vSMV-G7d population (not shown). These data demonstrate that the sequences of pSMV-G7d are very similar, but not identical, to the consensus sequences of the SMV-G7d population and presumably it represents a variant sequence (Domingo and Holland, 1997).

Table 2
Sequences of sense and antisense oligonucleotide primers used for reverse transcriptase reactions and PCR amplifications

Primer	Sequences[a] (5′-3′)	Position[b]	Restriction site
601a	*GGTCTCCAGTATTTTGTT*	601–584	
412s	*GTTTGACCTTGCGTCGCT*	412–429	
237a	*GTTTTGCCATCTGAACTGCTT*	237–217	
3dta	GTTTCTAGAGCTCAGCGGCCGCATCGA$(T)_{20}$		
9269s	*GCACACTGCAAGGGATGT*	9269–9286	
3dt-core	GTTTCTAGAGCTCAGCGGCCGC**ATCGAT**TT		*Cla*I
CI3a	GC**GGATCC***ATCTGTAATTGGACTGCATTCA*	5526–5506	*Bam*HI
CI5s	GCGGATCC*AGTGTTGATGAGATTCAGA*	3624–3642	
G75′d	GGTAGGCC**TTT*AAA***TTAAAACTACCTATAAAGAC	1–23	*Dra*I
Ca35S	ACGCACAATCCCACTATC		
Nosa	AGACCGGCAACAGGATTCA		
1988s	*GCGCCATTTGGTTATTGG*	1988–2005	
3263a	*TGTATTGATAATACCTTGC*	3263–3245	
4048a	*CCAAACTTGCTCAATCCTCTC*	4048–4028	
3584s	*GGCATTTTTCAGCACAATG*	3584–3602	
214s	*TTCAGGCAGTTCAGATGGCGAAACAA*	214–233	

[a] Nucleotide identical or complementary to SMV sequences are italicized; the recognition sequences for restriction enzymes used for cloning are shown in bold, and non-SMV sequences are underlined.

[b] The positions of oligonucleotides on the SMV genome are based on sequences of SMV strains G7 and G7d (GenBank Accession Nos. AY216010, and AY216987, respectively).

pSMV-G7d is phenotypically and genotypically a unique variant of SMV

According to the literature, SMV-G7a is the only known strain of SMV capable of inducing systemic mosaic without SHR in *Rsv1*-genotype soybean (Buzzell and Tu, 1984; Chen et al., 1994). To compare the phenotype of pSMV-G7d with that of SMV-G7a, two isolates of SMV-G7a (ATCC PV614 and ATCC PV724), which had been deposited by two independent sources with the American Type Culture Collection (ATCC), were obtained. However, none of 64 *Rsv1*-genotype soybean plants mechanically inocu-

Table 3
Nucleotide and amino acid variation among genomic regions of pSMV-G7, vSMV-G7d population, and pSMV-G7d

Nucleotide					Amino acid			
Region	Position	pSMV-G7	vSMV-G7d	pSMV-G7d	Position	pSMV-G7	vSMV-G7d	pSMV-G7d
P1 (132–1058)[a]								
	322 (2)[b]	A	G	G	64	Lys	Arg	Arg
	512 (3)	T	T	C[c]				
	650 (3)	A	A	G				
	872 (3)	T	C	C				
HC-Pro (1059–1614)								
	1142 (3)	C	T	T				
	1546 (2)	A	G	G	472	Gln	Arg	Arg
P3 (2430–3626)								
	2501 (3)	T	C	C				
	2598 (1)	G	A	A	823	Val	Met	Met
	2615 (3)	T	C	C				
	2874 (1)	A	A	G	915	Met	Met	Val
	2988 (1)	A	G	G	953	Lys	Glu	Glu
	3466 (2)	C	T	T	1112	Ala	Val	Val
NIb (8321–8534)								
	8363 (3)	A	G	G				
CP (8538–8721)								
	8655 (1)	G	A	A	2842	Val	Met	Met

[a] Numbers are the length of sequences determined for each region of vSMV-G7d population by direct sequencing of RT-PCR products and were used for comparison with the corresponding regions of pSMV-G7 and pSMV-G7d.

[b] The positions of mutated nucleotides and amino acids on the SMV genome are based on sequences of SMV strains G7 and G7d (GenBank Accession Nos. AY216010 and AY216987, respectively). Numbers in parentheses show position within the codon.

[c] Underlines indicate nucleotide or amino acid variation between vSMV-G7d population and its representative clone pSMV-G7d.

Table 4
Percentage nucleotide sequence identity of P1[a] and coat protein (CP) regions among SMV strains

Isolates	pSMV-G7	pSMV-G7d	SMV-N
SMV-G7a (ATCC PV614)	95.2 (94.8)[b]	94.7 (94.7)	99.2 (99.4)
pSMV-G7		99.5 (99.9)	95.5 (95.0)
pSMV-G7d			95.1 (94.8)

[a] The nucleotide (nt) sequences of P1 region of SMV-G7a PV614 (GenBank Accession No. AH008455) was limited to the 3′ 873 of 927 nts; therefore, only this region of P1 was used in all the comparisons.

[b] Numbers outside and inside parentheses represent percentage nucleotide identity among P1 and CP regions, respectively.

lated with SMV-G7a ATCC PV614 or 76 with ATCC PV724 showed infection on the basis of symptom expression and DAS-ELISA. This was irrespective of using PI 96983 (*Rsv1*) or L78-379 (*Rsv1*) lines. Under similar conditions, 41 of 41 and 28 of 46 Williams 82 (*rsv1*) soybean plants mechanically inoculated with ATCC PV614 and ATCC PV724, respectively, were systemically infected.

To determine genetic similarity of SMV-G7a to SMV-G7d, the published full-length sequences of CP and the partial sequences of the P1 regions of SMV-G7a ATCC PV614 corresponding to nucleotides 186–1058 of the genome (GenBank Accession Nos. AF200572 and AH008455, respectively) were compared with the homologous regions of pSMV-G7, pSMV-G7d, and SMV-N (GenBank Accession No. D00507). The percentage sequence identity indicated that SMV-G7a ATCC PV614 was most similar to SMV-N (ATCC PV728) (Table 4), an isolate of strain SMV-G2 (Cho and Goodman, 1979). The comparison of the respective deduced full-length amino acid sequences of the CP also showed a similar pattern (not shown). To genetically compare the two isolates of SMV-G7a with each other, a portion of the P1 region of SMV-G7a ATCC PV724, corresponding to nucleotides 764–1055, was RT-PCR amplified, sequenced (GenBank Accession No. AY216988), and compared with the corresponding region of SMV-G7a ATCC PV614. The sequence alignment showed that both were identical (not shown), which suggests that the two SMV-G7a isolates are closely related. The data demonstrate pSMV-G7d represents a unique variant of SMV both phenotypically and genotypically.

Discussion

In *R*-genotype plants, the recognition of pathogens as intruders is a key for elicitation of *R*-dependent defense responses, which are often, but not always, associated with localized HR and efficient containment of the pathogen (Heath, 2000; Shirasu and Schulze-Lefert, 2000). In this study, it was demonstrated that long-term passage of large populations of the progeny of the molecularly cloned genome of SMV-G7 (pSMV-G7) in *Rsv1*-genotype soybean resulted in emergence of a mutant population (vSMV-G7d) capable of evading *Rsv1*-mediated recognition. An infectious clone of an individual genome from this population (pSMV-G7d), despite having limited sequence differences from the consensus sequences of vSMV-G7d population, demonstrated similar capability. The ability of pSMV-G7d to evade *Rsv1*-mediated recognition was associated with an unknown proportion of the 17 mutations, with the majority in transitions, in the entire genome resulting in a total of seven amino acid substitutions in four of nine proteins.

The “low” rate of mutation accumulation in the genome of pSMV-G7d is not surprising, because the virus evolved under strong selection pressure, and probably negative selection prevented the survival of certain mutations. *Tobacco mosaic virus* also accumulated a low level of mutations when its replication was subjected to a selective disturbance (Kearney et al., 1999). In the absence of selection pressure, it has been shown that taxonomically different plant viruses were capable of generating a tremendous amount of heterogeneity (Schneider and Roossinck, 2001); however, the biological significance was not demonstrated.

The founder population from which vSMV-G7d was derived originated from a pSMV-G7 biolistically inoculated Williams 82 (*rsv1*) soybean plant. The original inoculum was homogenous DNA, so the mutation could not be present in the inoculum. Thus, all the accumulated mutations in vSMV-G7d genome must have been generated de novo as a result of virus RdRp error. However, the involvement of recombination during the evolution of vSMV-G7d cannot be excluded. Recombination is not uncommon among members of *Potyviridae* (Bousalem et al., 2000; Cervera et al., 1993; Glais et al., 2002; Moury et al., 2002; Ohshima et al., 2002; Revers et al., 1996). It has been proposed that recombination is a mechanism utilized by RNA viruses to counterbalance their high replication error rate and to rescue functional parental sequences from the mutated sequences (Nagy and Simon, 1997; Simon and Bujarski, 1994). Nevertheless, as for any population (Drake et al., 1998; Tolou et al., 2002), nucleotide substitutions were the initial source of variation in progenies of pSMV-G7.

The demonstration that a molecularly cloned genome of a plant RNA virus can evolve experimentally to evade an *R*-mediated recognition supports the notion that RdRp error together with large population size and short generation times coupled with selection pressure is one of the mechanisms responsible for emergence of such viral mutants in plants. Minor modification of the *Avr* signal by a point mutation is a common strategy among plant viruses to evade *R*-mediated recognition (Culver et al., 1994; Padgett and Beachy, 1993). This is mostly due to multifunctionality of viral genes; hence, point mutation is the only affordable strategy. Other pathogens, such as bacteria and fungi, introduce diverse mutations into their *Avr* genes, including point mutations, insertions, and deletions to avoid triggering *R*-mediated resistance responses (Leach et al., 2001).

A large part of the SMV-G7d genome, including CI, 6K,

NIa-Vpg, and 3′-UTR, remained mutation free. Except for 6K, the remaining regions of the genome are not conserved among SMV strains (Jayaram et al., 1992); hence, the virus tolerates mutations in these regions. Mutation during replication of a RNA genome is probably a random event, which indicates that in the course of the evolution of SMV-G7d, the selection pressure imposed by the *Rsv1*-genotype, acted against accumulation of mutations in these regions. The perfect sequence identities of these regions with those of its progenitor (pSMV-G7), however, suggest that these regions are not involved in induction of *Rsv1*-mediated LSHR. Among the regions of the pSMV-G7d genome, P3 and P1 accumulated the highest number of mutations. This is not surprising as these two regions are the least conserved among SMV strains (Jayaram et al., 1992), and among other potyviruses (Urcuqui-Inchima et al., 2001).

The current study was concerned with whether the mutations result in emergence of new variants capable of evading *Rsv1*-mediated recognition. Thus, more complex questions such as the mutation rate of the SMV polymerase, the dynamic of SMV mutation, and the complexity of the quasispecies structure of the virus were not evaluated. However, nearly all RNA viruses have extremely high mutation rates (Drake, 1993; Drake and Holland, 1999; Malpica et al., 2002), and the ability of taxonomically different plant RNA viruses as well as satellite RNAs and viroids to generate quasispecies from molecularly cloned genomes, but not of known biological importance, has been demonstrated (Gora-Sochacka et al., 1997; Kearney et al., 1999; Kurath and Palukaitis, 1990; Kurath and Dodds, 1995; Roossinck, 1997; Schneider and Roossinck, 2001). Such studies, however, have not been conducted with any member of the genus *Potyvirus*, which constitutes the largest and economically most important genus of plant viruses (Shukla et al., 1994), in part because the synthesis of infectious clones of potyviruses has been recalcitrant (Jakab et al., 1997; Johansen, 1996).

The observation that a majority of the mutations in the pSMV-G7d genome were also present in the consensus sequence of the vSMV-G7d population suggests that these mutations have been fixed in the population. With positive selection, fixation of certain mutations in an evolving RNA viral population is expected (Smith et al., 1997). However, of three silent mutations in the P1 gene of pSMV-G7d, two were not present in the consensus sequence of the vSMV-G7d population. Also a G substitution for A in position 2874 of the pSMV-G7d genome was not detected in the consensus sequence of the vSMV-G7d population or in two independent clones examined. This mutation is responsible for change of a predicted valine to methionine in position 915 of the pSMV-G7d polypeptide located in the P3 region. High-fidelity *Taq* polymerase was used for all the RT-PCR amplifications in this study; thus, it is unlikely that all three mutations originated in vitro. The observation that no other mismatches were found elsewhere between pSMV-G7d and vSMV-G7d consensus sequences, and a large portion of pSMV-G7d genome is mutation free, supports this conclusion. Therefore, the nucleotide differences between vSMV-G7d population and pSMV-G7d are more likely indicative of the quasispecies nature of the vSMV-G7d population. According to the quasispecies theory, the most adapted master sequence(s) and closely related variants represent the fittest members(s) of the swarm of the mutants in any defined selective environment (Holland and Domingo, 1998). The observation that both vSMV-G7d and pSMV-G7d are capable of evading *Rsv1*-mediated recognition suggests that none of these three mutations are critical for *Rsv1*-mediated evasion.

The data presented in this communication must be interpreted with caution in regard to the durability of *R*-genes against plant viruses. The vSMV-G7d emerged as a result of experimental evolution of pSMV-G7 after continuous large population passages in *Rsv1*-genotype soybean plants. Large population passage is known to favor the selection of novel mutants, which have the highest relative fitness (Clarke et al., 1993; Novella et al., 1995). However, an evolving plant virus in the environment is continuously subjected to different genetic bottlenecks. The spread of SMV under field conditions is dependent on infected seeds or viruliferous aphids (Hill et al., 1980). SMV-G7 induced LSHR in PI 96983 prematurely kills the infected plants. Thus under field conditions, the survival of an evolving population of SMV-G7 in an *Rsv1*-genotype soybean plant, with a genetic makeup similar to that of PI 96983, depends solely on aphid transmission. In contrast to a large population passage, aphid transmission results in the transmission of a few infectious genomes best adapted to the vector regardless of their fitness to the plant hosts. Thus, in nature, an emerging virus mutant is faced with adaptive fitness in both the host and the vector (Pirone and Blanc, 1996). Plant viruses also often use alternate hosts for survival. These factors present an additional challenge for evolving viral mutants. Experimental evidence suggests that the quasispecies structure of plant viruses is host-dependent (Kearney et al., 1999; Schneider and Roossinck, 2001). Furthermore, viral mutants evolved to evade *R*-mediated recognition are not as competitive as wild-type viruses in susceptible hosts (Jenner et al., 2002; Goulden et al., 1993). As a result of complex interactions among plants, viruses, and their vectors, it is not surprising that the majority of *R*-genes deployed against plant viruses have proven to be relatively durable (Harrison, 1981; Hull, 2002).

Materials and methods

Soybean genotypes, virus sources, inoculation, and propagation

The soybean (*Glycine max*) cultivars Williams 82 (*rsv1*), line PI 96983 (*Rsv1*), and line L78-379 (*Rsv1*), which is a near isoline of Williams with the *Rsv1* allele from line PI

96983 (Bernard et al., 1991), were used in all experiments. Seeds were obtained from virus-indexed plants and grown in a greenhouse with supplemental lighting during the winter at 22 to 25°C or in a growth chamber operating at 20°C with a photoperiod of 18 h. The infectious cDNA clone of SMV-G7, driven by the 35S promotor (pSMV-G7) (Eggenberger and Hill, 1997), was used as the virus source. The plasmid DNA was coated (3–4 μg of DNA) onto 1.6-μm gold particles (Bio-Rad Laboratories, Hercules, CA) as described by Sanford et al. (1993). Hypocotyls of the soybean Williams 82 (*rsv1*), an SMV-susceptible soybean cultivar (Bernard and Cremeens, 1988), were bombarded with the particles using a PDS-1000 helium gun (Bio-Rad). Biolistic delivery was done using 1100-PSI rupture disks (Bio-Rad) and a 6-cm distance to target. Infectious sap from one of the infected plants was used to mechanically inoculate the carborundum-dusted (600 mesh) soybean line PI 96983 (*Rsvl*) as previously described (Hajimorad and Hill, 2001). Large population transfers of the virus were done under greenhouse conditions by inoculating primary leaves of at least four young (2 weeks old) PI 96983 seedlings with sap from systemically infected PI 96983 leaf tissues every 3–4 weeks. In each inoculation, sap from systemically infected trifoliolate leaves of one of the infected plants served as inoculum. This practice continued until a stable virus population (vSMV-G7d) incapable of inducing LSHR evolved (about 18–20 months). Passage of vSMV-G7d in PI 96983 continued for an additional 2 months before the virus was propagated for purification. SMV was propagated in PI 96983; virions were purified, and the preparation was dialyzed against 0.05 M Na-borate, pH 8.0 (Hajimorad and Hill, 2001). The concentration of virions was determined spectrophotometrically and purified virus was stored at 4°C. Two isolates of SMV-G7a (ATCC PV614 and ATCC PV724) were obtained from L.L. Domier (University of Illinois, USA) and our laboratory collection, respectively.

RNA isolation

Viral RNA was isolated by dissociation of purified SMV virions in the presence of 1% SDS and 0.5 mg/ml proteinase-K (Boehringer Mannheim GmbH, Germany) at 37°C for 1 h (Vance and Beachy, 1984). The RNA was then purified using an RNeasy purification kit (Qiagen, Valencia, CA) and stored at −85°C. Total soybean RNA was isolated from liquid nitrogen-frozen leaf tissues kept at −85°C, as described by Pawlowski et al. (1994). The concentrations of the isolated RNAs were determined spectrophotometrically.

Immunoassay

Squash-immunoblotting was done as described previously (Hajimorad and Hill, 2001). For Western immunoblotting, SMV-infected or mock-inoculated soybean leaf tissues were pulverized in liquid nitrogen, extracted in five volumes (w/v) of a buffer containing 0.625 M Tris–HCl, pH 6.0, 2% (w/v) SDS, 10% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol, and analyzed by 12% SDS–PAGE as described (Hajimorad et al., 1996). Separated proteins were detected by staining with Coomassie brilliant blue or were transferred to nitrocellulose membranes for immunoblot analysis (Hajimorad et al., 1996). Detection of SMV CP in infected soybean leaf tissues was also done by double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) as described previously (Hajimorad and Hill, 2001).

Probes and slot-blot hybridization

A cloned fragment of a soybean PR-1 protein gene (GenBank Accession No. AI930866) was PCR-amplified with vector-specific T7 and T3 primers, gel-purified, and reamplified in the presence of [^{32}P]dCTP to generate a cDNA-labeled probe as described by Hajimorad and Hill (2001). A probe against soybean 18S ribosomal RNA (Eckenrode et al., 1984) was synthesized with a random-primed DNA labeling kit (Life Technologies) according to the manufacturer's instructions. The SMV probe was PCR synthesized in the presence of [^{32}P] dCTP using SMV specific primers 3584s and 4048a (Table 2) and supercoiled plasmid pSMV-G7d as a template. This region of SMV genome is identical between pSMV-G7 and pSMV-G7d. Unincorporated nucleotides were removed using Probe Quant G-50 Microcolumns (Pharmacia, Piscataway, NJ). RNA denaturation, slot-blot hybridization, and detection of hybridization signals were performed as described (Hajimorad and Hill, 2001). Images were reformatted for publication with Adobe PhotoShop (Adobe Systems, San Jose, CA, USA).

The vSMV-G7d RNA 5′ and 3′-end sequences

A rapid amplification of cDNA ends kit (5′-full RACE core set kit, Takara Shuzo Co., Ltd., Japan) was used to determine the 5′-end sequences of vSMV-G7d RNA. Purified virion RNA was primed with 5′-phosphorylated primer 601a (Table 2) and reverse transcribed according to instructions of the manufacturer. Following RNase H treatment and ethanol precipitation, the first-strand cDNA product was self-ligated using T4 RNA ligase and used as a template for PCR amplification in the presence of primers 412s and 237a (Table 2) and LA *Taq* polymerase (Takara). The gel-purified (Sambrook et al., 1989) PCR product was ligated into pGEM-T (Promega, Madison, WI, USA) and cloned. Plasmid DNA from two independent clones was sequenced with vector-specific T7 and Sp6 primers. To determine the 3′ -end sequences, purified vSMV-G7d RNA was reverse transcribed in the presence of primer 3dta (Table 2) using Superscript reverse transcriptase (Gibco-BRL) according to the manufacturer's recommendation. Following RNase H treatment, the first-strand cDNA was PCR amplified in the presence of the primers 9269s and 3dt-core (Table 2) using LA *Taq* polymerase. The gel-purified PCR product was

ligated into pGEM-T and cloned and plasmid DNA from two independent clones were sequenced as above.

Consensus sequences of the viral population

The genomic regions of interest were reverse transcribed (RT) in the presence of antisense SMV-specific primers using as templates viral RNA isolated from purified virions or total RNA isolated from systemically infected soybean leaf tissues. The RT products were subsequently used as templates for PCR amplification of the targeted regions in the presence of SMV-specific primers. The PCR products were purified by using a Qiaquick PCR purification kit (Qiagen) and directly sequenced using SMV-specific primers.

Synthesis of full-length infectious clone of SMV-G7d (pSMV-G7d) and screening for infectivity

The plasmid vector used for cloning SMV-G7d was pBR322 (Bolivar et al., 1977), modified to contain the 35S promoter and NOS terminator from pAGUS1 (Skuzeski et al., 1990). The polylinker between the promoter and terminator contained a *StuI* site at the start of transcription, followed by *Bam*HI, *Dra*I, and *Cla*I sites.

Two overlapping cDNA fragments were reverse transcribed in the presence of primers 3dta and CI3a (Table 2) using SuperScript reverse transcriptase. The 5′-region corresponding to nucleotides 1–5526 was PCR amplified using primers G75′d and CI3a (Table 2). The product was digested with *DraI* and *Bam*HI and ligated into *Stu*I/*Bam*HI-digested plasmid vector. ElectroMax DH5α-E cells (Gibco-BRL) were transformed by electroporation using a MicroPulser (Bio-Rad). Positive transformants were identified by PCR amplification using primers Ca35S (a sense primer specific to the 35S promoter) and 237a (Table 2) and verified by restriction digest analyses. The 3′-region of the genome, corresponding to nucleotides 3624–9588 and the poly(A) tail, was PCR amplified using primers C15s and 3dt-core (Table 2), digested by *Sal*I and *Cla*I, and ligated into a pool of similarly digested plasmid vector downstream of the 5′-region. Primers 9269s and Nosa (a reverse nopaline synthase transcription terminator primer; Table 2) were used for PCR screening of the transformants. Selected plasmids containing the full-length genome of SMV-G7d (pSMV-G7d) were purified using QiaPrep Spin MiniPrep Kit (Qiagen) and tested for infectivity on PI 96983 by particle bombardment as described above. The presence of SMV in symptomatic plants was verified by squash-immunoblotting (Hajimorad and Hill, 2001).

Sequencing, assembly, and analysis

Sequencing was done at the Iowa State University DNA Sequencing and Synthesis Facility. Both strands of supercoiled plasmid of pSMV-G7 and pSMV-G7d were sequenced using primers Nosa and Ca35S (Table 2) to initiate sequencing of (+) and (−) strands, respectively. Other primers were designed and synthesized as information emerged and sequencing was completed using a primer walking approach. The sequences were edited by Factura (Applied Biosystem, MA, USA), assembled by Autoassembler software (Applied Biosystem), and analyzed by using the DNA Star package and the Wisconsin package version 10.2 (Genetic Computer Group, Madison, WI, USA).

The sequences of pSMV-G7 and pSMV-G7d are in GenBank as Accession Nos. AY216010 and AY216987, respectively. Partial sequences of P1 and HC-Pro regions of SMV-G7a isolate ATCC PV724 are also in GenBank as Accession Number AY216988.

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References

Banerjee, D., Zhang, X., Bent, A.F., 2001. The leucine-rich repeat domain can determine effective interaction between *RPS2* and other host factors in Arabidopsis *RPS2*-mediated disease resistance. Genetics 158, 439–450.

Bendahmane, A., Kanyuka, K., Baulcombe, D.C., 1999. The *Rx* gene from potato controls separate virus resistance and cell death responses. Plant Cell 11, 781–791.

Bernard, R.L., Cremeens, C.R., 1988. Registration of "Williams 82" soybean. Crop Sci. 28, 1027–1028.

Bernard, R.L., Nelson, R.L., Cremeens, C.R., 1991. USDA soybean genetic collection: isoline collection. Soybean Genet. Newsl. 18, 27–57.

Bolivar, F., Rodriguez, R.L., Greene, P.J., Betlach, M.C., Heyneker, H.L., Boyer, H.W., 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene 2, 95–113.

Bonas, U., Lahaye, T., 2002. Plant disease resistance triggered by pathogen-derived molecules: refined models of specific recognition. Curr. Opin. Microbiol. 5, 44–50.

Bousalem, M., Douzery, E.J.P., Fargette, D., 2000. High genetic diversity, distant phylogenetic relationships and intraspecies recombination events among natural populations of *Yam mosaic virus*: a contribution to understanding potyvirus evolution. J. Gen. Virol. 81, 243–255.

Buzzell, R.I., Tu, J.C., 1984. Inheritance of soybean resistance to soybean mosaic virus. J. Hered. 75, 82.

Cervera, M.T., Riechmann, J.L., Martin, M.T., Garcia, J.A., 1993. 3′-Terminal sequence of the plum pox virus PS and Ô6 isolates: evidence for RNA recombination within the potyvirus group. J. Gen. Virol. 74, 329–334.

Chackerian, B., Rudensey, L.M., Overbaugh, J., 1997. Specific N-linked and O-linked glycosylation modifications in the envelope VI domain of Simian immunodeficiency virus variants that evolve in the host alter recognition by neutralizing antibodies. J. Virol. 71, 7719–7727.

Chen, P., Buss, G.R., Roane, C.W., Tolin, S.A., 1994. Inheritance in soybean of resistant and necrotic reactions to soybean mosaic virus strains. Crop Sci. 34, 414–422.

Cho, E.-K., Goodman, R.M., 1979. Strains of soybean mosaic virus: classification based on virulence in resistant soybean cultivars. Phytopathology 69, 467–470.

Clarke, D.K., Duarte, E.A., Moya, A., Elena, S.F., Domingo, E., Holland, J., 1993. Genetic bottlenecks and population passages cause profound fitness differences in RNA viruses. J. Virol. 67, 222–228.

Cohn, J., Sessa, G., Martin, G.B., 2001. Innate immunity in plants. Curr. Opin. Immunol. 13, 55–62.

Culver, J.N., Lindbeck, A.G.C., Dawson, W.O., 1991. Virus-host interactions: induction of chlorotic and necrotic responses in plants by tobamoviruses. Annu. Rev. Phytopathol. 29, 193–217.

Culver, J.N., Stubbs, G., Dawson, W.O., 1994. Structure-function relationship between tobacco mosaic virus coat protein and hypersensitivity in *Nicotiana sylvestris*. J. Mol. Biol. 242, 130–138.

Dangl, J.L., Jones, J.D.G., 2001. Plant pathogens and integrated defense responses to infection. Nature 411, 826–833.

De Jager, C.P., Wesseling, J.B.M., 1981. Spontaneous mutations in cowpea mosaic virus overcoming resistance due to hypersensitivity in cowpea. Physiol. Plant Pathol. 19, 347–358.

Dinesh-Kumar, S.P., Tham, W.-H., Baker, B.J., 2000. Structure-function analysis of the tobacco mosaic virus resistance gene *N*. Proc. Natl. Acad. Sci. USA 97, 14789–14794.

Domingo, E., Biebricher, C.K., Eigen, M., Holland, J.J., 2001. Quasispecies and RNA Virus Evolution: Principles and Consequences. Landes Bioscience, Georgetown, TX.

Domingo, E., Holland, J.J., 1997. RNA virus mutations and fitness for survival. Annu. Rev. Microbiol. 51, 151–178.

Domingo, E., Martínez-Salas, E., Sobrino, F., de la Torre, J.C., Portela, A., Ortín, J., López-Galindez, C., Pérez-Breña, P., Villanueva, N., Nájera, R., VandePol, S., Steinhauer, D., DePolo, N., Holland, J., 1985. The quasispecies (extremely heterogeneous) nature of viral RNA genome populations: biological relevance—a review. Gene 40, 1–8.

Drake, J.W., 1993. Rates of spontaneous mutation among RNA viruses. Proc. Natl. Acad. Sci. USA 90, 4171–4175.

Drake, J.W., Charlesworth, B., Charlesworth, D., Crow, J.F., 1998. Rates of spontaneous mutation. Genetics 148, 1667–1686.

Drake, J.W., Holland, J.J., 1999. Mutation rates among RNA viruses. Proc. Natl. Acad. Sci. USA 96, 13910–13913.

Eckenrode, V.K., Arnold, J., Meagher, R.B., 1984. Comparison of the nucleotide sequence of soybean 18S rRNA with the sequences of other small-subunit rRNAs. J. Mol. Evol. 21, 259–269.

Eggenberger, A.L., Hill, J.H., 1997. Analysis of resistance-breaking determinants of soybean mosaic virus. Phytopathology 87, S27.

Eigen, M., 1996. On the nature of virus quasispecies. Trends Microbiol. 4, 216–218.

Feuer, R., Boone, J.D., Netski, D., Morzunov, S.P., St.Jeor, S.C., 1999. Temporal and spatial analysis of sin nombre virus quasispecies in naturally infected rodents. J. Virol. 73, 9544–9554.

Flor, H.H., 1971. Current status of the gene-for-gene concept. Annu. Rev. Phytopathol. 9, 275–296.

García-Arenal, F., Palukaitis, P., Zaitlin, M., 1984. Strains and mutants of tobacco mosaic virus are both found in virus derived from single-lesion passaged inoculum. Virology 132, 131–137.

Glais, L., Tribodet, M., Kerlan, C., 2002. Genomic variability in *Potato potyvirus Y* (PVY): evidence that $PVY^{N}W$ and PVY^{NTN} variants are single to multiple recombinants between PVY^{O} and PVY^{N} isolates. Arch. Virol. 147, 363–378.

Gora-Sochacka, A., Kierzek, A., Candresse, T., Zagorski, W., 1997. The genetic stability of potato spindle tuber viroid (PSTVd) molecular variants. RNA 3, 68–74.

Goulden, M.G., Kohm, B.A., Santa Cruz, S., Kavanagh, T.A., Baulcombe, D.C., 1993. A feature of the coat protein of potato virus X affects both induced virus resistance in potato and viral fitness. Virology 197, 293–302.

Hajimorad, M.R., Ding, X.S., Flasinski, S., Mahajan, S., Graff, E., Haldeman-Cahill, R., Carrington, J.C., Cassidy, B.G., 1996. NIa and NIb of peanut stripe potyvirus are present in the nucleus of infected cells, but do not form inclusions. Virology 224, 368–379.

Hajimorad, M.R., Hill, J.H., 2001. *Rsvl*-mediated resistance against *Soybean mosaic virus*-N is hypersensitive response-independent at inoculation site, but has the potential to initiate a hypersensitive response-like mechanism. Mol. Plant Microbe Interact. 14, 587–598.

Hammond-Kosack, K.E., Jones, J.D.G., 1996. Resistance gene-dependent plant defense responses. Plant Cell 8, 1773–1791.

Harrison, B.D., 1981. Plant virus ecology: ingredients, interactions and environmental influences. Ann. Appl. Biol. 99, 195–209.

Heath, M.C., 2000. Hypersensitive response-related death. Plant Mol. Biol. 44, 321–334.

Hill, J.H., Lucas, B.S., Benner, H.I., Tachibana, H., Hammond, R.B., Pedigo, L.P., 1980. Factors associated with the epidemiology of soybean mosaic virus in Iowa. Phytopathology 70, 536–540.

Holland, J., Domingo, E., 1998. Origin and evolution of viruses. Virus Genes 16, 13–21.

Hull, R., 2002. Matthew's Plant Virology. Academic Press, NY.

Jakab, G., Droz, E., Brigneti, G., Baulcombe, D., Malnoe, P., 1997. Infectious in vivo and in vitro transcripts from a full-length cDNA clone of PVY-N605, a Swiss necrotic isolate of Potato virus Y. J. Gen. Virol. 78, 3141–3145.

Jayaram, C.H., Hill, J.H., Miller, W.A., 1992. Complete nucleotide sequences of two *Soybean Mosaic Virus* strains differentiated by response of soybean containing the *Rsv* resistance gene. J. Gen. Virol. 73, 2067–2077.

Jenner, C.E., Wang, X., Ponz, F., Walsh, J.A., 2002. A fitness cost for *Turnip mosaic virus* to overcome host resistance. Virus Res. 86, 1–6.

Johansen, I.E., 1996. Intron insertion facilitates amplification of cloned virus cDNA in *Escherichia coli* while biological activity is reestablished after transcription in vivo. Proc. Natl. Acad. Sci. USA 93, 12400–12405.

Jones, R.A.C., 1982. Breakdown of potato virus X resistance gene Nx: selection of a group four strain from strain group three. Plant Pathol. 31, 325–331.

Kearney, C.M., Thomson, M.J., Roland, K.E., 1999. Genome evolution of tobacco mosaic virus populations during long-term passaging in a diverse range of hosts. Arch. Virol. 144, 1513–1526.

Khan, I.A., Jones, G.E., 1989. Selection for a specific tobacco mosaic virus variant during bolting of *Nicotiana sylvestris*. Can. J. Bot. 67, 88–94.

Kim, C.-H., Palukaitis, P., 1997. The plant defense response to cucumber mosaic virus in cowpea is elicited by the viral polymerase gene and affects virus accumulation in single cells. EMBO J. 16, 4060–4068.

Kurath, G., Dodds, J.A., 1995. Mutation analyses of molecularly cloned satellite tobacco mosaic virus during serial passage is plants: evidence for hotspots of genetic change. RNA 1, 491–500.

Kurath, G., Palukaitis, P., 1990. Serial passage of infectious transcripts of a cucumber mosaic virus satellite RNA clone results in sequence heterogeneity. Virology 176, 8–15.

Leach, J.E., Vera Cruz, C.M., Bai, J., Leung, H., 2001. Pathogen fitness penalty as a predictor of durability of disease resistance genes. Annu. Rev. Phytopathol. 39, 187–224.

Lech, W.J., Wang, G., Yang, Y.L., Chee, Y., Dorman, K., McCrae, D., Lazzeroni, L.C., Erickson, J.W., Sinsheimer, J.S., Kaplan, A.H., 1996. In vivo sequence diversity of the protease of human immunodeficiency virus type 1: presence of protease inhibitor-resistant variants in untreated subjects. J. Virol. 70, 2038–2043.

Lim, S.M., 1985. Resistance to soybean mosaic virus in soybeans. Phytopathology 75, 199–201.

Macnell, B.H., Boxall, M., 1974. The evolution of a pathogenic strain of tobacco mosaic virus in tomato: a host-passage phenomenon. Can. J. Bot. 52, 1305–1307.

Malpica, J.M., Fraile, A., Moreno, I., Obies, C.I., Drake, J.W., Garcia-Arenal, F., 2002. The rate and character of spontaneous mutation in an RNA virus. Genetics 162, 1505–1511.

Mortara, L., Letourneur, F., Gras-Masse, H., Venet, A., Guillet, J.-G., Bourgault-Villada, I., 1998. Selection of virus variants and emergence of virus escape mutants after immunization with an epitope vaccine. J. Virol. 72, 1403–1410.

Moury, B., Morel, C., Johansen, E., Jacquemond, M., 2002. Evidence for diversifying selection in *Potato virus Y* and in the coat protein of other potyviruses. J. Gen. Virol. 83, 2563–2573.

Nagy, P.D., Simon, A.E., 1997. New insights into the mechanisms of RNA recombination. Virology 235, 1–9.

Najera, I., Holguin, A., Quinones-Mateu, M.E., Munoz-Fernandez, M.A., Najera, R., Lopez-Galindez, C., Domingo, E., 1995. *pol* Gene quasispecies of human immunodeficiency virus: mutations associated with drug resistance in virus from patients undergoing no drug therapy. J. Virol. 69, 23–31.

Novella, I.S., Duarte, E.A., Elena, S.F., Moya, A., Domingo, E., Holland, J.J., 1995. Exponential increases of RNA virus fitness during large population transmissions. Proc. Natl. Acad. Sci. USA 92, 5841–5844.

Nurnberger, T., Scheel, D., 2001. Signal transmission in the plant immune response. Trends Plant Sci. 6, 372–379.

Ohshima, K., Yamaguchi, Y., Hirota, R., Hamamoto, T., Tomimura, K., Tan, Z., Sano, T., Azuhata, F., Walsh, J.A., Fletcher, J., Chen, J., Gera, A., Gibbs, A., 2002. Molecular evolution of *Turnip mosaic virus*: evidence of host adaptation, genetic recombination and geographical spread. J. Gen. Virol. 83, 1511–1521.

Padgett, H.S., Beachy, R.N., 1993. Analysis of a tobacco mosaic virus strain capable of overcoming *N* gene-mediated resistance. Plant Cell 5, 577–586.

Paguio, O.R., Kuhn, C.W., Boerma, H.R., 1988. Resistance-breaking variants of cowpea chlorotic mottle virus in soybean. Plant Dis. 72, 768–770.

Pawlowski, K., Kunze, R., De Vries, S., Bisseling, T., 1994. Isolation of total poly (A) and polysomal RNA from plant tissues, in: Glevin, S.B., Schilperoort, R.A. (Eds.), Plant Molecular Biology Manual, Kluwer Academic, Dordrecht, The Netherlands, pp. D5/1–D5/4.

Pawlotsky, J.-M., 2000. Hepatitis C virus resistance to antiviral therapy. Hepatology 32, 889–896.

Pawlotsky, J.-M., Germanidis, G., Neumann, A.U., Pellerin, M., Frainais, P.-O., Dhumeaux, D., 1998. Interferon resistance of Hepatitis C virus genotype 1b: relationship to nonstructural 5A gene quasispecies mutations. J. Virol. 72, 2795–2805.

Pirone, T.P., Blanc, S., 1996. Helper-dependent vector transmission of plant viruses. Annu. Rev. Phytopathol. 34, 227–247.

Revers, F., Le Gall, O., Candresse, T., Le Romancer, M., Dunez, J., 1996. Frequent occurrence of recombinant potyvirus isolates. J. Gen. Virol. 77, 1953–1965.

Roossinck, M.J., 1997. Mechanisms of plant virus evolution. Annu. Rev. Phytopathol. 35, 191–209.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning: A Laboratory Manual, second ed. Cold Spring Laboratory Press, Cold Spring Harbor, NY.

Sanford, J.C., Smith, F.D., Russell, J.A., 1993. Optimizing the biolistic process for different biological applications. Methods Enzymol. 217, 483–509.

Santa Cruz, S., Baulcombe, D.C., 1993. Molecular analysis of potato virus X isolates in relation to the potato hypersensitivity gene *Nx*. Mol. Plant-Microbe Interact. 6, 707–714.

Schneider, W.L., Roossinck, M.J., 2001. Genetic diversity in RNA virus quasispecies is controlled by host-virus interactions. J. Virol. 75, 6566–6571.

Shirasu, K., Schulze-Lefert, P., 2000. Regulators of cell death in disease resistance. Plant Mol. Biol. 44, 371–385.

Shukla, D.D., Ward, C.W., Brunt, A.A., 1994. The *Potyviridae*. CAB International, Wallingford, UK.

Simon, A.E., Bujarski, J.J., 1994. RNA-RNA recombination and evolution in virus-infected plants. Annu. Rev. Phytopathol. 32, 337–362.

Skuzeski, J.M., Nichols, L.M., Gesteland, R.F., 1990. Analysis of leaky viral translation termination codons in vivo by transient expression of improved β-glucuronidase vectors. Plant Mol. Biol. 15, 65–79.

Smith, D.B., McAllister, J., Casino, C., Simmonds, P., 1997. Virus "quasispecies:" making a mountain out of a molehill. J. Gen. Virol. 78, 1511–1519.

Steinhauer, D.A., Domingo, E., Holland, J.J., 1992. Lack of evidence for proofreading mechanisms associated with an RNA virus polymerase. Gene 122, 281–288.

Takken, F.L.W., Joosten, M.H.A.J., 2000. Plant resistance genes: their structure, function and evolution. Eur. J. Plant Pathol. 106, 699–713.

Tolou, H., Nicoli, J., Chastel, C., 2002. Viral evolution and emerging viral infections: what future for the viruses? A theoretical evaluation based on informational spaces and quasispecies. Virus Genes 24, 267–274.

Urcuqui-Inchima, S., Haenni, A.-L., Bernardi, F., 2001. Potyvirus proteins: a wealth of functions. Virus Res. 74, 157–175.

Vance, V.B., Beachy, R.N., 1984. Translation of soybean mosaic virus RNA in vitro: evidence of protein processing. Virology 132, 271–281.

Yu, Y.G., Saghai Maroof, M.A., Buss, G.R., Maughan, P.J., Tolin, S.A., 1994. RFLP and microsatellite mapping of a gene for soybean mosaic virus resistance. Phytopathology 84, 60–64.