Available online at www.sciencedirect.com

SCIENCE DIRECT

Virology 345 (2006) 156 – 166

VIROLOGY

www.elsevier.com/locate/yviro

Strain-specific P3 of *Soybean mosaic virus* elicits *Rsv1*-mediated extreme resistance, but absence of P3 elicitor function alone is insufficient for virulence on *Rsv1*-genotype soybean

M.R. Hajimorad [a,*], A.L. Eggenberger [b], J.H. Hill [b]

[a] *Department of Entomology and Plant Pathology, The University of Tennessee, 205 Ellington Plant Sciences Building, Knoxville, TN 37916, USA*
[b] *Department of Plant Pathology, Iowa State University, 351 Bessey Hall, Ames, IA 50011, USA*

Received 22 June 2005; returned to author for revision 10 August 2005; accepted 21 September 2005
Available online 2 November 2005

Abstract

When challenged by mechanical inoculation, the *Rsv1* gene of soybean invokes extreme resistance (ER) against *Soybean mosaic virus* (SMV) strain N, but not SMV-G7 and its experimentally evolved variant, SMV-G7d. SMV-G7 provokes a lethal systemic hypersensitive response (LSHR), whereas SMV-G7d induces systemic mosaic. Thus, for *Rsv1*-genotype soybean, SMV-G7 and SMV-G7d are both virulent virus strains. The elicitor function of SMV-G7 provoking *Rsv1*-mediated LSHR was recently mapped to P3, and the influence of amino acids 823, 953, and 1112 of the precursor polypeptide of SMV-G7d on evasion of *Rsv1*-mediated recognition provoking LSHR was demonstrated. We have now extended this study to SMV-N. Initially, amino acids corresponding to those of SMV-G7d at these positions were substituted, individually or in combinations. All the mutants remained replication competent on *rsv1*-genotype soybean; however, none lost the elicitor function provoking *Rsv1*-mediated ER. Subsequently, P3 of SMV-N was precisely replaced with P3 of SMV-G7 or SMV-G7d and vice versa. All the chimeras were replication competent on *rsv1*-genotype soybean, but surprisingly SMV-N/G7P3 and SMV-N/G7dP3 failed to gain virulence on *Rsv1*-genotype soybeans. However, SMV-G7/NP3 and SMV-G7d/NP3 lost virulence, and this loss of virulence function was mapped to the N-terminus domain of SMV-N P3. The data indicate that SMV strain-specific P3 provokes *Rsv1*-mediated ER; however, virulence on *Rsv1*-genotype soybean is not solely a consequence of the absence of the P3 elicitor functions provoking *Rsv1*-mediated ER and LSHR.

Keywords: Chimeric viruses; Plant viruses; Avirulence; Dominant monogenic resistance (*R*) gene; Resistance response

Introduction

It has been well documented that resistance mediated by monogenic dominant resistance (*R*) genes against plant viruses, similar to the other plant pathogens, operates on the basis of a "gene-for-gene" hypothesis (Flor, 1971; Hull, 2002). If an invading virus harbors a matching avirulence (*avr*) gene, then a host bearing a complementary *R*-gene activates an effective defense response and consequently confines it to the point of entry (Hammond-Kosack and Jones, 1996; Nimchuk et al., 2003). *R*-dependent elicitor function for a number of plant viral genes has been illustrated (Culver, 1997; Hull, 2002).

Phenotypically, *R*-mediated recognition of plant viruses harboring complementary *avr* genes results in expression of extreme resistance (ER) or hypersensitive response (HR) (Hull, 2002), both of which are considered the consequence of the same recognition event (Bendahmane et al., 1999). In HR-expressing tissues, the defense responses include rapid calcium and ion fluxes, an extracellular oxidative burst, salicylic acid production, transcriptional programming within and around the infection site and, often, but not always, localized programmed cell death (Greenberg and Yao, 2004; Heath, 2000; Shirasu and Schulze-Lefert, 2000). It is presumed that the additive effect of these events leads to the suppression of the invading virus, and its confinement to the point of entry (Hammond-Kosack and Jones, 1996). On the other hand, in ER-expressing tissues, the arrest of an avirulent virus is not associated with any visible symptoms or virus accumulation (Bendahmane et al., 1999; Hajimorad and Hill, 2001), and at least in *Potato virus X* (PVX)/*Rx* pathosystem, it is not associated with any new host RNA synthesis (Gilbert et al., 1998). Currently, the underlying

* Corresponding author. Fax: +1 865 974 4744.
E-mail address: mrh@utk.edu (M.R. Hajimorad).

0042-6822/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2005.09.055

molecular and biochemical mechanisms leading to the expression of ER instead of HR are not understood.

Plant viruses have evolved mechanisms to circumvent and/or suppress *R*-mediated surveillance systems. One tactic involves minor modification of *avr* genes (Berzal-Herranz et al., 1995; Goulden et al., 1993; Karasawa et al., 1999; Kim and Palukaitis, 1997; Meshi et al., 1988; Padgett and Beachy, 1993; Tsuda et al., 1998). How these modifications affect avoidance of an *R*-mediated surveillance system remains an enigma; it is not known if the evasion is a passive or an active process. Mutations in viral *avr* genes, however, occasionally result in emergence of evolutionary intermediate strains of viruses that escape to distant tissues, but provoke systemic HR (SHR). In some instances, SHR progresses to lethal SHR (LSHR) (Culver et al., 1991; Hajimorad et al., 2005; Kim and Palukaitis, 1997; Santa Cruz and Baulcombe, 1993).

The viral strains with differential interactions with *R*-genotype plants have been widely utilized for mapping the elicitors of *R*-mediated defense responses. This is commonly achieved by construction of artificial chimeras followed by phenotypic analyses (Culver et al., 1991; Goulden et al., 1993; Hajimorad et al., 2005; Jenner et al., 2003; Karasawa et al., 1999; Kavanagh et al., 1992; Kim and Palukaitis, 1997; Kiraly et al., 1999; Malcuit et al., 1999; Meshi et al., 1988, 1989; Padgett et al., 1997; Querci et al., 1995; Santa Cruz and Baulcombe, 1993; Tsuda et al., 1998; Weber et al., 1993). These studies have shown that virulence on *R*-genotype plants is associated with the absence of the elicitor function provoking *R*-mediated defense responses. The majority of these studies, however, involve HR-dependent pathosystems as exemplified by viruses such as *Tobacco mosaic virus* (TMV), PVX and *Cauliflower mosaic virus* (CaMV) (Culver, 1997; Kiraly et al., 1999). TMV and PVX have in common the expression of mature proteins from single open reading frames (ORF) (Hull, 2002). The P6 of CaMV, the elicitor of HR in *Nicotiana clevelandii*, also is expressed via a monocistronic mRNA (Hull, 2002; Kiraly et al., 1999). However, similar studies on pathosystems involving viruses with polyprotein gene expression strategy, such as potyviruses, are limited (Hajimorad et al., 2005; Jenner et al., 2003). The genome of viruses in the genus *Potyvirus* consists of a single ORF that encodes for a single polypeptide, which is subsequently cleaved proteolitically under the control of a number of regulatory mechanisms to produce 8–10 mature proteins (Dougherty and Semler, 1993; Hull, 2002).

In soybean, the *Rsv1*-gene invokes ER against *Soybean mosaic virus* (SMV) strain N, but not SMV-G7 and its experimentally evolved variant, SMV-G7d (Hajimorad and Hill, 2001; Hajimorad et al., 2003). This genetically mapped *R*-gene (Yu et al., 1994) was originally identified in soybean line PI 96983 (*Rsv1*) conferring ER against SMV-strain groups G1-G6 and C14, but not G7 (Cho and Goodman, 1979; Kiihl and Hartwig, 1979, Lim, 1985). The classical genetic studies subsequently established the presence of a single *R*-gene against SMV in PI 96983 (*Rsv1*) (Kiihl and Hartwig, 1979; Chen et al., 1991, 1994). Although the phenotype of SMV-N (Vance and Beachy, 1984), a group G2 isolate of SMV (Cho and Goodman, 1979), on mechanically inoculated PI 96983 (*Rsv1*) leaf tissues is ER, restricted SHR is induced when the virus is introduced continuously into *Rsv1*-bearing scions from infected *rsv1*-genotype soybean rootstocks (Hajimorad and Hill, 2001). In contrast, SMV-G7 and SMV-G7d are not limited by ER-tier of *Rsv1*-mediated resistance response in mechanically inoculated PI 96983 (*Rsv1*) and provoke LSHR and systemic mosaic, respectively (Hajimorad et al., 2003). We proposed elsewhere that, analogous to the PVX/*Rx* pathosystem (Bendahmane et al., 1999), *Rsv1*-mediated resistance against SMV operates based on a two-tiered mechanism of resistance (Hajimorad and Hill, 2001). Primary resistance or ER (HR independent) is operational at the point of inoculation and secondary resistance (HR mediated) activates when the ER-tier of resistance is bypassed and the HR elicitor is allowed to accumulate. Thus, we hypothesized that the SMV-N elicitor of ER and restricted SHR are encoded by the same virus cistron, and that the corresponding region of SMV-G7, albeit modified, is the elicitor of *Rsv1*-mediated LSHR (Hajimorad et al., 2005). By taking the advantage of differential interactions of SMV-G7 and SMV-G7d with *Rsv1*-genotype soybeans (Hajimorad et al., 2003), the SMV-G7 elicitor of *Rsv1*-mediated LSHR was recently mapped to P3, and the amino acid residues involved were identified (Hajimorad et al., 2005).

To identify the SMV elicitor of *Rsv1*-mediated ER, we have now extended our study to SMV-N, and by site-directed mutagenesis substituted, individually or in combination, amino acids corresponding to those of SMV-G7d involved in evasion of *Rsv1*-mediated recognition provoking LSHR. Furthermore, we precisely replaced P3 of SMV-N with P3 of SMV-G7 or SMV-G7d and vice versa. We demonstrate in this paper that neither amino acid substitutions nor P3 replacement confers virulence on *Rsv1*-genotype soybean to SMV-N. Moreover, we present evidence that the P3 of SMV-N provokes *Rsv1*-mediated ER, and the elicitor function resides on the N-terminus.

For the purpose of this paper, we have defined virulence as the capability of a viral strain to evade *R*-mediated recognition provoking ER or HR irrespective of its phenotype (Shaner et al., 1992). Based on this definition, SMV-G7 and SMV-G7d represent virulent strains of the virus with respect to *Rsv1*-genotype soybeans.

Results

P3 of SMV-N has significant genetic differences with P3 of SMV-G7 and SMV-G7d

The genetic differences between P3 of an infectious full-length cDNA clone of SMV-N (pSMV-N) and those of SMV-G7 (pSMV-G7) and SMV-G7d (pSMV-G7d) were determined by comparison of pSMV-N sequences (GenBank accession No. D00507) corresponding to nucleotides 2427–3623 with the homologous genomic regions of pSMV-G7 and pSMV-G7d (GenBank AY216010 and AY216987, respectively), representing nucleotides 2430–3626. At the nucleotide level, P3 of pSMV-N shares 92.9 and 92.4% sequence identity with those

of pSMV-G7 and pSMV-G7d, respectively, and at the amino acid level 94.2 and 93.2%, respectively. There are a total of 85 and 91 nucleotide substitutions between P3 of pSMV-N with P3 of pSMV-G7 and pSMV-G7d, respectively. These nucleotide differences result in a total of 23 and 27 amino acid substitutions in P3 of pSMV-N as compared to P3 of pSMV-G7 and pSMV-G7d, respectively (Fig. 1). It is interesting to note that all the six nucleotide substitutions identified earlier in P3 of pSMV-G7d as compared to pSMV-G7 (Hajimorad et al., 2003) are identical between pSMV-N and pSMV-G7. Similarly, the four amino acid differences between pSMV-G7 and pSMV-G7d are also identical between pSMV-N and pSMV-G7 (Fig. 1). It has been shown that reciprocal amino acid substitutions between pSMV-G7 and pSMV-G7d at the position 915 did not affect the elicitor function provoking *Rsv1*-mediated LSHR (Hajimorad et al., 2005).

The elicitor function of SMV-N provoking Rsv1-mediated ER is not altered by substitutions of the amino acids involved in induction of Rsv1-dependent LSHR or SHR

It has been demonstrated that substitution of amino acids 823, 953, and 1112 of the precursor polypeptide of pSMV-G7 with those corresponding to pSMV-G7d at these positions abolished pSMV-G7 elicitor function provoking *Rsv1*-mediated LSHR (Hajimorad et al., 2005). To find out if similar substitutions have any impact on the elicitor function of pSMV-N provoking *Rsv1*-mediated ER, the corresponding amino acids of pSMV-N were substituted by site-directed mutagenesis with those of pSMV-G7d, and consequently pSMV-N_{V822M}, pSMV-N_{K952E}, and pSMV-N_{A1111V} were synthesized. Inoculation of the mutants onto Williams 82 (*rsv1*) showed that all three gave rise to systemic infection and were thus replication competent. However, when progenies were sap inoculated onto PI 96983 (*Rsv1*) or L78-379 (*Rsv1*), based on absence of symptoms and lack of detection of the viruses by DAS-ELISA, all retained the elicitor function provoking *Rsv1*-mediated ER (data not shown). The amino acids located at positions 823 and 953 of pSMV-G7 act in concert in abolishing the elicitor function of pSMV-G7 provoking *Rsv1*-mediated SHR (Hajimorad et al., 2005). To find out if concomitant incorporation of the two amino acids in P3 of pSMV-N has any influence on the loss of elicitor function provoking *Rsv1*-mediated ER, pSMV-$N_{V822M+K952E}$ was synthesized. The mutant remained infectious on Williams 82 (*rsv1*), but failed to infect PI 96983 (*Rsv1*) or L78-379 (*Rsv1*) (data not shown). An additional pSMV-N derived mutant, pSMV-$N_{V822M+K952E+A1111V}$, containing all three substitutions was also synthesized. Similar to all the other pSMV-N derived mutants, it retained infectivity on Williams 82 (*rsv1*); however, the mutant did not gain virulence on PI 96983 (*Rsv1*) and L78-379 (*Rsv1*) (data not shown).

The stability of all the mutations was confirmed by RT-PCR amplification of P3 sequences recovered from Williams 82 (*rsv1*) infected plants followed by direct sequencing. Only progenies derived from pSMV-N_{K952E}, pSMV-$N_{V822M+K952E}$ and pSMV-$N_{V822M+K952E+A1111V}$ contained a translationally silent mutation at nucleotide 3002. This mutation was originated during PCR as the corresponding plasmids harbored the same silent mutation. It has been shown that incorporation of translationally silent mutations in P3 did not influence the outcome of interaction of SMV with *Rsv1*-genotype soybeans

```
N    (766) GEVQQRMKCETALITSIFKPKRMIQILENDPYILLMGLVSPSILIHMYRMKHFEKGVELW
G7   (767) GEVQQRMKCETALITSIFKPKIMVQILENDPYVLLMGLVSPSILIHMYRMKHFEKGVELW
G7d  (767) GEVQQRMKCETALITSIFKPKIMVQILENDPYVLLMGLVSPSILIHMYRMKHFEKGMELW

N    (826) ISKEHSVAKIFIILEQLTKRVAANDVLLEQLEMISETSERFMSILEDCPQAEHSYKTAKD
G7   (827) ISKEHSVAKIFIILEQLTKRVAANDVLLEQLEMISETSERFMSILEDCPQASHSYKTAKD
G7d  (827) ISKEHSVAKIFIILEQLTKRVAANDVLLEQLEMISETSERFMSILEDCPQASHSYKTAKD

N    (886) LLTMYIERKASNNQLVENGFVDMNDKLYMAYEKIYSDRLKQEWRALSWLEKFSITWQLKR
G7   (887) LLTMYIERKASNNQLVENGFVDMNDKLYMAYEKIYSDRLKQEWRALSWLEKFSITWQLKR
G7d  (887) LLTMYIERKASNNQLVENGFVDMNDKLYVAYEKIYSDRLKQEWRALSWLEKFSITWQLKR

N    (946) FAPHTEKCLTKKVVEESSASSGNFASVCFMNAQSHLRNVRNTLFQKCDQVWTASVRAFVK
G7   (947) FTPHTEKCLTKKAVEENSASSGNFASVCFMNAQSHLRNVRNTLFQKCDQVWTASVRAFVR
G7d  (947) FTPHTEECLTKKAVEENSASSGNFASVCFMNAQSHLRNVRNTLFQKCDQVWTASVRAFVR

N    (1006) LIISTLHRCYSDIVYLVNICIIFSLLVQMTSVLQGIVNTVRRDKALLSGWKRKKEDEEAVI
G7   (1007) FIISTLHRCYSDIVYLVNICIIFSLLVQMTSVLQGIINTARRDKALLHSHKRREDEEAVI
G7d  (1007) FIISTLHRCYSDIVYLVNICIIFSLLVQMTSVLQGIINTARRDKALLHSHKRREDEEAVI

N    (1066) HLYEMCEKMEGGHPSIEKFLDHVKGVRPDLLPVAVSMTGQSEDVSAQAKTATQLQLEKIV
G7   (1067) HLYEMCEKMEGGHPSVEGFLNHVRGVRPDLLSVARNMTGQSEDVSAQVKTATQLQLEKIV
G7d  (1067) HLYEMCEKMEGGHPSVEGFLNHVRGVRPDLLSVARNMTGQSEDVSVQVKTATQLQLEKIV

N    (1126) AFMALLTMCIDNERSDAVFKVLSKLKAFFSTMGEDVKVQ
G7   (1127) AFMALLTMCIDNERSDAVFKVLSKLKAFFSTMGEDVKVQ
G7d  (1127) AFMALLTMCIDNERSDAVFKVLSKLKAFFSTMGEDVKVQ
```

Fig. 1. Alignment of the deduced primary amino acid sequences of the P3 of pSMV-N (N), pSMV-G7 (G7), and pSMV-G7d (G7d). The unique amino acids are highlighted. The numbering is based on predicted positions of P3 within the SMV polyprotein precursor of each of the viruses (Jayaram et al., 1992).

(Hajimorad et al., 2005). Thus, the data suggest that the amino acid involved in elicitation of *Rsv1*-mediated SHR or LSHR, in the context of pSMV-G7, do not influence the elicitor function of pSMV-N provoking *Rsv1*-dependent ER.

P3 of SMV-G7 or SMV-G7d does not confer virulence to SMV-N on Rsv1-genotype soybean

To examine the possibility that the tertiary structure of strain-specific P3 in its entirety is critical for elicitation of *Rsv1*-mediated ER, we precisely replaced P3 of pSMV-N with P3 of pSMV-G7 or pSMV-G7d. The pSMV-N/G7P3 and pSMV-N/G7dP3, which contain the precise P3 sequences of pSMV-G7 and pSMV-G7d, respectively, were synthesized from intermediate chimeras (Fig. 2). To synthesize pSMV-N/G7P3, a *Kpn*I/*Spe*I fragment containing nucleotide sequences 2430–3236 of pSMV-G7 was excised from pSMV-N/G7$_{(2430-3236)}$ and ligated into pSMV-N/G7$_{(3237-3626)}$ (Fig. 2). The pSMV-N/G7dP3 was synthesized similarly except pSMV-N/G7d$_{(2430-3236)}$ and pSMV-N/G7d$_{(3237-3626)}$ served as a donor and a recipient plasmid, respectively (Fig. 2). The P3 regions of all chimeras were sequenced and were found identical to the parental viruses. All the chimeras were replication competent in Williams 82 (*rsv1*) (Fig. 2); however, following sap inoculation of viral progenies onto PI 96983 (*Rsv1*) or L78-379 (*Rsv1*), based on absence of both symptoms and the virus as determined by squash-immunoblotting or DAS-ELISA, all failed to overcome the ER tier of *Rsv1*-mediated resistance response (Fig. 2). The P3 region of progenies derived from each of the chimeric viruses was recovered by RT-PCR from the infected Williams 82 (*rsv1*) plants, directly sequenced, and was found identical to the parental sequences.

P3 of SMV-N provokes Rsv1-mediated ER

To determine if P3 of pSMV-N alters the abilities of pSMV-G7 and pSMV-G7d to bypass the ER tier of *Rsv1*-mediated resistance, their corresponding P3 regions were precisely replaced with P3 of pSMV-N. In the course of constructing pSMV-G7/NP3 and pSMV-G7d/NP3, intermediate chimeras were synthesized (Fig. 2). To synthesize pSMV-G7/NP3, a *Kpn*I/*Spe*I fragment containing nucleotide sequences 2427–3233 of pSMV-N was excised from pSMV-G7/N$_{(2427-3233)}$ and ligated into pSMV-G7/N$_{(3234-3623)}$ (Fig. 2). The pSMV-G7d/NP3 was generated similarly except pSMV-G7d/N$_{(2427-3233)}$ and pSMV-G7d/N$_{(3234-3623)}$ served as a donor and a recipient plasmid, respectively (Fig. 2). The P3 regions of the chimeras were sequenced and found identical to the parental viruses. All

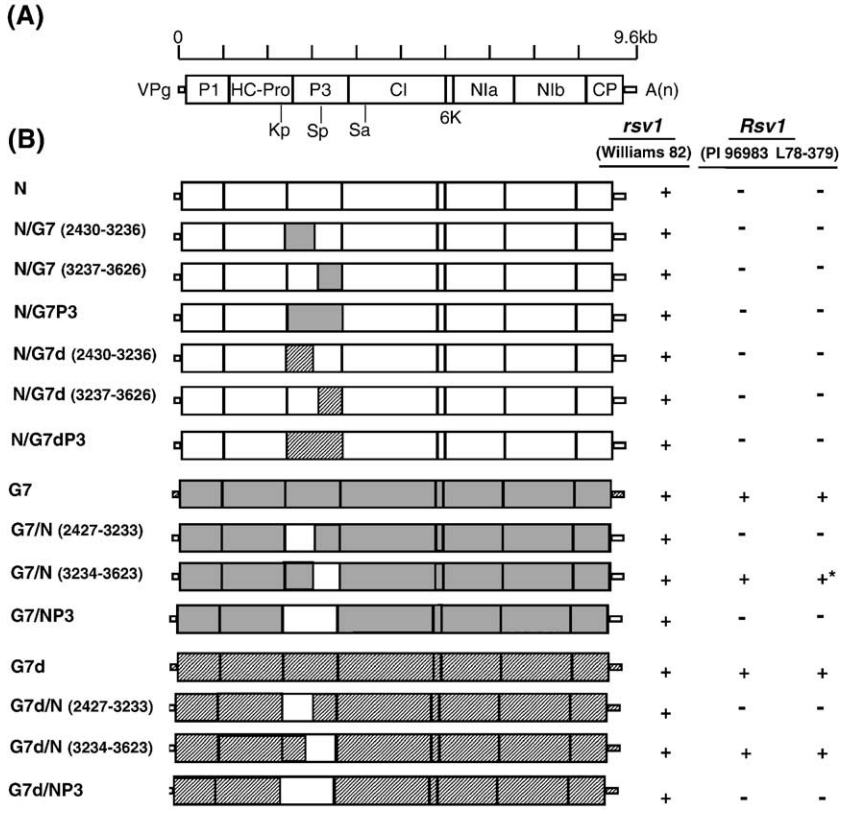

Fig. 2. Schematic representation of parental viruses, their derivative chimeras and their potential to infect *rsv1* (Williams 82) and *Rsv1* (PI 96983 or L78-379) soybean genotypes. (A) The genomic map of *Soybean mosaic virus* (SMV) and the positions of the single restriction sites *Kpn*I (Kp), *Spe*I (Sp), and *Sal*I (Sa) common among the three strains. (B) Schematic representation of pSMV-N (N), pSMV-G7 (G7), and pSMV-G7d (G7d), their derivative chimeras and the responses of soybean genotypes to sap inoculation containing progenies of parental or chimeric viruses. Following inoculation, the plants were maintained in a growth chamber (20 °C) until evaluated 6 weeks post-inoculation. Samples were collected and evaluated for the presence (+) or absence (−) of the viruses by squash-immunoblotting. The asterisk (*) indicates that infection in L78-379 (*Rsv1*) soybean genotype was associated with a single point mutation in P3 changing the encoded amino acid from alanine to threonine at position 896 precursor polypeptide.

the chimeras remained replication competent in Williams 82 (*rsv*1) (Fig. 2). However, based on absence of symptoms (Fig. 3) and lack of virus detection immunologically (Fig. 2), or by slot-blot hybridization (Fig. 4), the progenies of both pSMV-G7/NP3 and pSMV-G7d/NP3 gained the elicitor function provoking *Rsv1*-mediated ER when inoculated onto PI 96983 (*Rsv1*). Similar results were obtained when L78-379 (*Rsv1*) plants were inoculated with progenies of both the chimeras (Fig. 2). These data indicate that P3 of pSMV-N provokes *Rsv1*-mediated ER.

Analyses of the other pSMV-G7 and pSMV-G7d derived chimeras on PI 96983 (*Rsv1*) showed that progenies derived from pSMV-G7/$N_{(3234-3623)}$ and pSMV-G7d/$N_{(3234-3623)}$ did not elicit *Rsv1*-mediated ER as they both induced systemic infection (Fig. 3) and the viruses were detected in the inoculated plants immunologically (Fig. 2) and by slot-blot hybridization (Fig. 4). The symptoms, however, differed from the parental viruses (Fig. 3). The pSMV-G7/$N_{(3234-3623)}$, unlike pSMV-G7, failed to provoke *Rsv1*-mediated LSHR and elicited progressive SHR instead. On the other hand, the progeny of pSMV-G7d/$N_{(3234-3623)}$, unlike pSMV-G7d, induced moderate stunting (Fig. 3). The response of L78-379 (*Rsv1*) plants to inoculation with progeny of pSMV-G7d/$N_{(3234-3623)}$ was similar to the response of PI 96983 (*Rsv1*) as a total of 24 out of 32 inoculated plants showed systemic mosaic and moderate leaf distortion (not shown). In contrast, under similar conditions, in a number of independent experiments where 41 L78-379 (*Rsv1*) soybean plants were inoculated with progeny derived from pSMV-G7/

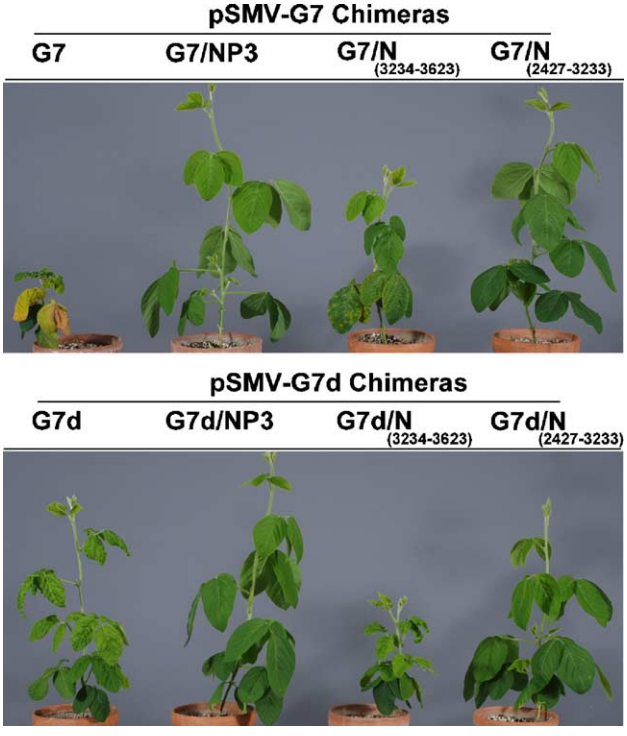

Fig. 3. Phenotypic differences in response of soybean line PI 96983 (*Rsv1*) to inoculation with infectious sap containing progenies of pSMV-G7 (G7), pSMV-G7d (G7d), or their derivative chimeras. Following inoculation, the plants were maintained in a growth chamber (20 °C) until photographed about 5 weeks post-inoculation.

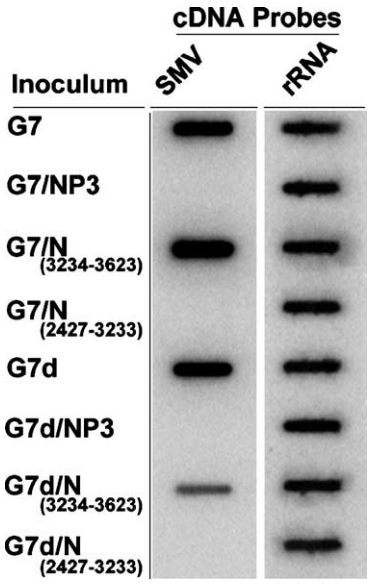

Fig. 4. Slot-blot hybridization analysis of accumulation of *Soybean mosaic virus* (SMV) RNA, and soybean 18S ribosomal RNA (rRNA) in soybean trifoliolate leaves. Primary leaves of PI 96983 (*Rsv1*) were mechanically inoculated with infectious sap containing progenies of pSMV-G7 (G7), pSMV-G7d (G7d) or their derivative chimeras. Following inoculation, plants were maintained in a growth chamber (20 °C) until a leaflet from trifoliolates 3–4 of infected plants was collected about 6 weeks post-inoculation. Samples from corresponding trifoliolate leaflets of four independent replicate plants were combined; total RNA isolated, denatured, 10 μg slot-blotted onto a membrane, and hybridized with ^{32}P-labeled cDNA probes.

$N_{(3234-3623)}$, none were infected. However, when progeny of pSMV-G7/$N_{(3234-3623)}$ containing a single mutation at position 2817 (G to A) were used as inoculum, four out of 17 inoculated L78-379 (*Rsv1*) exhibited very mild SHR on the lower and mosaic on the upper trifoliate leaves. P3 was recovered by RT-PCR from each of the infected plants and sequencing showed in all cases only a single G to A substitution at nucleotide 2817 changing the corresponding encoded amino acid at position 896 precursor polypeptide from alanine to threonine. This mutant was initially originated in a single infected PI 96983 (*Rsv1*) soybean plant inoculated with progenies derived from pSMV-G7/$N_{(3234-3623)}$, which unlike all the other infected PI 96983 (*Rsv1*) with the same inoculum (Fig. 3), was exhibiting severe stunting and strong SHR (not shown). Sub-inoculation of progeny containing G2817A mutation onto PI 96983 (*Rsv1*) also resulted in severe stunting together with strong SHR in 11 out of the 12 inoculated plants. The differential responses between PI 96983 (*Rsv1*) and L78-379 (*Rsv1*) to inoculation with pSMV-G7 and the phenotypic impact of amino acid substitutions in P3 of the virus have been documented (Hajimorad et al., 2003, 2005).

The progenies derived from pSMV-G7/$N_{(2427-3233)}$ and pSMV-G7d/$N_{(2427-3233)}$, however, both failed to bypass the *Rsv1*-mediated ER tier of resistance when inoculated onto PI 96983 (*Rsv1*) (Figs. 2–4) or L78-379 (*Rsv1*) (Fig. 2). When the P3 region of viral progenies derived from each of the chimeras was recovered by RT-PCR from Williams 82 (*rsv1*), PI 96983 (*Rsv1*), or L78-379 (*Rsv1*) infected soybeans and sequenced, analyses showed that the P3 sequences of the

progenies of all viruses, except those derived from pSMV-G7/$N_{(3234-3623)}$, were identical to the parental plasmids.

Discussion

The objectives of this study were to test the hypothesis that strain-specific P3 of SMV is the elicitor of *Rsv1*-mediated ER, and to demonstrate that, analogous to the interactions of viruses other than potyviruses with *R*-genotype plants, virulence of SMV on *Rsv1*-genotype soybean is a consequence of the absence of P3 elicitor functions provoking *Rsv1*-mediated ER and LSHR. We have shown previously that SMV strain-specific P3 provokes *Rsv1*-mediated LSHR (Hajimorad et al., 2005).

In pathosystems expressing HR-dependent resistance against plant viruses, perturbations of *avr* genes alter the localized HR to SHR (Culver et al., 1991; Kim and Palukaitis, 1997). *R*-mediated resistance response against potyviruses, however, is commonly expressed as ER, and localized HR leading to the arrest of the invading virus at the inoculation site has been reported in a few cases (Dogimont et al., 1996; Hinrichs-Berger et al., 1999). On the other hand, *R*-mediated SHR is a common occurrence against potyviruses (Collmer et al., 2000; Dogimont et al., 1996; Fellers et al., 2002; Hajimorad et al., 2003; Jenner et al., 2000, 2002, 2003; Jones, 1990; Kyle and Provvidenti, 1993; Patel, 1982; Valkonen et al., 1998; Vidal et al., 2002). Thus, analogous to HR-expressing pathosystems, one might expect that in SMV/*Rsv1* pathosystem, the elicitor of *Rsv1*-mediated ER and LSHR are modified forms of P3.

In the current study, the differential interactions of SMV-N, SMV-G7 and SMV-G7d with *Rsv1*-genotype soybeans were exploited to explore the role of P3 of SMV-N in elicitation of *Rsv1*-mediated ER. The SMV-N possesses the elicitor functions provoking *Rsv1*-mediated ER and restricted SHR (Hajimorad and Hill, 2001). In contrast, SMV-G7d represents a highly evolved strain of the virus as it has lost the elicitor functions provoking *Rsv1*-mediated ER as well as LSHR (Hajimorad et al., 2003). The SMV-G7d was evolved experimentally following a series of rapid high population transfer of progeny of pSMV-G7 on PI 96983 (*Rsv1*) over a relatively long period of time (Hajimorad et al., 2003). On the other hand, the SMV-G7 represents an evolutionary intermediate strain of SMV, which lacks the elicitor function provoking *Rsv1*-mediated ER but elicits *Rsv1*-mediated LSHR (Hajimorad et al., 2005).

However, the substitutions of the three amino acids of P3 of pSMV-N with those corresponding to pSMV-G7d with influence on evasion of *Rsv1*-mediated recognition provoking LSHR, individually or in combination, failed to alter the elicitor function of the virus provoking *Rsv1*-mediated ER. This is not surprising as all the three amino acids are identical between pSMV-G7 and pSMV-N (Fig. 1), and yet pSMV-G7 is capable of bypassing the ER-tier of *Rsv1*-mediated resistance response (Hajimorad et al., 2003). Furthermore, incorporation of the amino acids corresponding to pSMV-G7 into pSMV-G7d, individually or in combinations, did not abolish the ability of pSMV-G7d to bypass the ER-tier of *Rsv1*-mediated resistance response, but pSMV-G7d derived mutants provoked *Rsv1*-mediated SHR or LSHR (Hajimorad et al., 2005). If *Rsv1*-mediated recognition provoking ER is mediated by direct interaction with P3, then possibly these three amino acids do not participate in the elicitor site. Alternatively, their incorporation into P3 of pSMV-N do not alter the structure of the elicitor site or perhaps the overall tertiary structure of P3 to the extent necessary to evade *Rsv1*-mediated recognition provoking ER. Excluding these three amino acids, there are a total of 23 additional amino acids differences between P3 of pSMV-N and that of pSMV-G7 or pSMV-G7d (Fig. 1), which contribute to the elicitor function of P3 of pSMV-N provoking *Rsv1*-mediated ER.

The finding that precise replacement of P3 of pSMV-G7 and pSMV-G7d with P3 from pSMV-N rendered their derivative chimeras avirulent on *Rsv1*-genotype soybean (Figs. 2–4) indicates that P3 of pSMV-N provokes *Rsv1*-mediated ER. TMV- and PVX-based vectors expressing strain-specific CP of PVX or *avrPto* also were rendered avirulent upon inoculation onto *Rx*- and *Pto*-bearing leaf tissues, respectively (Bendahmane et al., 1999; Tobias et al., 1999). The elicitor site on P3 of pSMV-N provoking *Rsv1*-mediated ER was further narrowed to the N-terminus domain comprising 271 amino acids. This region of P3 of pSMV-N differs by 9 and 12 amino acids from the corresponding regions of pSMV-G7 and pSMV-G7d, respectively. The observation that progeny derived from pSMV-G7/$N_{(3234-3623)}$ gained virulence on L78-379 (*Rsv1*) only after accumulation of a point mutation at position 896 within the N-terminus, also confirm the importance of this domain of P3 in the elicitation of *Rsv1*-mediated ER. It is interesting to note that the corresponding domain of pSMV-G7 also serves as the elicitor of *Rsv1*-mediated LSHR (Hajimorad et al., 2005). The finding that SMV-strain specific P3 provokes *Rsv1*-mediated ER and LSHR is not surprising. The coat protein (CP) of PVX and movement protein (MP) of *Tomato mosaic virus* (ToMV) each also serves as the elicitor of both ER and HR mediated by *Rx* and *Tm-2*^{2} genes, respectively (Bendahmane et al., 1999; Weber et al., 1993; Weber and Pfitzner, 1998).

The observation that pSMV-N/G7P3 and pSMV-N/G7dP3 remained avirulent on *Rsv1*-genotype soybeans is somewhat unexpected. Similar experiments with HR-dependent pathosystems have resulted in gain of virulence by avirulent viral strains (Berzal-Herranz et al., 1995; Diveki et al., 2004; Karasawa et al., 1999; Kim and Palukaitis, 1997; Malcuit et al., 1999; Padgett et al., 1997; Querci et al., 1995; Santa Cruz and Baulcombe, 1993; Tsuda et al., 1998). Experiments on ER-dependent pathosystems, such as ToMV/*Tm-1,* ToMV/*Tm-2*, and ToMV/*Tm-2*^{2} and using a similar approach, also have resulted in gain of virulence by avirulent strains (Meshi et al., 1988, 1989; Weber et al., 1993). However, similar experiments on the PVX/*Rx* pathosystem have yielded somewhat unusual results. A single point mutation in CP of an avirulent PVX strain allowed the mutant to overcome *Rx*-mediated resistance at the protoplast level, but failed to replicate *in planta* suggesting that *Rx*-mediated resistance is complex (Goulden

et al., 1993; Goulden and Baulcombe, 1993). Nevertheless, exchanges of CP between an "*Rx*-sensitive" and an "*Rx*-insensitive" strain of PVX resulted in gain of virulence by the "*Rx*-sensitive" strain *in planta* (Kavanagh et al., 1992; Goulden et al., 1993; Querci et al., 1995).

In contrast to tobamovirus and potexvirus pathosystems where knowledge on *R*-mediated elicitor function of viral genes is advanced, only limited studies have been reported on *R*-mediated elicitor functions of potyviruses (Revers et al., 1999). The elicitor function of the nuclear inclusion a-protein (NIa) domain of PVY provoking *Ry*-mediated HR has been illustrated only in a transient expression assay system (Mestre et al., 2000, 2003). However, ER is considered as the primary mechanism of *Ry*-mediated resistance (Mestre et al., 2000). Thus, it is not known if NIa provokes *Ry*-mediated ER *in planta*. The elicitor function of strain-specific nuclear inclusion b-protein (NIb) of PVY provoking *Rk*-mediated SHR also has been illustrated in a transient expression assay system (Fellers et al., 2002). Nevertheless, strain-specific cytoplasmic inclusion (CI) cistron of *Turnip mosaic virus* (TuMV) was identified as the elicitor of *TuRB01*-mediated ER; however, the virulent viruses provoked SHR (Jenner et al., 2000). Thus, it is not known if CI is also the elicitor of *TuRB01*-mediated SHR. Similarly, for overcoming the resistance responses in *Brassica napus* line 165 by an avirulent strain of TuMV, mutations in both P3 and CI citrons were required (Jenner et al., 2002). This was attributed, however, to the existence of two independent *R*-genes against the virus in this host (Jenner et al., 2002). In contrast, strain-specific P3 of TuMV has been identified as the elicitor of both *TuRB03*-mediated ER and SHR (Jenner et al., 2003). It is interesting to note that the genetic determinant of potyvirus *Tobacco etch virus* (TEV) eliciting vascular necrosis in Tobasco pepper was mapped to 3′ of P3 and 5′ of CI cistrons, and the presence of both regions were found essential for avoiding the necrosis response (Chu et al., 1997). Similarly, the viral determinants of a strain of potyvirus *Lettuce mosaic virus* inducing systemic wilting in some lettuce cultivars have been mapped to P1 and CI cistrons (Krause-Sakate et al., 2005).

It is unlikely that PI 96983 (*Rsv1*) and L78-379 (*Rsv1*) each contains more than one *R*-gene against SMV-N, a G2 strain of the virus. Classical genetic studies on PI 96983 (*Rsv1*) by independent research groups have established the presence of a single *R*-gene against SMV (Kiihl and Hartwig, 1979; Chen et al., 1991, 1994). Furthermore, in a high resolution mapping population resulting from a cross of PI 96983 (*Rsv1*) and Lee 68 (*rsv1*), a homozygous recombinant F4 soybean line containing only one *Rsv1* resistance gene candidate from PI 96983 (*Rsv1*), expressed phenotypically "symptomless resistance" (ER) against SMV-G2 and "systemic necrosis" (SHR) against SMV-G7 (Hayes et al., 2004). This observation provide additional evidence that a single *R*-gene in PI 96983 (*Rsv1*) provokes both ER and LSHR against specific strains of SMV.

The inability of P3 derived from pSMV-G7 and pSMV-G7d to confer virulence to pSMV-N on *Rsv1*-genotype soybeans points to the involvement of additional SMV factor(s) in virulence. It has been demonstrated that the mechanism of virulence of viruses on *R*-genotype plants is the absence of the elicitor function provoking *R*-mediated resistance responses (Berzal-Herranz et al., 1995; Diveki et al., 2004; Karasawa et al., 1999; Kim and Palukaitis, 1997; Malcuit et al., 1999; Padgett et al., 1997; Querci et al., 1995; Santa Cruz and Baulcombe, 1993; Tsuda et al., 1998). However, an alternative mechanism such as the presence of a virus encoded specificity determinant suppressing the effect of avirulence also has been speculated (Goulden et al., 1993; Goulden and Baulcombe, 1993; Querci et al., 1995). The loss of virulence of pSMV-G7/NP3 and pSMV-G7d/NP3 on *Rsv1*-genotype soybean, however, indicates that strain-specific P3 of SMV is involved in virulence, but it is not sufficient by itself to confer virulence to an avirulent SMV strain. Hence, in addition to the modifications of P3, pSMV-G7 and pSMV-G7d must have evolved additional tactic(s), such as evoking a susceptible response, to overcome the ER-tier of *Rsv1*-mediated resistance response.

Although we have now established that strain-specific P3 of SMV provokes *Rsv1*-mediated ER and LSHR, the nature of the elicitor itself remains unknown. The single ORF of SMV is expressed as a precursor polypeptide, which is subsequently cleaved co-translationally and post-translationally by three virus-encoded proteases to produce 8–10 mature proteins (Hull, 2002). Thus, the mature P3 is produced following proteolysis of the virus precursor polypeptide. In the case of TEV, the amino-proximal end of P3 is cleaved *in cis* by helper-component proteinase (HC-Pro); however, its carboxy-proximal end is processed by NIa *in trans* (Carrington et al., 1989a, 1989b). Thus, it seems likely that at any given time P3 is present in many different polypeptide contexts within the infected cells (Rodriguez-Cerezo and Shaw, 1991). It is not known, however, which polypeptide context serves as the elicitor of *Rsv1*-mediated ER or LSHR. If the elicitor function of P3 is context dependent, then P3 would not be the sole target of recognition by the *Rsv1*-mediated surveillance system.

Materials and methods

Virus strains, soybean genotypes, inoculation, and SMV detection

Plasmids containing infectious full-length cDNA clones of SMV-G7 (pSMV-G7), SMV-G7d (pSMV-G7d) and SMV-N (pSMV-N) served as the virus sources (Eggenberger and Hill, 1997; Hajimorad et al., 2003). The soybean (*Glycine max*) cultivar Williams 82 (*rsv1*) (Bernard and Cremeens, 1988), line PI 96983 (*Rsv1*) (Kiihl and Hartwig, 1979), and line L78-379 (*Rsv1*), which is a near isoline of Williams (Bernard and Lindahl, 1972) with the *Rsv1* allele derived from line PI 96983 [Williams (6) x PI 96983] (Bernard et al., 1991), were used in this study. All seeds were obtained from virus-indexed greenhouse grown plants. To establish infection, plasmid DNA was bombarded onto hypocotyls of Williams 82 (*rsv1*) soybean by biolistic delivery (Hajimorad et al., 2003). Sap containing viral progenies from the infected plants was used to mechanically inoculate, in multiple replicate experiments, carborundum-dusted (600 mesh) soybeans (Hajimorad and Hill, 2001). The inoculated plants were maintained in a growth

chamber operating at 20 °C with a photoperiod of 18 h. The SMV detection was done by squash-immunoblotting or double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) (Hajimorad and Hill, 2001).

Site directed mutagenesis

The megaprimer PCR based mutagenesis method (Sambrook and Russell, 2001) was used for the introduction of point mutations into pSMV-N. To generate pSMV-N_{V822M} and pSMV-N_{K952E}, the targeted P3 regions were PCR amplified in the presence of pSMV-N with antisense mutagenic primers N2607a and N3003a, respectively, and the forward primer G2274s (Table 1). Each of the mutagenized amplified PCR products was then used as a forward megaprimer in the presence of pSMV-N together with primer N3264a (Table 1). The fragments were digested with *Kpn*I and *Spe*I, and ligated into pSMV-N. To generate pSMV-N_{A1111V}, the targeted P3 region was PCR amplified in the presence of pSMV-N with mutagenic primer N3480a and the forward primer N3157s (Table 1). The PCR product was then used as a forward megaprimer in the presence of primer G3843a (Table 1) and pSMV-N as a template. The PCR product was digested with *Spe*I and *Sal*I, and ligated into pSMV-N. To generate pSMV-$N_{V822M+K952E}$, the mutagenized PCR product amplified with primers N2607a and G2274s, was used as a forward megaprimer in the presence of the primer N3264a and pSMV-N_{K952E} as a template. This generated a mutagenized P3 fragment containing two point mutations that was then digested with *Kpn*I and *Spe*I and ligated into pSMV-N. For the synthesis of pSMV-$N_{V822M+K952E+A1111V}$, a *Spe*I and *Sal*I fragment was released from pSMV-N_{A1111V} and ligated into pSMV-$N_{V822M+K952E}$.

ElectroMax DH5α-E (Invitrogen, Carlsbad, CA, USA) cells were transformed by electroporation using a MicroPulser (Bio-Rad, Hercules, CA, USA) and positive transformants were identified by PCR. The plasmids were purified by using a QiaPrep Spin MiniPrep Kit (Qiagen, Valencia, CA, USA), and to ensure the absence of undesired PCR generated mutations, the entire PCR amplified regions were sequenced. The site-directed mutant viruses were tested for infectivity on Williams 82 (*rsv1*) by biolistic delivery (Hajimorad et al., 2003).

Table 1
Sequences of sense and antisense oligonucleotide primers used for synthesis of the site-directed mutants or replacement of P3

Name	Sequences[a] (5′–3′)	Position[b]
N2430a	CACCACCAACTCTGTAGAATTT	2430–2409
N2607a	TCCACAACTCCA***T***CCCTTTCTCAAA	2607–2583
N3003a	CTTTCTTTGTCAAACATT***C***TTCCGTATGTGGAG	3003–2971
N3157s	CAACACTTCACAGGTGCTACAGTGATATAG	3157–3186
N3264a	CTGTGTTGACAATGCCCTGC	3264–3245
N3480a	TTGCTGTTTTGGCCTGT***A***CGGAGACATCTTCTG	3480–3448
N3623a	CTGCACTTTAACATCCTCACCCATTGTGC	3623–3595
G2274s	ATTTTGGTTGACCATGCGT	2274–2292
G2433a	CACCACCAACTCTATAGAATTT	2433–2412
G2919s	CGCGCATTAAGCTGGTTG	2919–2936
G3266a	TGTATTGATAATACCTTGC	3266–3248
G3626a	CTGCACCTTAACATCCTCACCCATTGTGC	3626–3598
G3843a	CCAAATTTGCAATTTTGGCTGCTG	3843–3820
G3910a	AAACCTGTTGATTTCCCTGAGCC	3910–3888

[a] Non-identical nucleotides with the homologous strain are bold and italicized.

[b] The position of oligonucleotides on the SMV genome are based on sequences of SMV strains G7, G7d, and N (GenBank Accession Nos. AY216010, AY 216987, and D00507, respectively).

Precise replacement of P3 of SMV-N with P3 of SMV-G7 or SMV-G7d and vice versa

To replace precisely the entire P3, two convenient single restriction sites *Kpn*I and *Sal*I, which are common among the three plasmids, were utilized (Fig. 5). In the context of SMV-G7 and SMV-G7d genomes these sites are located at nucleotide 2338 and 3784, respectively, whereas, in SMV-N, these are located at nucleotides 2335 and 3781, respectively (Fig. 5). The P3 of all the three strains is 1197 nucleotides long. However, SMV-N lacks a codon (GGT) in the P1 region, which corresponds to nucleotides 723–725 in the context of SMV-G7 and SMV-G7d. Hence, the genomic position of P3 of SMV-N differs from those of SMV-G7 and SMV-G7d. The P3 sequences extend from nucleotides 2427–3623 in SMV-N and 2430–3626 in both SMV-G7 and SMV-G7d. The nucleotide sequences 2338–2430 and 3626–3784 are identical between SMV-G7 and SMV-G7d (Fig. 5); however, the corresponding regions of SMV-N differ from the SMV-G7 and SMV-G7d by 4 and 18 nucleotides, respectively (Fig. 5). As a result, P3 could not be precisely replaced by simply exchanging *Kpn*I and *Sal*I fragments among the viral genomes and consequently the megaprimer PCR-based strategy was adopted (Sambrook and Russell, 2001). The precise exchange of P3 was achieved in three steps and by taking the advantage of *Spe*I, which is positioned at nucleotides 3236 on SMV-G7 and SMV-G7d genomes and at nucleotide 3234 in the context of SMV-N (Fig. 5).

In the first step, two sets of megaprimers, representing 3′-termini sequences of HC-Pro cistron of pSMV-N or pSMV-G7d were PCR amplified (Fig. 5). The pSMV-G7d derived megaprimer was PCR amplified in the presence of primers G2274s and G2433a (Table 1), and used as a forward megaprimer in a PCR reaction in the presence of N3264a (Table 1) and pSMV-N as a template. The PCR product was digested with *Kpn*I and *Spe*I and ligated into pSMV-G7 and pSMV-G7d to generate pSMV-G7/$N_{(2427-3233)}$ and pSMV-G7d/$N_{(2427-3233)}$, respectively (Fig. 2). The pSMV-N derived megaprimer was amplified as above except using primer N2430a (Table 1) and pSMV-N as a template. It was then used in PCR reactions as a forward megaprimer in the presence of G3266a (Table 1) and pSMV-G7 or pSMV-G7d as templates. The products were digested with *Kpn*I and *Spe*I and ligated into pSMV-N to generate pSMV-N/$G7_{(2430-3236)}$ and pSMV-N/$G7d_{(2430-3236)}$ (Fig. 2).

In the second step, the sequences of pSMV-N at position 3157–3623 and those of pSMV-G7 and pSMV-G7d at positions 2919–3626 were PCR amplified and used as megaprimers. The pSMV-G7 and pSMV-G7d derived megaprimers were amplified in the presence of primers G3626a and

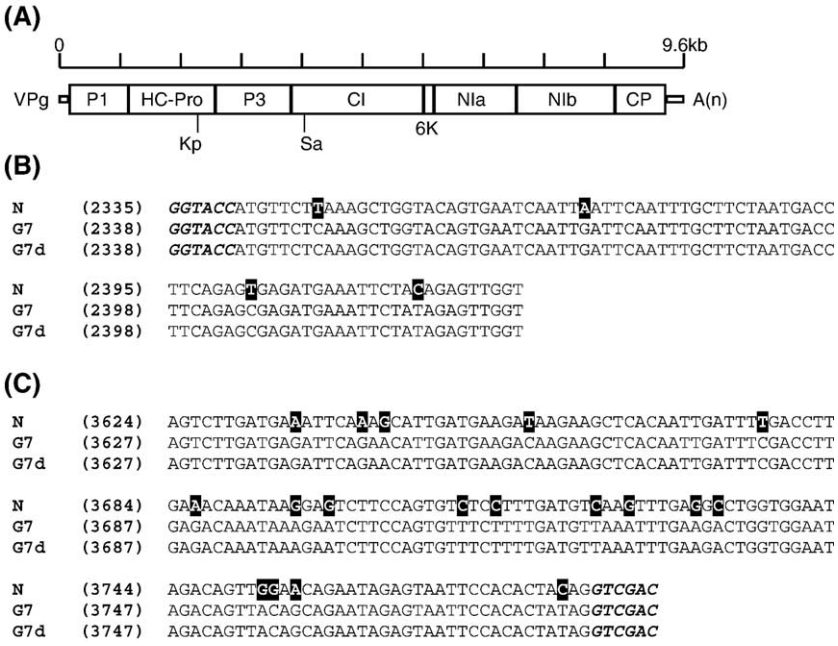

Fig. 5. (A) The genomic map of *Soybean mosaic virus* (SMV) and the position of the single restriction sites *Kpn*I (Kp), and *Sal*I (Sa) common to the three virus strains. (B) Alignments of nucleotide sequences of 3′-terminus of helper-component proteinase (HC-Pro) cistron, and (C) 5′-terminus of cytoplasmic inclusion (CI) cistron of pSMV-N (N), pSMV-G7 (G7), and pSMV-G7d (G7d). The recognition sequences of *Kpn*I and *Sal*I located in HC-Pro and CI, respectively, are italicized and bold.

G2919s (Table 1), and then used as forward megaprimers in the presence of primer G3910a and pSMV-N as a template. The amplified fragments were digested with *Spe*I/*Sal*I and ligated into pSMV-N to generate pSMV-N/$G7_{(3237-3626)}$ and pSMV-N/$G7d_{(3237-3626)}$. The pSMV-N derived megaprimer was amplified in the presence of N3157s and N3623a (Table 1), which served subsequently as a forward megaprimer in the presence of primer G3910a (Table 1) and pSMV-G7d as a template. The amplified PCR fragment was digested with *Spe*I/*Sal*I and ligated into pSMV-G7 and pSMV-G7d to generate pSMV-G7/$N_{(3234-3623)}$ and pSMV-G7d/$N_{(3234-3623)}$, respectively (Fig. 2).

To synthesize chimeras with precisely exchanged full-length P3 sequences, *Kpn*I/*Spe*I fragments were excised from the chimeras generated in the first stage and ligated into similarly digested chimeras obtained in the second stage. Thus, pSMV-N/G7P3, pSMV-N/G7dP3, pSMV-G7/NP3 and pSMV-G7d/NP3 were generated. Transformation of the bacterial cells, screening of the transformants, and purification of the recombinant plasmids were done as above. To ensure the absence of any undesired PCR-generated mutations, the entire PCR generated fragment from each of the chimeras was sequenced. The chimeric viruses were tested for infectivity on Williams 82 (*rsv1*) as described above.

RNA isolation, RT-PCR, and sequencing

Total soybean RNA was isolated from systemically-infected liquid nitrogen-frozen soybean leaf tissues kept at −85 °C by using an RNeasy Plant Mini Kit (Qiagen). The genomic regions of progeny viruses were reverse transcribed by using Super-Script reverse transcriptase (RT) (Invitrogen) and antisense SMV-specific primers. To confirm the identity of the progeny viruses as chimeras, two different regions, one representing the recipient strain and the other the P3 region from the donor strain, were RT-PCR amplified. RT-PCR amplification of progeny viruses derived from site-directed mutants targeted the mutated site flanked by surrounding sequences. Occasionally, another pair of nested PCR primers was used to re-amplify the PCR products. The PCR products were purified by using a QIAquick PCR purification kit (Qiagen) and directly sequenced. Sequencing was done at the Iowa State University DNA Sequencing and Synthesis Facility. The sequences were edited by Factura (Applied Biosystems, MA, USA) and analyzed using Auto-Assembler software (Applied Biosystems).

Probes and slot-blot hybridization

A probe against soybean 18S ribosomal RNA (Eckenrode et al., 1984) was synthesized with a random-primed DNA labeling kit (Invitrogen) according to the manufacturer's instructions. The SMV probe was PCR synthesized in the presence of ^{32}P-dCTP using SMV-specific primers and super-coiled plasmid pSMV-G7d as described previously (Hajimorad et al., 2003). 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).

Acknowledgments

The authors are grateful to Drs. M. Mazarei (Iowa State University) and K. L. Perry (Cornell University) for critical

reading of the manuscript and P. Chen (University of Arkansas) for helpful discussion on the genetics of *Rsv1*. This project was supported, in part, by the Iowa Soybean Promotion Board.

References

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., Lindahl, D.A., 1972. Registration of Williams soybean. Crop Sci. 12, 716.

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. Soyb. Genet. Newsl. 18, 27–57.

Berzal-Herranz, A., De La Cruz, A., Tenllado, F., Diaz-Ruiz, J.R., Lopez, L., Sanz, A.I., Vaquero, C., Serra, M.T., Garcia-Luque, I., 1995. The *Capsicum* L^3 gene-mediated resistance against the tobamoviruses is elicited by the coat protein. Virology 209, 498–505.

Carrington, J.C., Freed, D.D., Sanders, T.C., 1989a. Autocatalytic processing of the potyvirus helper component proteinase in *Escherichia coli* and in vitro. J. Virol. 63, 4459–4463.

Carrington, J.C., Cary, S.M., Parks, T.D., Dougherty, W.G., 1989b. A second proteinase encoded by a plant potyvirus genome. EMBO J. 8, 365–370.

Chen, P., Buss, G.R., Roane, C.W., Tolin, S.A., 1991. Allelism among genes for resistance to soybean mosaic virus in strain-differential soybean cultivars. Crop Sci. 31, 305–309.

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.

Chu, M., Lopez-Moya, J.J., Llave-Correas, C., Pirone, T.P., 1997. Two separate regions in the genome of the tobacco etch virus contain determinants of the wilting response of Tabasco pepper. Mol. Plant-Microbe Interact. 10, 472–480.

Collmer, C.W., Marston, M.F., Taylor, J.C., Jahn, M., 2000. The *I* gene of bean: a dosage-dependent allele conferring extreme resistance, hypersensitive resistance, or spreading vascular necrosis in response to the potyvirus *Bean common mosaic virus*. Mol. Plant-Microbe Interact. 13, 1266–1270.

Culver, J.N., 1997. Viral avirulence genes. In: Stacey, G., Keen, N.T. (Eds.), Plant-Microbe Interactions, vol. 2. Chapman and Hall, New York, pp. 196–219.

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.

Diveki, Z., Salanki, K., Balazs, E., 2004. The necrotic pathotype of the *Cucumber mosaic virus* (CMV) Ns strain is solely determined by amino acid 461 of the 1a protein. Mol. Plant-Microbe Interact. 17, 837–845.

Dogimont, C., Palloix, A., Daubze, A.-M., Marchoux, G., Selassie, K.G., Pochard, E., 1996. Genetic analysis of broad spectrum resistance to potyviruses using doubled haploid lines of pepper (*Capsicum annuum* L.). Euphytica 88, 231–239.

Dougherty, W.G., Semler, B.L., 1993. Expression of virus-encoded proteinases: functional and structural similarities with cellular enzymes. Microbiol. Rev. 57, 781–822.

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.

Fellers, J.P., Tremblay, D., Handest, M.F., Lommel, S.A., 2002. The *Potato virus* Y $M^S N^R$ NIb-replicase is the elicitor of a veinal necrosis-hypersensitive response in root knot nematode resistant tobacco. Mol. Plant Pathol. 3, 145–152.

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

Gilbert, J., Spillane, C., Kavanagh, T.A., Baulcombe, D.C., 1998. Elicitation of *Rx*-mediated resistance to PVX in potato does not require new RNA synthesis and may involve a latent hypersensitive response. Mol. Plant-Microbe Interact. 11, 833–835.

Goulden, M.G., Baulcombe, D.C., 1993. Functionally homologous host components recognize potato virus X in *Gomphrena globosa* and potato. Plant Cell 5, 921–930.

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.

Greenberg, J.T., Yao, N., 2004. The role and regulation of programmed cell death in plant-pathogen interactions. Cell. Microbiol. 6, 201–211.

Hajimorad, M.R., Hill, J.H., 2001. *Rsv1*-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.

Hajimorad, M.R., Eggenberger, A.L., Hill, J.H., 2003. 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. Virology 314, 497–509.

Hajimorad, M.R., Eggenberger, A.L., Hill, J.H., 2005. Loss and gain of elicitor function of *Soybean mosaic virus* G7 provoking *Rsv1*-mediated lethal systemic hypersensitive response maps to P3. J. Virol. 79, 1215–1222.

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

Hayes, A.J., Jeong, S.C., Gore, M.A., Yu, Y.G., Buss, G.R., Tolin, S.A., Saghai Maroof, M.A., 2004. Recombination within a nucleotide-binding-site/leucine-rich-repeat gene cluster produces new variants conditioning resistance to Soybean mosaic virus in soybeans. Genetics 166, 493–503.

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

Hinrichs-Berger, J., Harfold, M., Berger, S., Buchenauer, H., 1999. Cytological responses of susceptible and extremely resistant potato plants to inoculation with potato virus Y. Physiol. Mol. Plant Pathol. 55, 143–150.

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

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., Sanchez, F., Nettleship, S.B., Foster, G.D., Ponz, F., Walsh, J.A., 2000. The cylindrical inclusion gene of Turnip mosaic virus encodes a pathogenic determinant to the Brassica resistance gene *TuRB01*. Mol. Plant-Microbe Interact. 13, 1102–1108.

Jenner, C.E., Tomimura, K., Ohshima, K., Hughes, S.L., Walsh, J.A., 2002. Mutations in *Turnip mosaic virus* P3 and cylindrical inclusion proteins are separately required to overcome two *Brassica napus* resistance genes. Virology 300, 50–59.

Jenner, C.E., Wang, X., Tomimura, K., Ohshima, K., Ponz, F., Walsh, J.A., 2003. The dual role of the potyvirus P3 protein of *Turnip mosaic virus* as a symptom and avirulence determinant in Brassicas. Mol. Plant-Microbe Interact. 16, 777–784.

Jones, R.A.C., 1990. Strain group specific and virus specific hypersensitive reactions to infection with potyviruses in potato cultivars. Ann. Appl. Biol. 117, 93–105.

Karasawa, A., Okada, I., Akashi, K., Chida, Y., Hase, S., Nakazawa-Nasu, Y., Ito, A., Ehara, Y., 1999. One amino acid change in cucumber mosaic virus RNA polymerase determines virulent/avirulent phenotypes on cowpea. Phytopathology 89, 1186–1192.

Kavanagh, T., Goulden, M., Santa Cruz, S., Chapman, S., Barker, I., Baulcombe, D., 1992. Molecular analysis of a resistance-breaking strain of potato virus X. Virology 189, 609–617.

Kiihl, R.A.S., Hartwig, E.E., 1979. Inheritance of reaction to soybean mosaic virus in soybeans. Crop Sci. 19, 372–375.

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.

Kiraly, L., Cole, A.B., Bourque, J.E., Schoelz, J.E., 1999. Systemic cell death

is elicited by the interaction of a single gene in *Nicotiana clevelandii* and gene VI of Cauliflower mosaic virus. Mol. Plant-Microbe Interact. 12, 919–925.

Krause-Sakate, R., Redondo, E., Richard-Forget, F., Jadao, A.S., Houvenaghel, M.-C., German-Retana, S., Pavan, M.A., Candresse, T., Zerbini, F.M., Gall, O.L., 2005. Molecular mapping of the viral determinants of systemic wilting induced by a *Lettuce mosaic virus* (LMV) isolate in some lettuce cultivars. Virus Res. 109, 175–180.

Kyle, M.M., Provvidenti, R., 1993. Inheritance of resistance to potyviruses in *Phaeolus vulgaris* L.: II. Linkage relations and utility of a dominant gene for lethal systemic necrosis to soybean mosaic virus. Theor. Appl. Genet. 86, 189–196.

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

Malcuit, I., Marano, M.R., Kavanagh, T.A., De Jong, W., Forsyth, A., Baulcombe, D.C., 1999. The 25-kDa movement protein of PVX elicits *Nb*-mediated hypersensitive cell death in potato. Mol. Plant-Microbe Interact. 12, 536–543.

Meshi, T., Motoyoshi, F., Adachi, A., Watanabe, Y., Takamatsu, N., Okada, Y., 1988. Two concomitant base substitutions in the putative replicase genes of tobacco mosaic virus confer the ability to overcome the effects of a tomato resistance gene, *Tm*-1. EMBO J. 7, 1575–1581.

Meshi, T., Motoyoshi, F., Maeda, T., Yoshiwoka, S., Watanabe, H., Okada, Y., 1989. Mutations in the tobacco mosaic virus 30-kD protein gene overcome *Tm-2* resistance in tomato. Plant Cell 1, 515–522.

Mestre, P., Brigneti, G., Baulcombe, D.C., 2000. An *Ry*-mediated resistance response in potato requires the intact active site of the NIa proteinase from potato virus Y. Plant J. 23, 653–661.

Mestre, P., Brigneti, G., Durrant, M.C., Baulcombe, D.C., 2003. Potato virus Y NIa protease activity is not sufficient for elicitation of *Ry*-mediated disease resistance in potato. Plant J. 36, 755–761.

Nimchuk, Z., Eulgem, T., Holt III, B.F., Dangl, J.L., 2003. Recognition and response in the plant immune system. Annu. Rev. Genet. 37, 579–609.

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.

Padgett, H.S., Watanabe, Y., Beachy, R.N., 1997. Identification of the TMV replicase sequence that activates the *N* gene-mediated hypersensitive response. Mol. Plant-Microbe Interact. 10, 709–715.

Patel, P.N., 1982. Genetics of cowpea reactions to two strains of cowpea mosaic virus from Tanzania. Phytopathology 72, 460–466.

Querci, M., Baulcombe, D.C., Goldbach, R.W., Salazar, L.F., 1995. Analysis of the resistance-breaking determinants of potato virus X (PVX) strain HB on different potato genotypes expressing extreme resistance to PVX. Phytopathology 85, 1003–1010.

Revers, F., Le Gall, O., Candresse, T., Maule, A.J., 1999. New advances in understanding the molecular biology of plant/potyvirus interactions. Mol. Plant-Microbe Interact. 12, 367–376.

Rodriguez-Cerezo, E., Shaw, J.G., 1991. Two newly detected nonstructural viral proteins in potyvirus-infected cells. Virology 185, 572–579.

Sambrook, J., Russell, D.W., 2001. Molecular Cloning: A Laboratory Manual (3rd ed.). Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

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.

Shaner, G., Stromberg, E.L., Lacy, G.H., Barker, K.R., Pirone, T.P., 1992. Nomenclature and concepts of pathogenicity and virulence. Annu. Rev. Phytopathol. 30, 47–66.

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

Tobias, C.M., Oldroyd, G.E.D., Chang, J.H., Staskawicz, B.J., 1999. Plants expressing the *Pto* disease resistance gene confer resistance to recombinant PVX containing the avirulence gene *AvrPto*. Plant J. 17, 41–50.

Tsuda, S., Kirita, M., Watanabe, Y., 1998. Characterization of a pepper mild mottle tobamovirus strain capable of overcoming the *L3* gene-mediated resistance, distinct from the resistance-breaking Italian isolate. Mol. Plant-Microbe Interact. 11, 327–331.

Valkonen, J.P.T., Rokka, V.-M., Watanabe, K.N., 1998. Examination of the leaf-drop symptom of virus-infected potato using anther culture-derived haploids. Phytopathology 88, 1073–1077.

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

Vidal, S., Cabrera, H., Andersson, R.A., Fredriksson, A., Valkonen, J.P.T., 2002. Potato gene *Y*-1 is an *N* gene homolog that confers cell death upon infection with potato virus Y. Mol. Plant-Microbe Interact. 15, 717–727.

Weber, H., Pfitzner, A.J.P., 1998. *Tm*-2^2 resistance in tomato requires recognition of the carboxy terminus of the movement protein of tomato mosaic virus. Mol. Plant-Microbe Interact. 11, 498–503.

Weber, H., Schultze, S., Pfitzner, A.J.P., 1993. Two amino acid substitutions in the tomato mosaic virus 30-kilodalton movement protein confer the ability to overcome the *Tm*-2^2 resistance gene in the tomato. J. Virol. 67, 6432–6438.

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.