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

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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
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; Goudlen et al., 1993; Karasawa et al., 1999; Kim and Palukaitis, 1997; M.R. Hajimorad et al. / Virology 345 (2006) 156–166

The genetic differences between P3 of an infectious full-length cDNA clone of SMV-N (pSMV-N) and those of SMV-G7 and SMV-G7d (pSMV-G7 and 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 Rsvl-mediated LSHR (Hajimorad et al., 2005).

The elicitor function of SMV-N provoking Rsvl-mediated ER is not altered by substitutions of the amino acids involved in induction of Rsvl-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 Rsvl-mediated LSHR (Hajimorad et al., 2005). To find out if similar substitutions have any impact on the elicitor function of pSMV-N provoking Rsvl-mediated ER, the corresponding amino acids of pSMV-N were substituted by site-directed mutagenesis with those of pSMV-G7d, and consequently pSMV-NV822M, pSMV-NK952E, and pSMV-NA1111V were synthesized. Inoculation of the mutants onto Williams 82 (rsvl) showed that all three gave rise to systemic infection and were thus replication competent. However, when progenies were sap inoculated onto PI 96983 (Rsvl) or L78-379 (Rsvl), based on absence of symptoms and lack of detection of the viruses by DAS-ELISA, all retained the elicitor function provoking Rsvl-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 Rsvl-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 Rsvl-mediated ER, pSMV-NV822M+K952E was synthesized. The mutant remained infectious on Williams 82 (rsvl), but failed to infect PI 96983 (Rsvl) or L78-379 (Rsvl) (data not shown). An additional pSMV-N derived mutant, pSMV-NV822M+K952E+A1111V containing all three substitutions was also synthesized. Similar to all the other pSMV-N derived mutants, it retained infectivity on Williams 82 (rsvl); however, the mutant did not gain virulence on PI 96983 (Rsvl) and L78-379 (Rsvl) (data not shown).

The stability of all the mutations was confirmed by RT-PCR amplification of P3 sequences recovered from Williams 82 (rsvl) infected plants followed by direct sequencing. Only progenies derived from pSMV-NK952E, pSMV-NV822M+K952E and pSMV-NV822M+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 Rsvl-genotype soybeans.

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).
Thus, the data suggest that the amino acid involved in elicitation of \textit{Rsv1}-mediated SHR or LSHR, in the context of pSMV-G7, do not influence the elicitor function of pSMV-N provoking \textit{Rsv1}-dependent ER.

\textbf{P3 of SMV-G7 or SMV-G7d does not confer virulence to SMV-N on \textit{Rsv1}-genotype soybean}

To examine the possibility that the tertiary structure of strain-specific P3 in its entirety is critical for elicitation of \textit{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 \textit{KpnI}/\textit{SpeI} 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 (\textit{rsv1}) (Fig. 2); however, following sap inoculation of viral progenies onto PI 96983 (\textit{Rsv1}) or L78-379 (\textit{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 \textit{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 (\textit{rsv1}) plants, directly sequenced, and was found identical to the parental sequences.

\textbf{P3 of SMV-N provokes \textit{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 \textit{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 \textit{KpnI}/\textit{SpeI} 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.

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure2}
\caption{Schematic representation of parental viruses, their derivative chimeras and their potential to infect \textit{rsv1} (Williams 82) and \textit{Rsv1} (PI 96983 or L78-379) soybean genotypes. (A) The genomic map of \textit{Soybean mosaic virus} (SMV) and the positions of the single restriction sites \textit{KpnI} (Kp), \textit{SpeI} (Sp), and \textit{SalI} (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 (\textit{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.}
\end{figure}
the chimeras remained replication competent in Williams 82 (rsv1) (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/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 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 96283 (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 genes, respectively (Bendahmane et al., 1999; Weber et al., 1993; Weber and Pfützner, 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; Divinek et al., 2004; Karasawa et al., 1999; Kim and Palukaitis, 1997; Malecuit 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-22 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 (Nla) 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 Nla provokes Ry-mediated ER in planta. The elicitor function of strain-specific nuclear inclusion b-protein (Nb) 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 cistrons 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 LSRH 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 (Benzal-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 Nla 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 isolate 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
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 KpnI and SpeI, 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 SpeI and SalI, 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 KpnI and SpeI and ligated into pSMV-N. For the synthesis of pSMV-N_V822M_K952E_A1111V a SpeI and SalI 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).

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 KpnI and SalI, 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 KpnI and SalI 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 SpeI, 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 megaprimer, 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 KpnI and SpeI and ligated into pSMV-G7 and pSMV-G7d to generate pSMV-G7/N(K247–3233) and pSMV-G7d/N(K247–3233), 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 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 KpnI and SpeI and ligated into pSMV-G7 and pSMV-G7d to generate pSMV-G7/N(K247–3233) and pSMV-G7d/N(K247–3233), 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 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 KpnI and SpeI and ligated into pSMV-G7 and pSMV-G7d to generate pSMV-G7/N(K247–3233) and pSMV-G7d/N(K247–3233), respectively (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 megaprimer. The pSMV-G7 and pSMV-G7d derived megaprimer were amplified in the presence of primers G3266a and

Table 1

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

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequencesa (5′–3′)</th>
<th>Positionb</th>
</tr>
</thead>
<tbody>
<tr>
<td>N2430a</td>
<td>CACCAACCAACCTCTGTAAGATT</td>
<td>2430–2409</td>
</tr>
<tr>
<td>N2607a</td>
<td>TCCCAACATCCATCCCTTCTCAA</td>
<td>2607–2583</td>
</tr>
<tr>
<td>N3003a</td>
<td>CTTTCTTTGTCACAAACATCTGCTTGAG</td>
<td>3003–2971</td>
</tr>
<tr>
<td>N3157s</td>
<td>CAACACTCCACAGGTGCTACAGTGATATAG</td>
<td>3157–3186</td>
</tr>
<tr>
<td>N3264a</td>
<td>CTGTTGTTGACATGCTTCG</td>
<td>3264–3245</td>
</tr>
<tr>
<td>N3480a</td>
<td>TTGCTGTGTTGGCCTGTGACGACAGACATCTGCTG</td>
<td>3480–3448</td>
</tr>
<tr>
<td>N3623a</td>
<td>CGTCACATTTACACTCTACCCGTTGTCG</td>
<td>3623–3595</td>
</tr>
<tr>
<td>G2274s</td>
<td>ATTTGGTTGACATGCTGGT</td>
<td>2274–2292</td>
</tr>
<tr>
<td>G2433a</td>
<td>CACCAACCAACCTTATAAGATT</td>
<td>2433–2412</td>
</tr>
<tr>
<td>G2919s</td>
<td>CGGCGGATAACCTGGTTG</td>
<td>2919–2936</td>
</tr>
<tr>
<td>G2366a</td>
<td>TGTATTGATAAACTTCTG</td>
<td>2366–2348</td>
</tr>
<tr>
<td>G3266a</td>
<td>CTGCACCTAATCTCCACCCGTTGTCG</td>
<td>3266–3598</td>
</tr>
<tr>
<td>G3266a</td>
<td>CCAATTTGCAATTTTGCTGGCTG</td>
<td>3843–3820</td>
</tr>
<tr>
<td>G3910a</td>
<td>AAACCTGTTGATTTCCCTGAGCC</td>
<td>3910–3888</td>
</tr>
</tbody>
</table>

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, AY216987, and D00507, respectively).
(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 protease (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 megaprimer 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 SuperScript 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, Piscataway, NJ) 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 AutoAssembler 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 supercoiled 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).

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