Strain-Specific Cylindrical Inclusion Protein of *Soybean mosaic virus* Elicits Extreme Resistance and a Lethal Systemic Hypersensitive Response in Two Resistant Soybean Cultivars

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Submitted 6 January 2009. Accepted 17 April 2009.

In the *Soybean mosaic virus* (SMV)–soybean pathosystem, three independent genes (*Rsv1*, *Rsv3*, and *Rsv4*) conferring resistance to SMV have been identified. Recently, we constructed infectious cDNA clones of SMV G7H and G5H strains and found that these two strains differ in their ability to infect soybean genotypes possessing different SMV resistance genes despite a difference of only 33 amino acids. In particular, pSMV-G7H induced mosaic symptoms systemically in L29 (*Rsv3*) and provoked a lethal systemic hypersensitive response (LSHR) in Jinpumkong-2, whereas pSMV-G5H could not infect these soybean genotypes. To identify the responsible pathogenic determinants of SMV, we exploited the differential responses of pSMV-G7H- and pSMV-G5H-derived chimeric viruses and amino acid substitution mutant viruses in several soybean genotypes and demonstrated that cylindrical inclusion (CI) protein is the elicitor of *Rsv3*-mediated extreme resistance and a pathogenic determinant provoking LSHR in Jinpumkong-2. A single amino acid substitution in CI was found to be responsible for gain or loss of elicitor function of CI. Our finding provides a role for CI as a pathogenic determinant in the SMV–soybean pathosystem, and increases the understanding of the basis of the different disease responses of SMV strains.

In plants, resistance to infection by viruses is established through diverse mechanisms (Soosaar et al. 2005). One well-characterized mechanism for recognizing invading pathogens, including viruses, is mediated by resistance (*R*) genes (Kang et al. 2005). *R*-gene-mediated resistance is triggered by either direct or indirect interactions between products of *R* genes and the corresponding invading pathogen avirulence (*Avr*) genes (Bonas and Lahaye 2002; Flor 1971). Any protein component of a virus can function as the specific elicitor inducing the *R*-gene-mediated defense response (Culver 1997). However, be-

cause of the error-prone replication of plant RNA viruses resulting in amino acid substitutions that do not significantly compromise natural activity of the protein, variant viruses capable of evading *R*-gene-mediated resistance emerge continuously (Hammond-Kosack and Jones 1997).

The typical resistance responses of *R*-gene-mediated recognition of plant viruses include extreme resistance (ER) and hypersensitive response (HR) (Hull 2002), both of which are considered to be a consequence of the defense response (Bendahmane et al. 1999). ER is characterized by the arrest of avirulent viruses at the inoculation site without any visible symptoms or virus accumulation (Bendahmane et al. 1999; Hajimorad and Hill 2001). In contrast, HR results in localization of the virus to the inoculation site by rapid death of infected and neighboring cells. HR has been analyzed extensively at the molecular level through: induction of a variety of defense-related genes, including pathogenesis-related proteins; increased levels of defense signaling molecules, such as salicylic acid and jasmonic acid; and a wide range of physiological changes (Enyedi et al. 1992; Hammond-Kosack and Jones 1996; Soosaar et al. 2005). Occasionally, perturbation of *R*–*Avr* recognition can result in escape and spread of the virus to distant tissues, provoking a systemic HR (SHR). In some instances, rapid progression of SHR results in lethal SHR (LSHR) (Dinesh-Kumar et al. 2000; Hajimorad et al. 2003, 2005; Kim and Palukaitis 1997). Although it is still unknown what mechanism induces SHR other than localized HR, it has been suggested that SHR is a consequence of delayed occurrence of HR-associated events (Dinesh-Kumar et al. 2000).

Soybean mosaic virus (SMV), one of the most common viruses in soybean, is a member of the genus *Potyvirus* (Mayo and Pringle 1998). As in all *Potyvirus* spp., the SMV genome encodes one large polyprotein, which is cleaved to yield at least 10 mature proteins by three virus-encoded proteases: P1, HC-Pro, and NIa (Riechmann et al. 1992). Since the first classification of SMV into seven strains (G1 to G7) based on disease reactions on resistant soybean cultivars (Cho and Goodman 1979), emergence of variant SMV strains has been repeatedly reported (Cho et al. 1983; Choi et al. 2005; Hajimorad et al. 2003; Kim and Lee 1991; Kim et al. 2003; Lim 1985). Concomitantly, three distinct *R* genes, *Rsv1*, *Rsv3*, and *Rsv4*, conferring resistance to SMV have been reported (Buss et al. 1997; Chen et al. 1991; Hayes et al. 2000; Jeong et al. 2002; Kiihl and Hartwig 1979). *Rsv1*, which was first identified in soybean plant introduction (PI)96983, confers resistance to

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Accession numbers for nucleotide sequences used for sequence alignments are as follows: pSMV-G5H (FJ376388), pSMV-G7H (FJ807700), and SMV G7H (AY294045).

*The e-Xtra logo stands for “electronic extra” and indicates that Figure 5 appears in color online and a supplementary table is published online.

SMV strains G1 to G6 but not G7 (Chen et al. 1991; Cho and Goodman 1979; Lim 1985). Additional alleles at the *RsvI* locus were also found in cvs. York, Marshall, Kwanggyo, and Ogden (Chen et al. 1991). *Rsv3* confers susceptibility to G1 to G4 but resistance to G5 to G7 (Gunduz et al. 2002; Jeong et al. 2002), and *Rsv4* confers resistance to all known SMV strains (Gunduz et al. 2004; Hayes et al. 2000). Although elicitor functions for SMV viral proteins in the *Rsv*-mediated resistance response are poorly understood, strain-specific P3 protein was recently identified as the elicitor of *RsvI*-mediated ER and LSHR based on differential interactions of SMV G7, G7d, and N strains with *RsvI*-genotype soybean (Hajimorad et al. 2005, 2006).

In Korea, SMV G5H became the most prevalent strain (approximately 65%) in soybean fields in the late 1980s (Kim 2000). However, while the relative incidence of G5H decreased gradually with the cultivation of resistant soybean cultivars, a new SMV strain, G7H, causing systemic mosaic symptoms or LSHR in certain soybean cultivars that are resistant to G5H, emerged in the late 1990s (Kim 2000; Kim et al. 2003). Recently, we constructed infectious clones of SMV G7H (pSMV-G7H; FJ80770) and G5H (pSMV-G5H; FJ376388) strains, and their molecular characteristics and disease reactions on resistant soybean cultivars were reported (Seo et al. 2009a and b). Interestingly, we found that G7H shares 98.9% amino acid sequence homology with G5H (Seo et al. 2009b) and that the genome sequence of pSMV-G7H differs from that of pSMV-G5H by 33 amino acid substitutions (Table 1). Thus, to identify the pathogenic determinants causing different disease reactions in soybean cultivars infected with either SMV G7H or G5H, we exploited the differential responses of chimeric viruses between G7H and G5H strains and amino-acid-substituted mutant

viruses in several *Rsv*-genotype soybean lines. In this study, we demonstrated that the cylindrical inclusion (CI) gene is the elicitor of *Rsv3*-mediated ER as well as a pathogenic determinant that causes emergence of the resistance-breaking SMV-G7H strain, and the causal amino acid residue of CI was identified.

RESULTS

Characterization of disease reactions of SMV-G7H and SMV-G5H.

pSMV-G7H induced mosaic symptoms systemically in Lee 68 (*rsv*) and L29 (*Rsv3*) and provoked LSHR in Jinpungkong-2 (*Rsv* genotype has not yet been characterized) but could not infect PI96983 (*RsvI*) and V94-5152 (*Rsv4*) cultivars (Figs. 1 and 2) (Kim 2000). In contrast, pSMV-G5H induced mosaic symptoms, which were indistinguishable from those induced by pSMV-G7H, in Lee 68 but could not systemically infect PI96983, V94-5152, L29, or Jinpungkong-2 (Figs. 1 and 2) (Kim 2000). It was shown that *RsvI* conferred ER against avirulent strain SMV-N and mediated LSHR against SMV-G7 (Hajimorad and Hill 2001); therefore, we conducted experiments to characterize disease responses induced by either SMV-G7H or G5H on *Rsv*-genotype soybean lines. Infectious saps of SMV-G7H and G5H were mechanically inoculated on primary leaves of Lee 68 (*rsv*), PI96983 (*RsvI*), L29 (*Rsv3*), V94-5152 (*Rsv4*), and Jinpungkong-2. Two weeks after inoculation, total RNAs were extracted from either inoculated leaves or upper uninoculated leaves and subjected to reverse-transcription polymerase chain reaction (RT-PCR) to detect virus accumulation and upregulation of pathogenesis-related gene (*PR-1*) transcripts as a common molecular marker of HR (Hajimorad and Hill 2001; Hajimorad et al. 2005). When RT-PCR was performed to detect SMV, virus accumulations were detected in both G7H-inoculated and upper uninoculated leaves of Lee 68, L29, and Jinpungkong-2, while no PCR amplified signal was observed in either G7H-inoculated or upper uninoculated leaves of PI96983 and V94-5152 (Fig. 3). In the case of G5H inoculation, virus RNA accumulations were only detected in inoculated leaves of Lee 68 and Jinpungkong-2 and upper uninoculated leaves of Lee 68 (Fig. 3). Concomitantly, we assessed by RT-PCR to determine whether *PR-1* transcripts were upregulated in inoculated plants. Upregulation of *PR-1* transcripts was detected only in G7H-inoculated and upper uninoculated leaves of Jinpungkong-2 and G5H-inoculated leaves of Jinpungkong-2 (Fig. 3). Furthermore, when saps of G7H-inoculated leaves of PI96983 and V94-5152 and G5H-inoculated leaves of PI96983, L29, and V94-5152 were back-inoculated to Lee 68, virus infections were not detected by RT-PCR (data not shown). Altogether, these results show that *RsvI* and *Rsv4* conferred ER against G7H and G5H, whereas *Rsv3* conferred ER against G5H but not to G7H, and verified that G7H provoked LSHR in Jinpungkong-2 and G5H induced HR on inoculated leaves of Jinpungkong-2.

Disease responses of the chimeric viruses in resistant soybean cultivars.

Because *R*-gene-mediated disease responses have generally been interpreted as gene-for-gene interactions at the protein level (Bonas and Lahaye 2002), we hypothesized that the amino acid substitutions in one or more mature viral proteins resulted in pathogenic differences between SMV G7H and G5H strains in L29 and Jinpungkong-2. Thus, to identify the viral determinants mediating ER in L29 and LSHR in Jinpungkong-2, we constructed several chimeras utilizing common available restriction enzyme sites between pSMV-G7H and pSMV-G5H for exchanging reciprocal regions (Fig. 1). These chimeras were inoculated onto soybean seedlings by rub inocu-

Table 1. Amino acid differences between pSMV-G7H and pSMV-G5H^a

Region	Amino acid position	pSMV-G7H	pSMV-G5H
P1	128	Glu	Asp
	143	Phe	Ser
	154	Tyr	His
	167	Ala	Thr
	173	Asn	Lys
	187	Lys	Arg
	236	Ser	Asn
HC-pro	301	Glu	Lys
	374	His	Gln
	721	Cys	Gly
	763	Tyr	Cys
CI	1,365	Ile	Leu
	1,382	His	Gln
	1,532	Ile	Val
	1,539	Val	Ile
	1,612	Ser	Pro
	1,636	Val	Ile
	1,646	Ile	Val
	1,674	Thr	Ile
	1,687	Val	Ile
	1,733	Ala	Ser
	1,754	Lys	Arg
	1,763	Val	Ile
VPg	1,871	Ile	Val
N1b	2,552	Val	Ile
	2,644	Asn	Ser
	2,645	Asp	Glu
	2,683	Lys	Gln
	2,690	Ile	Val
	2,722	Ser	Ala
	2,771	Ile	Thr
	2,777	Ala	Thr
CP	2,814	Gly	Asp

^a GenBank accession numbers pSMV-G7H (FJ807700) and pSMV-G5H (FJ376388).

Fig. 1. Schematic of chimeric viruses of *Soybean mosaic virus* (SMV) strains G7H and G5H and their disease responses on different soybean genotypes. Chimeric viruses were constructed by exchanging corresponding fragments between pSMV-G7H and pSMV-G5H. The positions of the restriction enzyme cleavage sites used to make the chimeric constructs are shown. pSMV-G7H- and pSMVG5H-derived regions are indicated by blank and hatched boxes, respectively. The parental and chimeric viruses were inoculated onto different soybean genotypes by rub inoculation with plasmid DNAs. Symptom appearances were monitored visually for 3 weeks postinoculation (–, symptomless; M, mosaic; LL, local lesion; LSHR, lethal systemic hypersensitive response). Format for symptom symbols: (reactions on inoculated leaves)/(reactions on upper uninoculated leaves). SMV infection was verified by reverse-transcription polymerase chain reaction using total RNA prepared from upper uninoculated leaves.

Fig. 2. Disease responses of L29 (*Rsv3*) and Jinpumkong-2 to inoculation with *Soybean mosaic virus* (SMV) strains pSMV-G7H, pSMV-G5H, or their derivative chimeras. Soybean seedlings were rub inoculated on their cotyledons with 10 µg of infectious cDNA plasmids. Representative photographs were taken approximately 2 weeks postinoculation.

lation with plasmid DNAs as described previously (Seo et al. 2009a). Symptom development was monitored for 3 weeks postinoculation and SMV infection was confirmed by RT-PCR using total RNA extracted from upper noninoculated leaves.

Fig. 3. Detection of *Soybean mosaic virus* (SMV) accumulation and upregulation of pathogenesis-related gene (*PR-1*) transcripts on inoculated and upper uninoculated leaves. Soybean cultivars were mechanically inoculated with inoculation buffer (mock) or infectious sap of SMV-G7H or -G5H. Total RNAs, isolated from either inoculated or upper uninoculated leaves of each of the soybean cultivars (indicated at the top of gel image), were analyzed 14 days postinoculation by reverse-transcription polymerase chain reaction (PCR) to detect virus accumulation and upregulation of *PR-1* transcripts. The *ubiquitin* gene (*Ubi*) was used as an internal control. The resulting PCR products were analyzed by 2% agarose gel electrophoresis. Lane M contains the PCR marker and arrows indicate the expected PCR products.

As with their parental viruses, all chimeric viruses were replication competent and induced mild mosaic symptoms in Lee 68 (*rsv*) (Fig. 1), and the identities of progenies derived from chimeric viruses in infected Lee 68 (*rsv*) were confirmed by direct sequencing of RT-PCR products of two different regions, one representing the recipient strain and the other representing the donor strain (data not shown). We also tested virulence and symptomatology of these chimeric viruses in PI96983 (*Rsv1*), L29 (*Rsv3*), V94-5152 (*Rsv4*), and Jinpungkong-2 cultivars. None of the chimeric viruses infected PI96983 and V94-5152, similar to results with parental viruses (Fig. 1). However, interestingly, pSMV-G7H/G5H(3237-6230) and pSMV-G7H/G5H(4627-5962), which are pSMV-G7H-derived chimeras containing central fragments from pSMV-G5H, lost the ability to infect L29 and Jinpungkong-2, while other pSMV-G7H-derived chimeras infected these cultivars and induced mosaic symptoms and LSHR, respectively (Figs. 1 and 2). Conversely, pSMV-G5H/G7H(4627-5962), which is a pSMV-G5H-derived chimera swapped with the central *AgeI-EcoRV* fragment from pSMV-G7H, gained virulence on L29 and Jinpungkong-2 and induced symptoms similar to those induced by pSMV-G7H (Figs. 1 and 2). Together, these results suggest that the reciprocal replacement of the region containing the central *AgeI-EcoRV* fragment (from nucleotides 4,627 to 5,962) between pSMV-G7H and pSMV-G5H was sufficient for gain or loss of pathogenicity of the chimeric virus on L29 and Jinpungkong-2.

The pathogenic determinant of SMV-G7H maps to CI.

The central *AgeI-EcoRV* region of the SMV genome encodes amino acids 1,499 to 1,943 of the polyprotein precursor, sequentially consisting of the C-terminal half of CI, 6K2, and the N-terminal half of VPg (Fig. 4). To determine genetic differences in this central region between pSMV-G7H and pSMV-G5H, we compared amino acid sequences of corresponding regions of pSMV-G7H and pSMV-G5H. Sequence alignment showed that there are a total of 11 amino acid substitutions, 10 amino acid substitutions in the C-terminal half of CI and an amino acid substitution in the N-terminal half of VPg (Fig. 4).

Fig. 4. Alignment of deduced amino acid sequences of the C-terminal half of CI, 6K2, and the N-terminal half of VPg from *Soybean mosaic virus* (SMV) strains pSMV-G7H and pSMV-G5H. Dark highlighted amino acids are identical. Numbers are amino acid positions on the polyprotein precursor. GenBank accession numbers pSMV-G7H (FJ807700) and pSMV-G5H (FJ376388).

The amino acid sequence of 6K2 of pSMV-G7H is identical to that of pSMV-G5H; thus, we excluded 6K2 as a candidate gene for the pathogenic determinant of pSMV-G7H. The N-terminal half of VPg differs between pSMV-G7H and pSMV-G5H by a single amino acid at position 1,871 (Fig. 4). Thus, to determine whether VPg is a pathogenic determinant of SMV-G7H, the amino acid residue (Ile) at position 1,871 of pSMV-G7H was replaced with the corresponding amino acid residue (Val) from pSMV-G5H by site-directed mutagenesis (Fig. 5). This mutant clone, pSMV-G7H_{I1871V}, was inoculated into Lee 68 (*rsv*), L29 (*Rsv3*), and Jinpungkong-2 seedlings. We observed disease responses similar to those induced by pSMV-G7H (Fig. 5). This reveals that amino acid substitution from Ile to Val at position 1,871 does not affect the pathogenicity of pSMV-G7H. Actually, this was expected, because the amino acid of the viral population of SMV-G7H (GenBank accession number AY294045) at this position is Val, and pSMV-G7H induces disease reactions typical of SMV-G7H in various soybean cultivars, including Lee 68 (*rsv*), L29 (*Rsv3*), and Jinpungkong-2 (Seo et al. 2009a). These results suggest that VPg is not a pathogenic determinant of pSMV-G7H and that the pathogenic determinant of SMV-G7H might map to CI.

A single amino acid substitution in CI alters pathogenicity of SMV G7H and G5H strains in resistant soybean cultivars.

The C-terminal half of CI of pSMV-G7H differs from that of pSMV-G5H by 10 amino acids (Fig. 4). To identify which of these 10 amino acid substitutions in the C-terminal half of CI affects pathogenicity of SMV-G7H in L29 (*Rsv3*) and Jinpungkong-2 cultivars, site-directed mutagenesis was performed to

introduce single or multiple amino acid changes into pSMV-G7H and pSMV-G5H. First, we divided the 10 different amino acids into two groups based on their biochemical properties and substitution frequency to reduce the number of candidate amino acids affecting pathogenicity of pSMV-G7H. The amino acid differences at positions 1,612, 1,674, 1,733, and 1,754 were placed into group A and the other six amino acid substitutions between Ile and Val, which have similar biochemical properties (Chou 1989; Hopp and Woods 1981), were placed in group B (Fig. 4). Then, we replaced amino acids belonging to group A with those of pSMV-G5H and the resulting mutant construct was named pSMV-G7H_{S1612P+T1674I+A1733S+K1754R} (Fig. 5). Construction of the mutant carrying amino acids of pSMV-G5H at positions of group B was done by introducing amino acid residues of pSMV-G7H at positions of group A into a pSMV-G7H/G5H(4627-5962) background. The resulting mutant construct was named pSMV-G7H/G5H(4627-5963)_{P1612S+I1674T+S1733A+R1754K} (Fig. 5).

To assess the effects of these amino acid changes on pathogenicity of pSMV-G7H, pSMV-G7H_{S1612P+T1674I+A1733S+K1754R} and pSMV-G7H/G5H(4627-5963)_{P1612S+I1674T+S1733A+R1754K} were inoculated into Lee 68 (*rsv*), L29 (*Rsv3*), and Jinpungkong-2 seedlings. Both mutant constructs were replication competent and induced mild mosaic symptoms in Lee 68 (*rsv*) (Fig. 5). However, G7H_{S1612P+T1674I+A1733S+K1754R} failed to infect L29 (*Rsv3*) and Jinpungkong-2, whereas pSMV-G7H/G5H(4627-5963)_{P1612S+I1674T+S1733A+R1754K} induced symptoms similar to those induced by pSMV-G7H in these cultivars (Figs. 5 and 6). These results indicate that the key amino acid residues important for pathogenicity of pSMV-G7H exist among four amino acids at positions 1,612, 1,674, 1,733, and 1,754.

Fig. 5. Schematic of Soybean mosaic virus (SMV) strains pSMV-G7H- or pSMV-G5H-derived amino acid substitution mutants and their disease responses on different soybean genotypes. Amino acid substitution mutants were constructed by polymerase chain reaction (PCR)-based, site-directed mutagenesis. The positions of substituted amino acids on the polyprotein precursor are indicated at the top of the amino acid sequences. The crucial amino acid position (1,754) for the virulence of viruses on L29 (*Rsv3*) and Jinpungkong-2 is shown by a dotted line. The parental and mutant viruses were inoculated onto different soybean genotypes by rub inoculation with plasmid DNAs. Symptom appearances were monitored visually for 3 weeks postinoculation (-, symptomless; M, mosaic; LL, local lesion; LSHR, lethal systemic hypersensitive response). Format for symptom symbols: (reactions on inoculated leaves)/(reactions on upper uninoculated leaves). SMV infection was verified by reverse-transcription PCR using total RNA prepared from upper uninoculated leaves.

Therefore, we constructed another four pSMV-G7H-derived mutant constructs by replacing each amino acid with that of pSMV-G5H (Fig. 5). These single substitutions did not affect virulence of pSMV-G7H on Lee 68 (*rsv*) (Fig. 5). However, pSMV-G7H_{K1754R} lost the ability to infect L29 (*Rsv3*) and Jinpumpkong-2, whereas pSMV-G7H_{S1612P}, pSMV-G7H_{T1674I}, and pSMV-G7H_{A1733S} remained virulent and induced symptoms similar to those induced by pSMV-G7H in these cultivars (Figs. 5 and 6). Furthermore, pSMV-G7H_{S1612P+T1674I+A1733S}, which contains three amino acid substitutions at positions 1,612, 1,674, and 1,733, preserved similar pathogenicity to pSMV-G7H (Figs. 5 and 6). These results demonstrate that a single amino acid substitution in CI is sufficient for loss of virulence of pSMV-G7H in L29 (*Rsv3*) and Jinpumpkong-2.

In addition, to determine whether the amino acid substitution from Arg to Lys at position 1,754 affects the pathogenicity of pSMV-G5H, we introduced the Lys residue into pSMV-G7H/G5H(4627-5962) and pSMV-G5H at position 1,754. The resultants were named pSMV-G7H/G5H(4627-5962)_{R1754K} and pSMV-G5H_{R1754K}, respectively (Fig. 5). pSMV-G7H/G5H(4627-5962) and pSMV-G5H cannot infect L29 (*Rsv3*) and Jinpumpkong-2 (Figs. 1 and 2). However, pSMV-G7H/G5H(4627-5962)_{R1754K} and pSMV-G5H_{R1754K} gained virulence and induced mosaic symptoms in L29 (*Rsv3*) and LSRH in Jinpumpkong-2 (Figs. 5 and 6). Therefore, the amino acid substitution from Arg to Lys at position 1,754 is sufficient for pSMV-G5H to gain virulence and for CI to lose its elicitor function.

To make sure that progeny viruses from the substitution mutants maintained the introduced mutations in their genomes, the sequences of the CI cistron of progeny viruses recovered by RT-PCR from Lee 68 and L29 were analyzed. The results showed that all introduced mutations were maintained in progenies and no new mutations occurred in the CI cistron during replication (data not shown). In particular, in the case of pSMV-G7H_{K1754R} and pSMV-G5H_{R1754K}, the full-length genomic sequences of progeny viruses were determined and no mutation-changing amino acid sequences except the introduced mutations were detected (data not shown).

DISCUSSION

The virulence and symptomatology of a particular virus strain are expressed differently depending on the specific host genotype. This genotype-specific disease response is often the result of complex interactions between host genes and viral genes, including *R-Avr* interactions. The SMV-soybean pathosystem is an important model for studying molecular interactions between virus and plant in genotype-specific disease responses, because SMV strains are well characterized based on disease reactions of several soybean cultivars (Chen et al. 1991; Cho and Goodman 1979; Kim et al. 2003). Although strain SMV-G5H could not infect L29 (*Rsv3*) and Jinpumpkong-2 cultivars, a newly emerging strain, SMV-G7H, infected these cultivars and induced mosaic symptoms and LSHR, respectively (Figs. 1 and 2) (Kim 2000). However, the molecular basis of this resistance breaking and corresponding elicitor identity is unknown. In this study, we have shown that SMV CI protein is the elicitor provoking *Rsv3*-mediated ER in L29 and the pathogenic determinant inducing LSHR in Jinpumpkong-2. We have also demonstrated that a single amino acid substitution in CI at position 1,754 of the polyprotein is sufficient for loss or gain of virulence of SMV on L29 (*Rsv3*) and Jinpumpkong-2 cultivars, which are resistant to SMV-G5H but susceptible to SMV-G7H. In the SMV-soybean pathosystem, the P3 protein of SMV-G7 has recently been identified as the elicitor provoking LSHR in the *Rsv1*-genotype soybean (Hajimorad et al. 2005). It has also been demonstrated that strain-specific P3 and HC-Pro proteins of SMV act independently as avirulence determinants eliciting *Rsv1*-mediated ER, and concurrent mutations in both cistrons are required to confer virulence to an avirulent SMV strain on *Rsv1*-genotype soybean (Eggenberger et al. 2008; Hajimorad et al. 2006). Taken together, these findings reveal that SMV encodes more than three avirulence determinants.

In the Turnip mosaic virus (TuMV)-*Brassica* spp. pathosystem, it was also shown that P3 and CI proteins of TuMV are the avirulence determinants for either *TuRB03* and *TuRB04* or

Fig. 6. Disease responses of L29 (*Rsv3*) and Jinpumpkong-2 to inoculation with *Soybean mosaic virus* (SMV) strains pSMV-G7H, pSMV-G5H, or amino acid substitution mutants. Soybean seedlings were rub inoculated on their cotyledons with 10 μ g of infectious cDNA plasmids. Representative photographs were taken approximately 2 weeks postinoculation.

TuRB01 and *TuRB05*, respectively (Jenner et al. 2000, 2002, 2003). In particular, TuMV P3 protein plays a dual role as an avirulence and pathogenic determinant in infecting brassicas (Jenner et al. 2003). We also showed that strain-specific CI of SMV acts as the avirulence determinant in L29 (*Rsv3*) and is the pathogenic determinant provoking LSHR in Jinpumpkong-2. It is generally assumed that most, if not all, viral proteins play multiple roles in the viral life cycle and some of them interact with more than one host protein. In this regard, dual roles of viral proteins as avirulence and pathogenic determinants are not surprising, although little is known about the precise roles of viral proteins and their underlying molecular basis in pathogenicity.

The CI protein of members of *Potyvirus* has NTPase, RNA helicase, and RNA binding activities, which appear to participate in RNA replication (Urcuqui-Inchima et al. 2001). Furthermore, ultrastructural studies have shown that the viral RNA is associated with CI, and this RNA-protein complex is located at the entrance of the plasmodesmata (Roberts et al. 1998; Rodriguez-Cerezo et al. 1997), indicating that CI protein is involved in cell-to-cell movement of *Potyvirus* spp. We introduced amino acid substitutions into the CI cistron at positions 1,612, 1,674, 1,733, and 1,754 of the polyprotein precursor, singly or in combination (Fig. 5). These mutant viruses were replication competent and induced mosaic symptoms on upper leaves of Lee 68 (*rsv*) of a severity similar to those induced by their parental viruses (Fig. 5 and data not shown), indicating that the introduced mutations did not significantly affect CI activities for viral replication and movement. Furthermore, conferring avirulence to SMV-G7H was achieved by the amino acid substitution from Lys to Arg at position 1,754 (Fig. 5). Both Lys and Arg have similar characteristics in hydrophobicity and conformational preference (Chou 1989; Hopp and Woods 1981). In addition, several studies have shown that substitutions of either Lys for Arg or Arg for Lys within conserved protein domains abolished their protein-protein interactions (Aukerman et al. 1991; Baldi et al. 1996; Craig et al. 1997; Defeo-Jones et al. 1989). Thus, it seems more likely that the mutations at position 1,754, K1754R and R1754K, affect direct or indirect interactions between CI and either Rsv or its downstream proteins rather than the tertiary structure of CI. Indeed, in many cases, viral elicitor functions are lost by single or multiple mutations at amino acid residues that do not significantly affect the protein activities (Hammond-Kosack and Jones 1997). These results indicate that viral elicitors have crucial and specific domains directly or indirectly recognized by corresponding *R* gene products and are consistent with a simplistic receptor-ligand model for R-Avr protein recognition (Bonas and Lahaye 2002). In soybean cv. Jinpumpkong-2, SMV-G7H induced LSHR, and a single mutation, R1754K, introduced into SMV-G5H also conferred the ability to induce LSHR, whereas SMV-G5H was avirulent (Figs. 1 and 5). Jinpumpkong-2 is resistant to SMV strains G1 to G7a, except G7H (Kim 2000), whereas its *Rsv* genotype has not yet been characterized. On the other hand, soybean cv. L29 carries *Rsv3*, which is a single dominant resistance gene (Jeong et al. 2002). As shown above, *Rsv3* conferred resistance to SMV-G5H by recognizing CI protein as the elicitor; however, the mutation, R1754K, appeared to abolish this recognition and conferred virulence to SMV-G5H. Thus, it is likely that both *Rsv3* and an unknown *Rsv* protein of Jinpumpkong-2 have a similar architecture for direct or indirect recognition of the Arg residue at position 1,754 in CI. However, it also seems likely that they possess different specificity in recognition of CI. Namely, SMV-G5H CI seems likely to be recognized strongly by *Rsv3* but weakly by the unknown *Rsv* protein of Jinpumpkong-2. Hence, this weak recognition between CI as the elicitor and

the unknown *Rsv* protein of Jinpumpkong-2 may result in virus escape to distant tissues and, finally, induces LSHR in Jinpumpkong-2, because it was suggested that one type of systemic necrosis results from the "weak elicitor" function of *Avr* genes (Culver et al. 1991). In fact, several SMV resistance genes were found to be alleles at the *Rsv1* locus and these alleles confer different reactions to SMV strains (Chen et al. 1991; Cho and Goodman 1979). In addition, sequence analysis shows that SMV-G2 inducing mosaic symptoms on *Rsv3*-genotype soybean lines carries a lysine residue at position 1,754, whereas SMV G5 and G7 strains, which cannot infect *Rsv3*-genotype soybean, carry an Arg residue at position 1,754 (Gunduz et al. 2002), supporting the suggestion that the Arg residue at position 1,754 is important for recognizing CI by *Rsv3*.

Analyses of the disease responses of chimeric viruses and amino-acid-substituted mutant viruses derived from SMV G7H and G5H clearly show that SMV CI is the pathogenic determinant provoking either ER or LSHR in resistant soybean cultivars. However, we cannot exclude the possibility that CI protein is recognized as some intermediate form, because SMV encodes a single polyprotein precursor which is cleaved to produce 10 mature proteins (Riechmann et al. 1992). It is also not known at this stage whether CI protein interacts directly or indirectly with *Rsv3* protein to activate resistance-signaling pathways. Further work is needed to specify the CI context serving as the elicitor and to characterize the interaction between CI and *Rsv3* proteins and the involvement of this interaction in resistance-signaling pathways.

MATERIALS AND METHODS

Plants, virus sources, inoculation, and RT-PCR.

Soybean cvs. Lee 68 (*rsv*), PI 96983 (*Rsv1*), L29 (*Rsv3*), V94-5152 (*Rsv4*), and Jinpumpkong-2 (*Rsv* genotype not yet characterized) were used in this study (Gunduz et al. 2002, 2004; Kim 2000). Soybean cultivars were grown in a growth chamber at 25°C under a photoperiod of 16 an 8 h. Soybean seedlings were selected for inoculation when the cotyledons were fully expanded. SMV G7H and G5H strains were isolated from soybean fields (Kim et al. 2003) and propagated in soybean cvs. Lee 74 (*rsv*) and Somyungkong. Infectious full-length cDNA clones of SMV-G7H (pSMV-G7H) (Seo et al. 2009a) and SMV-G5H (pSMV-G5H) (Seo et al. 2009b) were constructed and used as the virus sources. Plasmid DNAs of pSMV-G7H and pSMV-G5H and their chimeric and mutated derivatives were prepared using the Plasmid Maxi Kit (Qiagen, Valencia, CA, U.S.A.). Each cDNA plasmid was inoculated by direct rub inoculation as described previously (Seo et al. 2009a). Inoculation experiments, comprising at least 10 plants in total for each chimeric or substitution mutant virus, were carried out independently three times. Symptoms were assessed visually for 3 weeks postinoculation. To detect virus accumulation and upregulation of *PR-1* transcripts on inoculated and upper uninoculated leaves, cDNAs were synthesized using M-MLV reverse transcriptase (Promega Corp., Madison, WI, U.S.A.) and oligo(dT) primer. The resulting cDNAs were used to detect SMV (using primer pair 5'-TCAGGTAAGGAGAA GGAAGGA-3' and 5'-CTGCTGTGGACCCATGCC-3'), *PR-1* (using primer pair 5'-GTGGGTGAATGAGAAATCCAAGTA-3' and 5'-GTGGCAGCATAGTTCCAACCTAT-3'), and *ubiquitin* transcripts (using primer pair 5'-GGAAGGACCCTCGC TGACTAC-3' and 5'-CATCCAACATTACACAACGAGCTT-3'). The *ubiquitin* gene was used as an internal control gene to standardize the different samples. PCR reactions were performed for 35 cycles using Ex *Taq* polymerase (TaKaRa, Tokyo). PCR products were analyzed by 2% agarose gel electrophoresis.

Construction of SMV chimeras and amino acid substitution mutants.

Chimeras were constructed by exchanging corresponding fragments between pSMV-G7H and pSMV-G5H using available restriction enzyme sites common to the two cDNA constructs. The utilized restriction enzyme sites and their nucleotide positions in the SMV genome were *EcoRI* (197), *SpeI* (3,237), *AgeI* (4,627), *EcoRV* (5,963), *PmlI* (6,231), and *BstBI* (8,797) (Fig. 1). Manipulation of DNA fragments to produce chimeras was performed using standard molecular biological methods (Sambrook and Russell 2001). Construction of amino acid substitution mutants was carried out by PCR-based, site-directed mutagenesis as described previously (Nassal and Rieger 1990). Briefly, megaprimers were amplified using *Pfu* polymerase (iNtRON, Seongnam, Korea) and appropriate primer pairs (Supplementary Table S1): 4610(Age)-Fw and G7H-S1612P-Rv primers for the mutation S1616P, 4610(Age)-Fw and G7H-T1674I-Rv for the mutation T1674I, 4610(Age)-Fw and G7H-A1733S-Rv for the mutation A1733S, G7H-K1754R-Fw and 5980(EcoRV)-Rv for the mutation K1754R, G7H-I1871V-Fw and 5980(EcoRV)-Rv for the mutation I1871V, 4610(Age)-Fw and G5H-P1612S-Rv for the mutation P1612S, 4610(Age)-Fw and G5H-I1674T-Rv for the mutation I1674T, 4610(Age)-Fw and G5H-S1733A-Rv for the mutation S1733A, and G5H-R1754K-Fw and 5980(EcoRV)-Rv for the mutation R1754K. Megaprimers were gel purified and then used together with either 4610(Age)Fw or 5980(EcoRV)-Rv to amplify the mutagenic *AgeI*-*EcoRV* fragments (Fig. 1). To introduce multiple point mutations, the already constructed mutant clones served as templates to prepare megaprimers. To facilitate cloning of mutagenic PCR products of the *AgeI*-*EcoRV* region, the middle fragments of pSMV-G7H and pSMV-G5H containing the region from *SpeI* to *PmlI* sites (Fig. 1) were amplified using Ex *Taq* polymerase (TaKaRa) and a primer pair (SMV4F and SMV6R) and cloned into a pGEM-T easy vector (Promega Corp.). The resulting clones were named pTMF-G7H and pTMF-G5H, respectively. The mutagenized PCR fragments, which were amplified to contain the region from *AgeI* to *EcoRV* sites (Fig. 1), were digested with *AgeI* and *EcoRV* and inserted into either pTMF-G7H or pTMF-G5H, which were opened with *AgeI* and *EcoRV*. The resultant clones were digested with *SpeI* and *PmlI* and the released SMV middle fragments containing amino acid substitutions were replaced with those of either pSMV-G7H or pSMV-G5H, as appropriate. The sequences of all constructed chimeras and mutants were validated by DNA sequencing.

RNA extraction and sequence analysis.

Total RNAs were isolated from SMV-infected soybean leaves using the TRI reagent method (MRC, Cincinnati) according to the manufacturer's instructions. Two different regions of progenies derived from chimeric viruses, one representing the recipient strain and the other representing the donor strain, were amplified by RT-PCR using SMV-specific primers. The CI regions of progenies derived from amino acid substitution mutant viruses were also amplified by RT-PCR using an appropriate primer pair (5'-CAGGTGCTACAGTGATATAG-3' and 5'-TATCTGCCTCTTCTTCTTCTGA-3'). All fragments covering the full-length genome of progenies of pSMV-G7H_{K1754R} and pSMV-G5H_{R1754K} were amplified as described previously (Lim et al. 2003). The resultant PCR products were purified using a GENECLAN *Turbo* kit (Qbiogene, Carlsbad, CA, U.S.A.) and subsequently used as templates for nucleotide sequencing. DNA sequencing was performed using the dideoxynucleotide termination method and an ABI Prism 3730 XL DNA analyzer (Applied Biosystems, Foster City, CA, U.S.A.) at the National Instrumentation Center for Environmental

Management of Seoul National University, according to the manufacturer's instructions. The sequence data were analyzed using the LaserGene program (DNASTAR, Madison, WI, U.S.A.) and ClustalW.

ACKNOWLEDGMENTS

This research was supported in part by grants from the BioGreen21 Program (20080401-034-010-009-02-00), Rural Development Administration; the Korea Science and Engineering Foundation grant (number R01-2008-000-10087-0) funded by the Ministry of Education, Science and Technology (MEST); and the Research Center for Fruit Expert Promotion, funded by the Agriculture R&D Promotion Center. J.-K. Seo was supported by graduate fellowship from the MEST through the Brain Korea 21 Project.

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