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Infectious *in vivo* Transcripts from a Full-length Clone of *Soybean mosaic virus* Strain G5H

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An infectious full-length clone of *Soybean mosaic virus* (SMV) strain G5H was constructed under the control of the cauliflower mosaic virus 35S promoter. The cloned SMV G5H established infections upon simple rub-inoculation of soybean leaves with intact plasmid DNA. We demonstrated that this SMV G5H infectious DNA clone caused typical characteristic symptoms and virulence of SMV strain G5H in twelve tested soybean cultivars. Soybean cultivars Lee74, Somyungkong and Sowonkong developed systemic mosaic symptom while Kwanggyo, Taekwangkong, Hwangkeumkong and Geumjeongkong-1 showed systemic necrosis. In contrast, Geumjeongkong-2, Jinpumkong-2, L29, V94-5152 and Ogden showed resistant response against SMV-G5H infection. We also determined full-length sequence of cloned SMV-G5H. The phyogenetic analyses reveal that SMV-G5H is most closely related to SMV-G5, and support that SMV-G5H might be derived from SMV-G5 by recombination rather than mutation.

Keywords **:** SMV, Infectious clone, DNA-based vector

Soybean mosaic virus (SMV), a member of the genus *Potyvirus*, is one of the most common viral pathogens of soybean worldwide (Mayo and Pringle, 1998). SMV has a positive-stranded RNA genome of approximately 9.6 kilobases in length, a genome-linked viral protein (VPg) covalently bound to the 5' end, and a poly(A) tail at the 3' end (Urcuqui-Inchima et al, 2001). As in all potyviruses, SMV genome encodes one large polyprotein, which is cleaved to yield at least 10 mature proteins by three virus-encoded proteases including P1, HC-Pro and NIa (Urcuqui-Inchima et al., 2001). SMV causes systemic mosaic or lethal necrosis in many soybean cultivars, and is widely distributed and easily transmitted by aphids in fields, thus resulting in significant reductions in soybean yield and quality. Since the first classification of SMV into seven strains (G1-G7) based on the symptoms developed on the resistant soybean cultivars (Cho and Goodman, 1979), viral strain variations and their virulence on soybean cultivars have been studied extensively (Cho et al., 1983; Choi et al., 2005; Kim and Lee, 1991; Kim, 2000; Kim et al., 2003; Lim, 1985). SMV strain G5H was the most prevalent strain in Korea in the late 1980s, causing lethal necrosis on cultivars Kwanggyo, Taekwangkong, and Daewonkong or mosaic on cultivars such as Sowonkong, Somyeongkong, Danbaegkong, Keunolkong, and so forth (Kim, 2000; Kim et al., 2003).

Genetic manipulation of infectious cDNA clones of RNA viruses has proved to be of great importance for studying the molecular biology of RNA viruses. The methodology for production of infectious RNA upon *in vitro* transcription of full-length cDNA clones of RNA viruses was introduced by Ahlquist et al. (1984). In addition, Mori et al. (1991) have described the utility of the 35S promoter of *Cauliflower mosaic virus* (CaMV) for production of infectious *in vivo* transcripts of viral cDNA clones. These techniques have since been applied to many different plant RNA viruses (Boyer and Haenni, 1994). In the past two decades, infectious clones of several potyviruses have been constructed, under the control of either a promoter for *in vitro* transcription such as T7 and SP6 promoters or the CaMV 35S promoter (Domier et al., 1989; Gal-On et al., 1991; Gal-On et al., 1995; Jakab et al., 1997; Maiss et al., 1992; Puurand et al., 1996; Riechmann et al., 1990; Takahashi et al., 1997). In this paper, the construction of a plasmid containing a full-length clone of SMV strain G5H linked to CaMV 35S promoter and processing signals to produce infectious *in vivo* transcripts is described. Soybean cultivars mechanically inoculated with this plasmid produced symptoms that were indistinguishable from those shown by virus-infected plants. This offers the possibility of investigating gene expression, replication, and pathogenicity of SMV strain G5H.

SMV strain G5H was propagated in soybean cultivars

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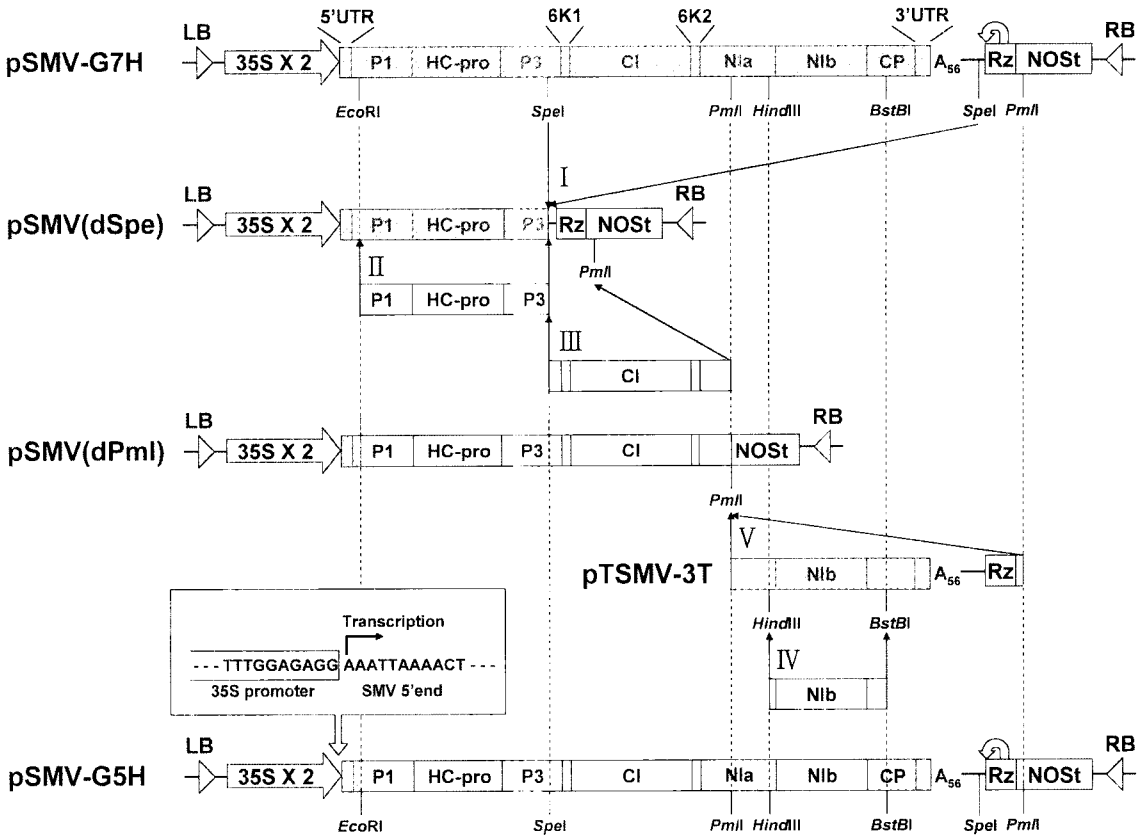

Fig. 1. Schematic representation of the construction of SMV G5H infectious clone. pSMV-G7H clone was utilized as backbone to construct pSMV-G5H. (I) pSMV-G7H was digested with *Spe*I, and self-ligated to creat pSMV(dSpe); (II) The PCR fragment of SMV G5H was inserted into pSMV(dSpe), which was opened with *Eco*RI and *Spe*I; (III) The middle fragment of SMV G5H was inserted into G5H-swapped pSMV(dSpe), which was digested with *Spe*I and *Pml*I; (IV) The 3' end fragment of SMV G5H was inserted into pTSMV-3T, which was cleaved with *Hin*dIII and *Bst*BI; (V) The 3' end fragment of SMV containing the region from *Pml*I to *Pml*I re-obtained from G5H-swapped pTSMV-3T was inserted into pSMV(dPml), which was opened with *Pml*I. pSMV-G5H contains the 5' and 3' terminus and a portion of NIa region, which are derived from pSMV-G7H as indicated by hatched boxes. SMV G5H full-length *in vivo* transcripts were produced under the control of the double 35S promoter of CaMV (35S×2), and the transcription initiation site of the promoter placed next to the first nucleotide of the SMV cDNA. The transcripts include a 56 nucleotide poly(A) tail and 55 nucleotide non-viral sequence at 3' end as a result of processing of a self-cleaving ribozyme sequence (Rz) and a nopaline synthase poly(A) signal (NOSt). The restriction enzyme cleavage sites used to make the constructs are shown.

Lee74 and Somyungkong. Total RNA extraction was carried out from SMV-G5H-infected soybeans using the TRI Reagent method (MRC, USA) according to the manufacturer's instructions. This total RNA was used for cDNA synthesis of SMV strain G5H. We have constructed an infectious full-length cDNA clone of SMV strain G7H (pSMV-G7H; Seo et al., 2009), and this pSMV-G7H clone has been utilized as backbone to construct an infectious full-length clone of SMV strain G5H (pSMV-G5H). The strategy for the construction of a full-length clone of SMV-G5H is outlined in Fig. 1. First, pSMV-G7H was digested with *Spe*I, and self-ligated to creat pSMV(dSpe), which deleted 3' large fragment of SMV genome. In pSMV(dSpe), *Eco*RI and *Spe*I sites are available as unique restriction sites. The PCR fragment of SMV G5H containing the corresponding region from *Eco*RI to *Spe*I sites was amplified (primers, 5'-GATTGGAAGCATGGCGATTT-3' and 5'-CCYTGCAG-YACACTAGTCATTTG-3') and subsequently digested with *Eco*RI and *Spe*I. The resulting fragment was inserted into pSMV(dSpe), which was digested with *Eco*RI and *Spe*I, to replace the region from *Eco*RI to *Spe*I sites of SMV-G7H with that of SMV-G5H. Next, the middle fragment of SMV-G5H containing the region from *Spe*I to *Pml*I sites was amplified (primers, 5'-CAGGTGCTACAGTGATATAG-3' and 5'-CCCAACCATACAAACCCGTTC-3'), digested with *Spe*I and *Pml*I, and inserted into G5H-swapped pSMV(dSpe), which was opened with *Spe*I and *Pml*I. The resultant clone was designated pSMV(dPml). To replace the 3' fragment of SMV G7H with G5H, the fragment from NIa to NOS terminator was amplified (primers, 5'-CAA-GTAGTTTCCAAGTCAGATG-3' and 5'-GAGCTGGTCA-CCTGTAATTCA-3') from pSMV-G7H as a template, and

cloned in to pGEM-T easy vector (Promega, USA). The resulting clone was named pTSMV-3T. Next, the 3' end fragment of SMV G5H containing the region from *Hin*dIII to *Bst*BI sites was amplified (primers, 5'-ATGTTTGGGG-TYGGCTATGG-3' and 5'-TTCCTCTCCATCCATCATC-AC-3'), and digested with *Hin*dIII and *Bst*BI. The resultant fragment was inserted into pTSMV-3T, which was cleaved with *Hin*dIII and *Bst*BI. Finally, the 3' end fragment of SMV containing the region from *Pml*I to *Pml*I was re-obtained from G5H-swapped pTSMV-3T by *Pml*I digestion and inserted into pSMV(dPml), which was opened with *Pml*I and treated with alkaline phosphatase. The resulting clone was named pSMV-G5H.

pSMV-G5H contains the 5' and 3' terminus and a portion of NIa region, which are derived from the backbone clone, pSMV-G7H. However, we found that G5H and G7H have the same amino acid sequences in these regions when we carried out sequence analysis of RT-PCR products of SMV-G5H (See also the GenBank accession numbers for the nt and the deduced aa sequences for G7H and G5H, AY294045 and 1141783, respectively). Consequently, SMV G5H full-length *in vivo* transcripts were produced under the control of the 35S promoter of CaMV, and these transcripts include a 56 nucleotide poly(A) tail and 55 nucleotide non-viral sequence at 3' end as a result of processing of a self-cleaving ribozyme sequence and a nopaline synthase poly(A) signal.

We have previously shown that the plasmid DNA of pSMV-G7H is infectious on soybeans when it was inoculated by mechanical rub-inoculation (Seo et al., 2008). Thus, to verify whether SMV G5H full-length *in vivo* transcripts generated from pSMV-G5H effectively infect soybeans and has the same virulence with viral SMV-G5H, the plasmid DNA of pSMV-G5H was inoculated on several soybean cultivars by rub-inoculation. The plasmid DNA of pSMV-G5H was prepared using the Plasmid Maxi Kit (QIAGEN, USA). Approximately 10 μg of DNA of pSMV-G5H was diluted in 50 mM potassium phosphate, pH 7.5, to a total volume of 80 μl, and inoculated onto two leaves per seedling by direct rub-inoculation dusted with the carborundum. Soybean plants were observed over a period of 3 weeks following the inoculation. At 7 to 10 days post-infiltration (dpi), soybean cultivars inoculated with pSMV-

Table 1. Disease reactions of soybean cultivars to pSMV-G5H clone

Soybean cultivars	Disease reaction[a]	
	vSMV-G5H[b]	pSMV-G5H
Lee74	M	M
Somyungkong	M	M
Sowonkong	M	M
Kwanggyo	N	N
Taekwangkong	N	N
Hwangkeumkong	N	N
Geumjeongkong-1	N	N
Geumjeongkong-2	–	–
L29	–	–
V94-5152	–	–
Jinpumkong-2	–	–
Ogden	–	–

[a]Symbols for symptoms: M, mosaic; N, necrosis; –, Simptomless and no viral RNA was detected in upper uninoculated leaves by RT-PCR.
[b]Inculation with the plant sap infected with viral SMV-G5H.

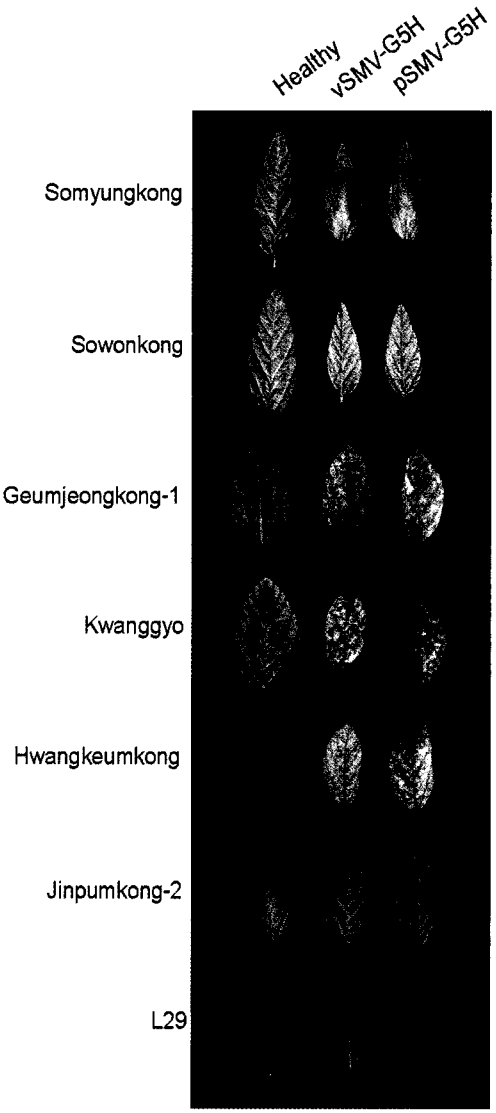

Fig. 2. Symptom appearance in different soybean cultivars infected with SMV-G5H-infected sap or pSMV-G5H. Soybean seedlings were inoculated on their cotyledons with either SMV-G5H-infected sap or with 10 μg of pSMV-G5H plasmid DNA. Soybean cultivar was designated on the left. Photographs of upper uninoculated leaves were taken at 14-18 days post-inoculation.

G5H plasmid DNA showed typical SMV G5H-induced disease reactions; Lee74, Somyungkong and Sowonkong developed systemic mosaic symptom, Kwanggyo, Taekwangkong, Hwangkeumkong and Geumjeongkong-1 showed systemic necrosis, and Geumjeongkong-2, Jinpumkong-2, L29, V94-5152 and Ogden reacted with resistance to pSMV-G5H (Table 1 and Fig. 2). These results demonstrate that pSMV-G5H plasmid is fully infectious and has the same virulence with viral SMV-G5H strain to twelve tested soybean cultivars.

To further confirm virus infection on soybean cultivars, we also carried out RT-PCR analysis to detect SMV viral RNAs. Total RNAs were prepared from upper leaves of healthy or SMV-G5H inoculated soybeans at 10 dpi. RT-PCR analysis was carried out using a SMV specific primer pair (5'-AAGCCAATCAATCTTTCCAG-3' and 5'-CCA-AAAGAGTCAATCACGTG-3') as described previously (Seo et al., 2009). PCR products were separated by agarose gel electrophoresis and visualized with ethidium bromide-staining. As shown in Fig. 3, a unique DNA bands with the predicted size (approximately 1.2 kbp) were amplified by RT-PCR using total RNA extracted from Lee74, Somyungkong, Sowonkong, Kwanggyo, Taekwangkong, Hwangkeumkong and Geumjeongkong-1 cultivars inoculated with pSMV-G5H. In contrast, no DNA bands were amplified from Geumjeongkong-2, Jinpumkong-2, L29, V94-5152 and Ogden cultivars inoculated with pSMV-G5H. Moreover, when the sap from pSMV-G5H-inoculated soybean was inoculated to new soybean seedlings, the inoculated plants showed indistinguishable symptoms compared to those developed by soybeans inoculated with virus (data not shown). This indicates that the progeny virus from pSMV-G5H DNA inoculated plants is not phenotypically different from the original virus.

In Korea, SMV-G5 was the most prevalent strain (about 80%) in the early 1980s. However, about 65% of the SMV damage was due to G5H strain in the late 1980s (Kim, 2000; Kim et al., 2003). SMV strains G5 and G5H cause similar disease reaction in the soybean differentials except for several cultivars (Kim et al., 2003), thus G5H is thought to be derived from G5 strain by mutation or recombination. We previously determined the complete nucleotide sequence of SMV-G5 strain (Accession No. AY294044; Lim et al., 2003). Thus, to verify the sequence identity between the previously reported sequence of SMV-G5 and pSMV-G5H, full-length sequence of pSMV-G5H was determined as described previously (Lim et al., 2003). The sequence alignment showed that the full-length nucleotide and deduced amino acid sequence identity between G5H and G5 are 97.7% and 99.1%, respectively, and these sequence identities between G5H and G5 are higher than those derived from between G5H and other SMV strains (Table 2). As summarized in Table 3, G5H differs by 27 amino acids from the reported sequence of G5.

In this regard, it is worth mentioning that P3 of SMV has been reported as an elicitor of *Rsv1*-mediated lethal systemic hypersensitive response (LSHR) and a single amino acid substitution of P3 (M to V at position 838) of SMV-G7d induces LSHR instead of mosaic in PI96983 cultivar (Hajimorad et al., 2005). In search of additional virulence determinants of SMV on *Rsv1*-genotype soybean, it was recently reported that SMV-N P3 amino acids are involved in elicitation of *Rsv1*-mediated extreme resistance and the C-terminus of the helper-component proteinase (HC-Pro) is also involved in the virulence of SMV on *Rsv1*-genotype soybean (Eggenberger et al., 2008). Moreover, they also present evidence that concurrent mutations in the C-terminus of HC-Pro and the N-terminus of P3 of SMV-N

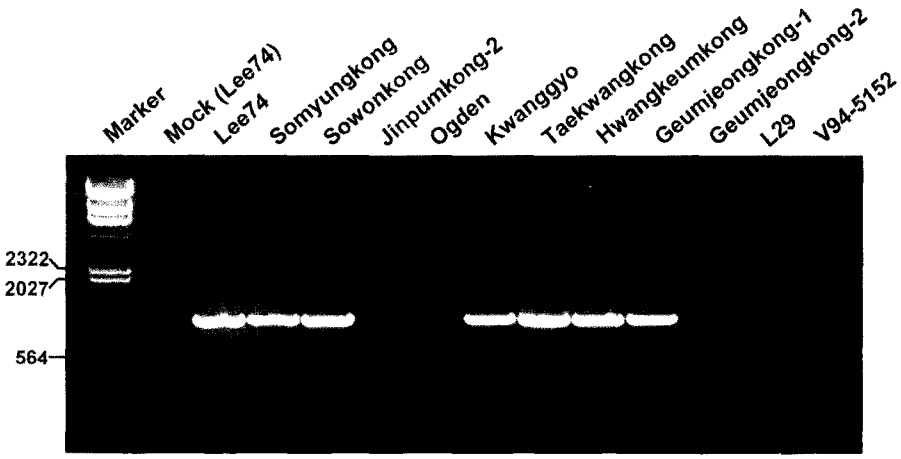

Fig. 3. Detection of SMV in soybean cultivars inoculated with pSMV-G5H by RT-PCR. Soybean cultivars (indicated at the top of gel image) were inoculated with pSMV-G5H plasmid DNA. Total RNAs, isolated from upper uninoculated leaves of each soybean plant, were analyzed at 18 dpi by RT-PCR.

Table 2. Full-length nucleotide and deduced amino acid sequence similarities (%) between SMV-G5H and known strains of SMV

	Strains	Nucleotide[a]										
		G5H	G5	G7H	G2	G7	G7d	Aa	Aa15-M2	N	HH5	severe
Amino acid	G5H	–	97.7	97.3	95.5	94.4	94.3	94.4	94.4	95.7	96.6	95.2
	G5	99.1	–	98.5	96.0	94.1	94.0	94.2	94.2	96.2	95.7	94.8
	G7H	98.9	99.3	–	96.8	93.6	93.4	93.7	93.6	97.1	95.7	94.8
	G2	97.3	97.5	98.1	–	93.6	93.4	93.8	93.8	98.9	94.8	94.0
	G7	97.6	97.5	97.3	96.4	–	99.8	96.3	96.2	93.6	95.0	93.6
	G7d	97.4	97.2	97.1	96.2	99.8	–	96.1	96.1	93.5	94.8	93.4
	Aa	97.8	97.6	97.5	96.7	98.4	98.2	–	99.9	93.9	94.9	93.7
	Aa15-M2	97.7	97.5	97.4	96.6	98.3	98.1	99.8	–	93.9	94.9	93.7
	N	98.2	98.3	98.9	98.6	97.1	96.9	97.5	97.4	–	95.1	94.2
	HH5	98.8	98.5	98.7	97.4	97.6	97.4	97.8	97.8	98.2	–	96.8
	serve	98.4	98.3	98.5	97.3	97.2	97.0	97.6	97.5	98.1	98.8	–

[a]GenBank accession numbers (G5H, 1141783; G5, AY294044; G7H, AY294045; G2, S42280; G7, AF241739; G7d, AY216987; Aa, AB100442; Aa15-M2, AB100443; N, NC-002634; HH5, AJ310200; severe, AJ312439)

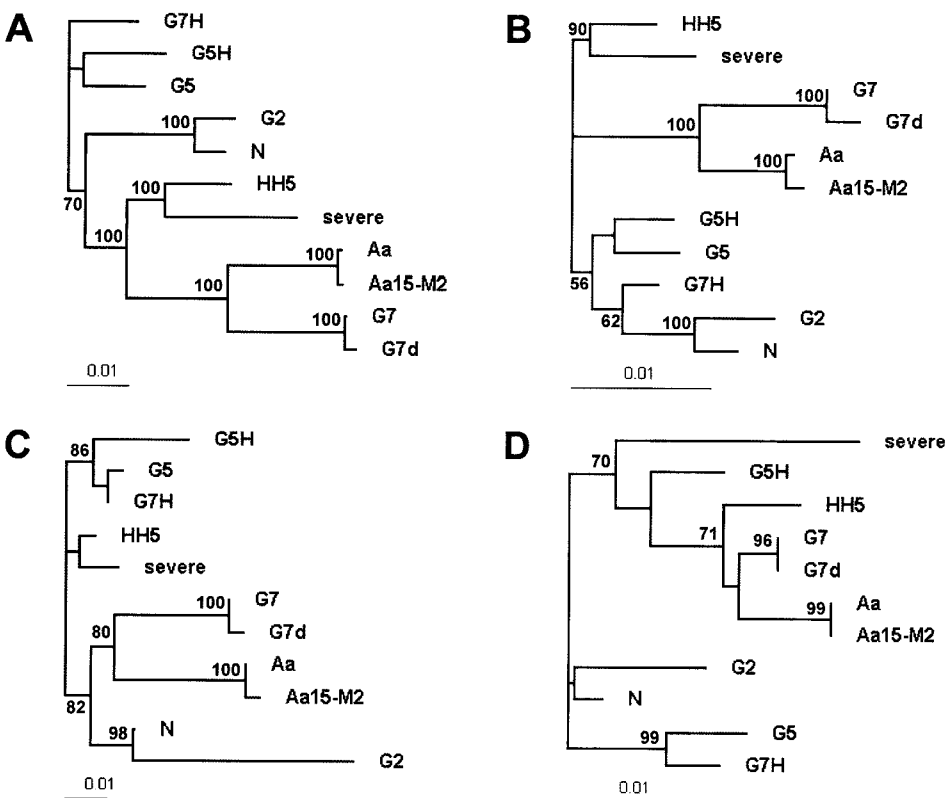

Fig. 4. Phylogenetic analyses for the genomic RNA sequences (A), the deduced polyprotein amino acid sequences (B), and the predicted P1 and NIb amino acid sequences (C and D, respectively) of 11 reported SMV strains. The phylogenetic trees were conducted by the PAUP version 4.0. The numbers on the branches indicate bootstrap percentages based on 1000 replications (only values >50% are shown). SMV sequence data were obtained from the NCBI database: G5H (1141783), G5 (AY294044), G7H (AY294045), G2 (S42280), G7 (AF241739), G7d (AY216987), Aa (AB100442), Aa15-M2 (AB100443), N (NC-002634), HH5 (AJ310200), and severe (AJ312439).

are necessary and sufficient for virulence of SMV-N on *Rsv1*-genotype soybean. Therefore, it is likely that some of 27 amino acid differences between G5 and G5H may play a role in elicitation of systemic necrosis by SMV G5H in certain soybean cultivars, which are resistant to the G5. To further assess which of the 27 amino acid differences is important for defense responses, we are currently examining by introducing amino acid substitutions into the pSMV-

Table 3. Amino acid differences between pSMV-G5H and the reported SMV G5 sequence

Region	Amino acid position	rSMV-G5[a]	pSMV-G5H
P1	97	Ser	Leu
	128	Glu	Asp
	143	Phe	Ser
	154	Tyr	His
	167	Ala	Thr
	173	Asn	Lys
	187	Lys	Arg
	236	Ser	Asn
	301	Glu	Lys
HC-pro	374	His	Gln
	405	Arg	Lys
CI	1267	Glu	Lys
VPg	1871	Ile	Val
	2015	Gly	Glu
NIa-pro	2115	Arg	Lys
NIb	2449	Arg	Lys
	2455	Pro	Ser
	2549	Ala	Val
	2552	Val	Ile
	2644	Asn	Ser
	2645	Asp	Glu
	2683	Lys	Gln
	2690	Ile	Val
	2722	Ser	Ala
	2771	Ile	Thr
	2777	Ala	Thr
CP	2814	Gly	Asp

[a]Previously reported SMV-G5 sequence (GenBank accession no. AY294044)

G5H construct.

We also performed phylogenetic analyses using PAUP version 4.0 program to determine the relationship between G5H and other SMV strains. Phylogenetic analysis of both full-length nucleotide and amino acid sequences showed that G5H is much more closely related to G7H and especially G5 than to other SMV strains (Fig. 4A and 4B). Meanwhile, interestingly, out of 27 different amino acids between G5H and G5, nine and eleven amino acid differences are located in P1 and NIb cistrons, respectively (Table 3). Thus, we carried out phylogenetic analyses for P1 and NIb amino acid sequences to examine the phylogenetic relationship of either P1 or NIb compared with that of full-length amino acid sequence among SMV strains. In phylogenetic trees of P1, G5H, G5 and G7H were clustered into one group, however, G5 showed relatively higher similarity to G7H than G5H (Fig. 4C). Furthermore, in the phylogenetic tree of NIb, G5H belongs to opposite branch with G5 although G5H and G5 show very higher similarity in phylogenetic tree of full-length amino acids (Fig. 4B and 4D).

In many viruses, abnormal biases of the sequence difference among natural virus populations have been observed (Aaziz and Tepfer, 1999; Edwards et al., 1992; Fraile et al., 1997; Simon and Bujarski, 1994). Computational and phylogenetic analyses have demonstrated that such kind of genetic exchanges might be results of interspecies recombination (Bousalem et al., 2000; Cervera et al., 1993; Desbiez and Lecoq, 2004; Revers et al., 1996). Our phylogenetic analyses also support the idea that G5H might be derived from G5 by recombination with another SMV strain(s) rather than accumulation of natural mutations and that SMV field isolates, like those of other potyviruses (Bousalem et al., 2000; Cervera et al., 1993; Desbiez and Lecoq, 2004; Gal-On et al., 1998; Ohshima et al., 2002), contain recombinants in their populations. Recombination events as well as accumulation of mutations are major evolutionary strategies for plant RNA virus diversity in relation to its host (Roossink, 1997; Simon and Bujarski, 1994). Since the prevalence of SMV G5 in early 1980s, several G5-resistant soybean cultivars had been cultivated in the fields in 1980s (Kim, 2000). Thus, it seems likely that such selection pressure resulted in the emergence of G5H, a resistant-breaking strain.

In this study, we constructed an infectious full-length clone of SMV strain G5H. Infectious *in vivo* transcripts of SMV-G5H were produced under the control of the 35S promoter of CaMV and processed by a self-cleaving ribozyme sequence and a nopaline synthase poly(A) signal to generate authentic 3' end of a inserted cDNA sequence. Utilizing these viral transcriptions and processing signals to produce infectious *in vivo* transcripts bypasses the difficulties of RNA transcription *in vitro*, especially the need for synthetic cap structures for many plant RNA viruses. This infectious clone of SMV-G5H may serve as a useful tool for elucidating the biological properties of the virus and understanding of genetic interactions between virus and soybean plant in defense responses.

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