Virology 400 (2010) 1–7

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Rapid Communication

Mutational analysis of the putative *pipo* of soybean mosaic virus suggests disruption of PIPO protein impedes movement

R.-H. Wen, M.R. Hajimorad *

Department of Entomology and Plant Pathology, The University of Tennessee, Knoxville, TN 37996, USA

ARTICLE INFO

Article history:
Received 25 November 2009
Returned to author for revision
30 December 2009
Accepted 17 January 2010
Available online 19 February 2010

Keywords:
Potyvirus
P3
Movement
Replication
RNA virus
GUS
Glycine max

ABSTRACT

The presence of a small open reading frame embedded in the P3 cistron of potyvirus turnip mosaic virus, termed "*pipo*," was recently discovered. We have now studied the putative *pipo* of soybean mosaic virus (SMV). Introduction of single, or multiple, stop codon mutations at different locations within *pipo*, without substitution in polyprotein amino acids, did not abolish replication, but restricted the virus to small cluster of cells within the inoculated leaves. Furthermore, extensive mutagenesis of the conserved GA_6 motif at the 5′ end of *pipo* also generated two out of five mutants that remained restricted to small foci of infected cells within the inoculated leaves. Long-distance movement function of the movement-defective PIPO-mutants was not restored following co-inoculation with competent SMV strains. Taken together, the data suggest that the putative *pipo* of SMV is essential for the virus movement; however, knock out of its expression does not abolish replication.

Introduction

Soybean mosaic virus (SMV), a single-stranded positive-sense RNA virus, belongs to the genus *Potyvirus* within the family *Potyviridae*, which encompasses the largest and economically most important plant viruses infecting major crops worldwide (Adams et al., 2005). The genome of SMV, similar to other members in the genus *Potyvirus*, contains an open reading frame (ORF) encoding a single large polypeptide that is cleaved by three virus-encoded proteinases to produce eight to nine mature proteins (Jayaram et al., 1992). The major host of SMV is soybean and a number of strains of the virus, based on phenotypic reactions on differential soybean cultivars, have been identified (Cho and Goodman, 1979).

The presence of a small ORF embedded in the P3 cistron of *Turnip mosaic virus* (TuMV; genus *Potyvirus*; family *Potyviridae*), termed *pipo* (Pretty Interesting *Potyviridae* ORF), was discovered recently (Chung et al., 2008). Sequence alignments of 48 viruses representing all genera in the family *Potyviridae*, including SMV, revealed the presence of the *pipo*. Furthermore, a small conserved motif, $G_{1-2}A_{6-7}$ was identified at the 5′ end of *pipo* of all 48 potyviral genomes that were examined (Chung et al., 2008).

The *pipo* of TuMV has the potential to encode a protein of ~7-kDa in the +2 frame relative to P3 (Chung et al., 2008). However, attempts to detect a ~7-kDa protein in TuMV-infected tissues by using two antibodies directed against two synthetic peptides corresponding to the deduced amino acids of the *pipo* encoded protein were unsuccessful, nevertheless, a protein of ~25-kDa was detected (Chung et al., 2008). Based on this observation Chung et al. (2008) concluded that PIPO protein is not expressed independently (i.e. via an internal ribosome entry site, shunting or transcriptional slippage), but rather as a fusion protein with the N-terminal portion of P3 (P3N + PIPO). However, the presence of P3 amino acid sequences in the detected protein, serologically or by sequencing, remains to be demonstrated.

Currently there is very limited experimental evidence available on the *pipo* and its precise role in the life cycle of potyviruses remains unknown. In the case of TuMV, it has been demonstrated that knock out of PIPO protein expression is lethal to the virus in *Nicotiana benthamiana* (Chung et al., 2008). The importance of the genomic region of *Wheat streak mosaic virus* (WSMV; genus *Tritimovirus*; Family *Potyviridae*) harboring the putative *pipo* of the virus in replication and movement was noted by Choi et al. (2005) prior to the discovery of *pipo*. Introduction of synonymous mutations into WSMV polyprotein frame by these authors, without alteration in the corresponding amino acids of P3, resulted in mutant viruses that remained restricted to small clusters of cells within the inoculated leaves and a reduction in their level of replication in protoplasts. Choi et al. (2005) attributed the altered functions to a P3 internal RNA sequence element, but not to a functional ORF. However, based on the lack of widely conserved secondary structure in the vicinity of *pipo* in

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

0042-6822/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2010.01.022

48 potyviral sequences, Chung et al. (2008) dismissed the idea of the involvement of an RNA secondary structure.

We have now studied the putative *pipo* of SMV by mutational analysis in order to examine its biological significance in the life cycle of the virus in soybean. Here we demonstrate that (i) mutations that knocked out the expression of PIPO protein of two SMV strains did not abolish replication, but restricted the resultant mutants to inoculated soybean leaves, (ii) the conserved GA_6 motif located at the 5′ end of SMV *pipo* influences virus movement, and (iii) long-distance movement function of PIPO cannot be complemented in *trans* by co-inoculation with movement-competent SMV strains. This report presents the first confirmation of the essential role of *pipo*, following its discovery, in the life cycle of a different potyvirus (SMV) in a different host (soybean). Furthermore, it is the first demonstration of the biological significance of the conserved motif of *pipo* for any potyvirus.

Results

The genomic position of the putative pipo of SMV

Based on alignment of the sequences of 48 potyviruses, including SMV-N, Chung et al. (2008) predicted that SMV *pipo* is 225 nucleotides long, encoding for 75 amino acids, and identified GA_6 as the conserved motif at its 5′ end. In light of this information, the position of *pipo* of SMV-N (GenBank accession no. D00507) maps to nucleotide sequences 2882–3106 (Figs. 1A, B). The highly conserved motif GA_6 is positioned at the nucleotide sequences 2880 to 2886 (Fig. 1B). We predict that AAA encoding lysine serves as the first codon for the putative SMV *pipo* encoded protein because of the presence of an adjacent upstream in-frame stop codon (TGA) at nucleotide positions 2879–2881 (Fig. 1B). According to Chung et al. (2008), this stop codon is present immediately upstream of the 5′ end of *pipo* in 16 out of 48 potyviral genomes. There is also another in-frame stop codon (TGA) upstream of the GA_6 motif located at nucleotide positions 2858–2860 of SMV-N (not shown). The putative *pipo* of two other well characterized biologically distinct strains of SMV, G7 and G7d (Hajimorad et al., 2003), similar to SMV-N *pipo*, also have two stop codons (TGA) preceding to their respective conserved GA_6 motifs. However, in contrast to SMV-N, in the contexts of SMV G7 and SMV-G7d genomes (AY216010, and AY216987, respectively), the position of the *pipo* is located at nucleotides 2885–3109. There is no difference in the size of *pipo* among these strains. However, SMV-N lacks a codon in the P1 cistron of the genome upstream of *pipo* (Hajimorad et al., 2006). Hence, the position of *pipo* of SMV-N differs by three nucleotides from those of SMV-G7 and SMV-G7d. Downstream of the GA_6 motif, the first in-frame stop codon (TGA) is located at nucleotide positions 3107–3109 in SMV-N genome (Fig. 1B), and we predict this serves as the terminal signal for the PIPO protein.

Knock out of PIPO protein expression

To investigate whether PIPO protein of SMV is essential for the life cycle of the virus, we knocked out its expression by introduction of a single, or multiple, stop codon mutations at different locations (Fig. 1B). In order to facilitate detection of low levels of virus replication, we introduced the stop codon mutations into SMV-NGUS (Wang et al., 2006). A total of three SMV-N-derived *pipo* stop codon mutants were constructed (Fig. 2). SMV-N_{A2894T}GUS (AGA→TGA; codon position 2894–2896; Fig. 1B) and SMV-N_{G2981T}GUS (GGA→TGA; codon position 2981–2983; Fig. 1B) each contained a single stop codon mutation. However, SMV-$N_{A2996T+A3008T+C3017T}$GUS, (AAA→TAA, AGA→TGA and CAG→TAG, codon positions 2996–2998, 3008–3010, and 3017–3019, respectively; Fig. 1B) contained three concurrent stop codon mutations. As it is known that the efficiency of *in vivo* translational termination is context dependent (McCaughan et al., 1995; Poole et al., 1995), we constructed the SMV-$N_{A2996T+A3008T+C3017T}$GUS to ensure the maximum disruption of *pipo* expression. All of these substitutions leading to the disruption of *pipo* expression are synonymous in relation to SMV polyprotein ORF (Fig. 1B).

When the stop codon mutants were biolistically inoculated into primary leaves of soybean cv. Williams82, all were surprisingly replication competent. However, replication remained restricted to small clusters of cells within the inoculated leaves as was evident by GUS expression (Figs. 2B–D), and no indication of systemic movement, based on symptoms, GUS expression or RT-PCR, was observed (data not shown). Under similar conditions, the inoculated leaves with SMV-NGUS showed extensive large foci of GUS expression that were extended to the veins (Fig. 2A) as well as to systemically infected leaves (data not shown). However, biolistic inoculation of SMV-NGUS into primary leaves of SMV-N resistant soybean line L78-379 (*Rsv1*), which is functionally immune to this strain of SMV (Hajimorad et al., 2008; Zhang et al., 2009), did not show any visible foci of GUS expression (Fig. 2E). Similar observation was made when a NGUS-derived polyprotein frameshift mutant ($N_{\Delta 2341A}$GUS), serving as a negative control, was inoculated to primary leaves of Williams82 (Fig. 2F).

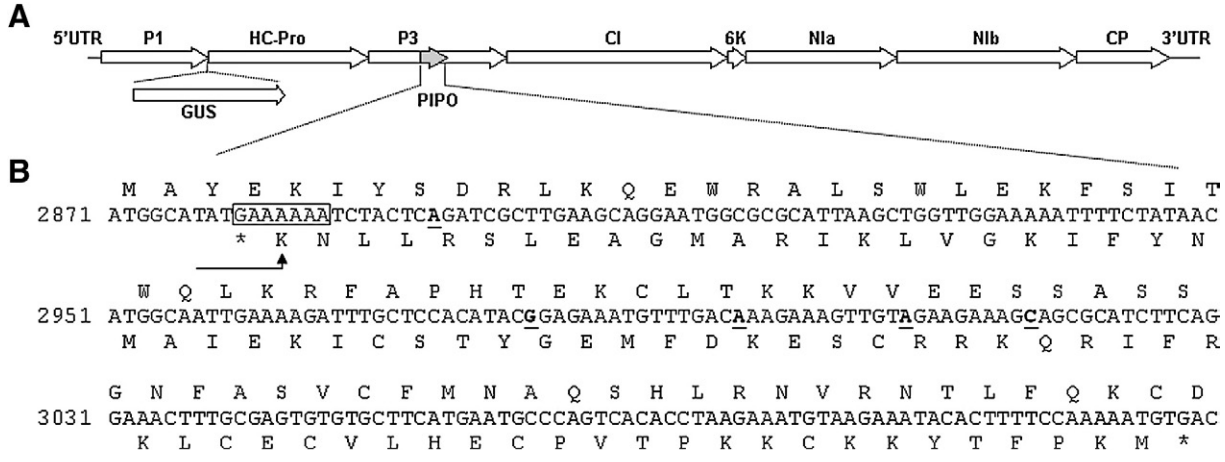

Fig. 1. (A) Schematic representation of the genome of soybean mosaic virus (SMV) showing the positions of the putative *pipo* within P3 cistron. GUS is fused between P1 and HC-Pro cistrons. (B) Partial genomic nucleotide sequences of SMV-N (GenBank accession no. D00507) containing the putative *pipo*. The deduced primary amino acid sequences of P3 and PIPO protein are shown above and below the nucleotide sequences, respectively. The arrow points to the first amino acid of the putative PIPO protein and the conserved motif (GA_6) at the 5′ end of *pipo* is boxed. The asterisks are in-frame stop codons at the beginning of the motif and at the 3′ end of *pipo*. The nucleotides targeted for the generation of *pipo* stop codon mutants, by substituting with a deoxythymidine "T" at each position, are bold and underlined.

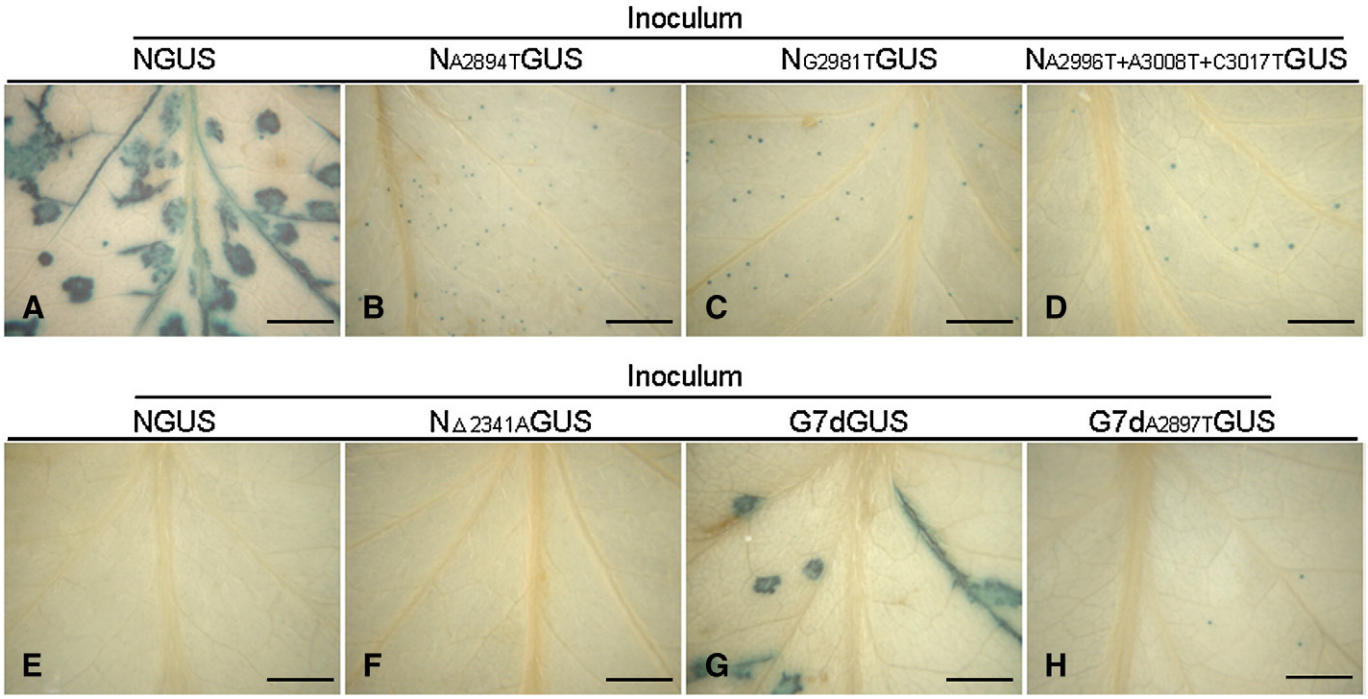

Fig. 2. The impact of introduction of stop codon mutations into *pipo* of SMV-NGUS (NGUS) or SMV-G7dGUS (G7dGUS) on movement of the viruses in biolistically inoculated primary leaves of soybean cv. Williams82 (*rsv1*) (A–D, F–H) or functionally immune line L78-379 (*Rsv1*) (E). Note N_{A2894T}GUS, N_{G2981T}GUS and $G7d_{A2897T}$GUS each contains a single (TGA) while $N_{A2996T+A3008T+C3017T}$GUS harbors three concurrent stop codon (TAA, TGA, TAG) mutations. The phenotypes of NGUS and a NGUS derivative polyprotein frameshift mutant ($N_{\Delta 2341A}$GUS), serving as negative controls, are also shown in biolistically inoculated L78-379 (*Rsv1*) and Williams82 (*rsv1*), respectively. The inoculated leaves shown were analyzed for the presence of GUS expression at 14 days postinoculation. Scale bar = 3 mm.

We also generated all the three *pipo* stop codon mutants in the context of SMV-N without GUS, and assayed the biolistically inoculated primary soybean leaves by RT-PCR. To eliminate the plasmids that contained the infectious cDNAs of the stop codon mutants (i.e. the inocula), the total RNA from the biolistically inoculated leaves was subjected to DNase I treatment. Data in Fig. 3A show that RT-PCR amplified SMV-N_{A2894T} sequences in the total RNA of plants only at 1 day postinoculation (dpi), but not at any other time points. More likely, the transcripts derived from the inoculated plasmid DNA at 1 dpi served as the template for RT-PCR where it was degraded at the later time points. However, nested-PCR detected SMV-N_{A2894T} sequences in the inoculated leaves at all the time points, but only if reverse transcriptase was present in the cDNA synthesis cocktail (Fig. 3A). The identity of SMV-N_{A2894T} in the PCR amplified fragments was verified following sequencing. In the absence of DNase I treatment, SMV-N_{A2894T} sequences were PCR amplified even if the reverse transcriptase was not present in the reaction, which suggests that the plasmid DNA, and not the replicated viral RNA, served as the template for PCR amplification (Fig. 3B).

To show that the debilitating impact of disruption of PIPO protein on movement of SMV-N-derived mutants is not strain-specific, we also introduced a stop codon (TGA) mutation into the putative *pipo* of SMV-G7dGUS and generated SMV-$G7d_{A2897T}$GUS (AGA→TGA, codon position 2897–2899). After biolistic delivery into primary leaves of Williams82, and assay of plants at 14 and 21 dpi, the pattern of GUS expression was indistinguishable from those of SMV-N-derived *pipo* stop codon mutants (Fig. 2, compare H with B–D). Under similar conditions, SMV-G7dGUS replicated and moved efficiently as evidenced by expression of GUS in large foci and veinal tissues of the inoculated leaf (Fig. 2G), as well as in the non-inoculated leaves (data not shown).

Mutagenic analysis of GA_6 motif

To investigate the biological significance of the conserved GA_6 motif, a total of five point mutations were introduced into the motif in the context of SMV-NGUS (Fig. 4). At first, we targeted the *pipo* in-frame stop codon (TGA) adjacent to the motif (Figs. 1B and 4A). When the stop codon was eliminated without any alteration in P3 amino

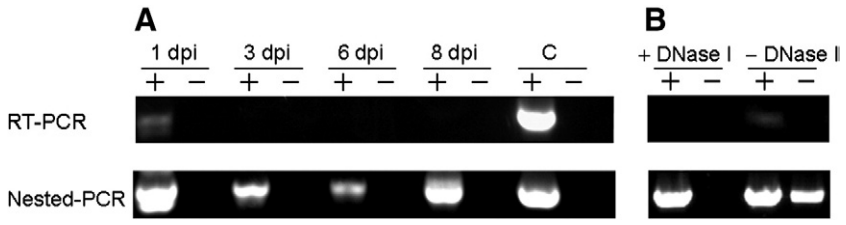

Fig. 3. RT-PCR detection of SMV-N-derived *pipo* stop codon mutant (SMV-N_{A2894T}) in biolistically inoculated primary leaves of soybean cv. Williams82. At each time point (days postinoculation; dpi) total RNA was extracted from two inoculated primary leaves. (A) DNase I-treated total RNA was subjected to cDNA synthesis in the presence (+) or absence (−) of reverse transcriptase, and the products subsequently served as templates for PCRs. The resultant products (RT-PCRs) were diluted 1:100 and served as a template in nested-PCRs. Total RNA extract from biolistically inoculated primary leaves of Williams82 with SMV-N harvested 8 dpi and treated similarly served as a positive control (C). (B) DNase I-treated (+) or DNase I-untreated (−) total RNA extract from biolistically inoculated Williams82 with SMV-N_{A2894T} harvested 8 dpi, was subjected to cDNA synthesis in the presence (+) or absence (−) of reverse transcriptase where subsequently the products served as templates in PCR. The resultant RT-PCR products were diluted 1:100 and served as template in nested PCR.

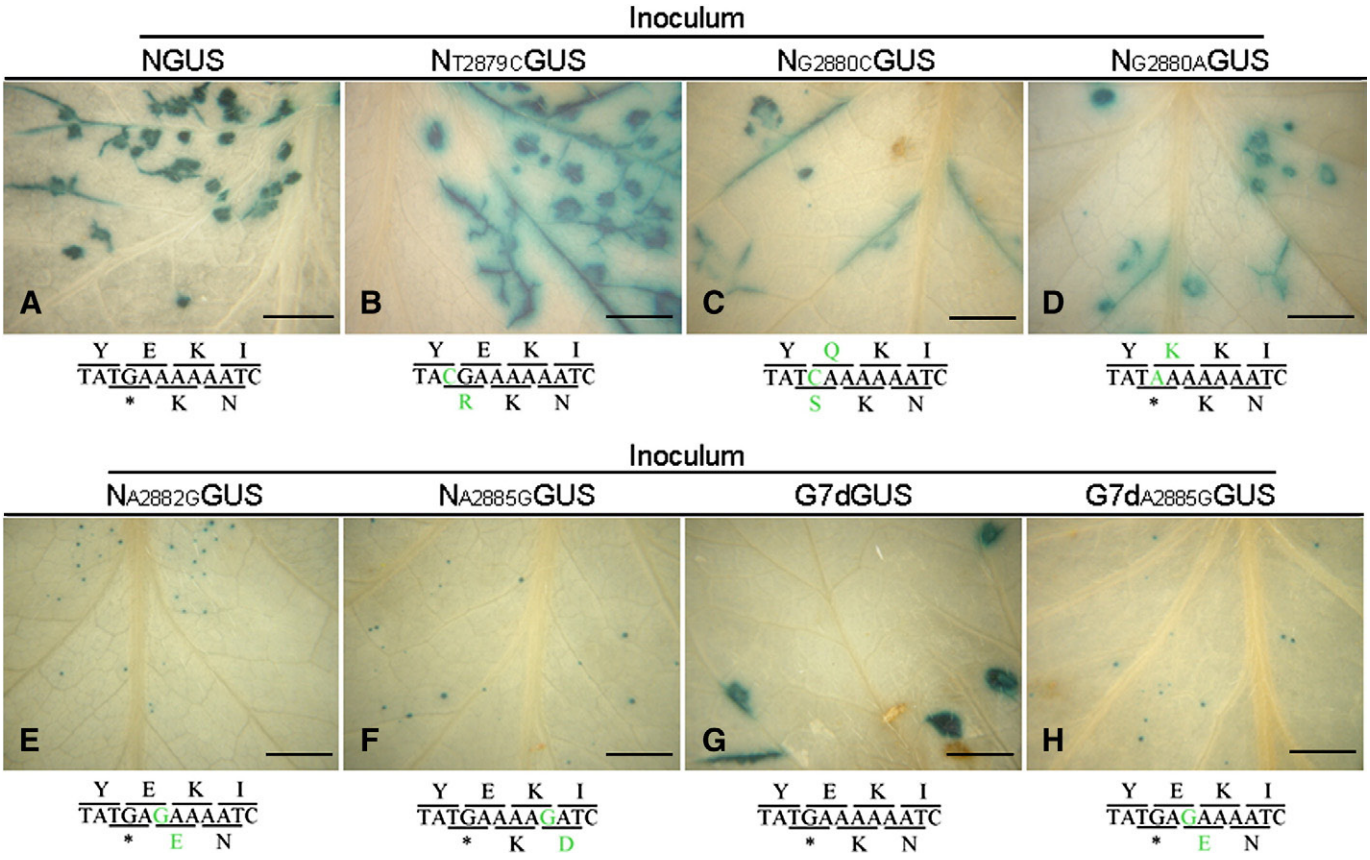

Fig. 4. The impact of point mutations in the conserved motif (GA_6) of *pipo* of SMV-NGUS (NGUS), or SMV-G7dGUS (G7dGUS) on movement of the resultant mutants in biolistically inoculated primary leaves of soybean cv. Williams82. The primary genomic sequences corresponding to the motif are shown and the primary amino acids of P3 and PIPO protein are shown above and below the nucleotides, respectively. The substituted nucleotides or amino acids are indicated in color. The inoculated leaves shown were analyzed for the presence of GUS expression at 14 days postinoculation. Scale bar = 3 mm.

acid by changing the TGA codon to CGA, which encodes arginine, the resultant mutant (SMV-N_{T2879C}GUS) efficiently replicated in the inoculated leaves (Fig. 4B) similar to the parental SMV-NGUS (Fig. 4A), and moved systemically (data not shown). Similarly, when TGA was changed to TCA to encode serine, the mutant (SMV-N_{G2880C}GUS) also spread in the inoculated leaves (Fig. 4C), albeit to a lesser extent as compared with SMV-N_{G2879C}GUS (Fig. 4, compare C with B). Nevertheless, SMV-N_{G2880C}GUS was capable of establishing systemic infection (data not shown). This mutation, however, resulted in an amino acid substitution in P3 (glutamic acid to glutamine) as well, which replaces a negatively charged residue with a polar residue. When the stop codon was changed from TGA to TAA to create SMV-N_{G2880A}GUS, which restored the *pipo* in-frame stop codon, but changed the P3 encoded amino acid glutamic acid to lysine, the resultant mutant was still capable of efficient replication and movement locally (Fig. 4D) as well as systemically (data not shown). The substitution in P3 of this mutant, however, resulted in replacement of a negatively charged residue with a positively charged amino acid. Interestingly, the exchange of the two PIPO amino acids within the motif from lysine to glutamic acid (SMV-N_{A2882G}GUS) and asparagine to aspartic acid (SMV-N_{A2885G}GUS), without alteration in P3 amino acid, resulted in restriction of the movement of the two mutants to a small cluster of the cells within the inoculated leaves (Figs. 4E, F). Both these mutations altered the charge of the putative PIPO protein. Furthermore, the nucleotide substitutions leading to these mutations altered the GA_6 motif as well, which may be important for expression of *pipo* via ribosomal frameshifting or transcriptional slippage.

To show that the debilitating impact on virus movement of amino acid exchanges within the GA_6 motif is not SMV strain-specific, we also substituted lysine for glutamic acid within the putative *pipo* motif of SMV-G7dGUS and generated SMV-G7d_{A2885G}GUS with no alteration in P3. When it was inoculated into the primary leaves of Williams82, in contrast to the parental SMV-G7dGUS (Fig. 4G), the expression of GUS by the resultant mutant remained restricted to a small cluster of cells within the inoculated leaves (Fig. 4H) and no indication of systemic movement of the virus in the non-inoculated leaves was observed (data not shown).

To show that the *uidA* gene coding for β-glucuronidase (GUS) has no negative impact on the movement ability of the resultant mutants, all the mutations in GA_6 motif were introduced into untagged SMV-N and SMV-G7d and the inoculated plants were observed for symptom expression and assayed with RT-PCR. No differences between the movement phenotypes of the mutants in the presence or the absence of GUS were observed (data not shown).

Complementation of movement-defective pipo-mutants

To find out whether *pipo* functions in movement can be complemented in *trans*, we initially co-inoculated SMV-N_{A2882G}GUS containing a single mutation in the GA_6 motif (Fig. 5A) with SMV-N, either simultaneously or sequentially. In sequential inoculations, the mutant was delivered biolistically 24 h prior to biolistic inoculation with SMV-N and vice versa. Histochemical analysis of the leaves inoculated simultaneously with both viruses 14 dpi showed no apparent increase in the sizes of GUS foci (Fig. 5, compare B with A). Similar observations were made when the sequentially inoculated leaves with both viruses were assayed (data not shown). Analyses of the inoculated leaves at earlier time points than 14 dpi (i.e. 7, 10, 12 dpi) or later (i.e. 18 dpi) did not influence the results (data not

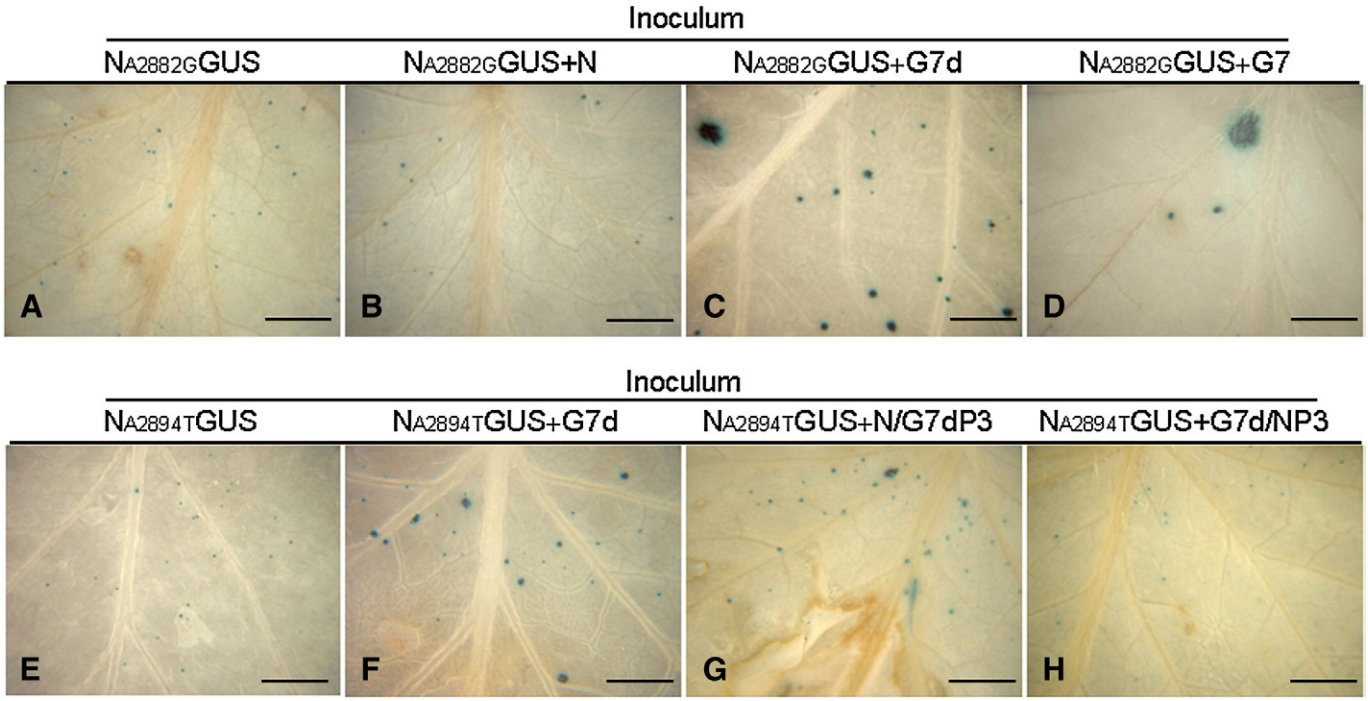

Fig. 5. Histochemical analysis of primary leaves of soybean cv. Williams82 for the presence of GUS expression following biolistic inoculation with SMV-NGUS (NGUS)-derived *pipo* mutants individually or in combination with SMV-N (N), SMV-G7 (G7), SMV-G7d (G7d) or their derivative P3-chimeras (N/G7dP3 and G7d/NP3). For co-inoculation, an equal quantity of each of the two plasmids was combined and delivered biolistically. The inoculated leaves shown were analyzed at 14 days postinoculation. Scale bar = 3 mm.

shown). Interestingly, when SMV-N_{A2882G}GUS was co-inoculated simultaneously with SMV-G7d, noticeable increases in the sizes of the GUS foci were observed (Fig. 5, compare C with A). Similar observations were made when SMV-G7 instead of SMV-G7d was used to complement the movement-defective SMV-N_{A2882G}GUS (Fig. 5, compare D with A). The inoculation of SMV-N_{A2894T}GUS that contained a stop codon mutation in *pipo* and remained restricted to the inoculated leaves (Fig. 5E) with SMV-G7d also resulted in increases in the sizes of GUS-expressing foci in the inoculated leaves (Fig. 5, compare F with E). In all the simultaneously co-inoculated plants, typical systemic symptoms of SMV-N, SMV-G7 and SMV-G7d were observed after 12 dpi.

To find out if P3 cistron of SMV-G7d is involved in the limited complementation of the local movement of the SMV-N-derived *pipo* mutants, we co-inoculated SMV-N_{A2894T}GUS with either SMV-N/G7dP3 or SMV-G7d/NP3 in which the precise P3 cistron had been exchanged between the two viruses (Hajimorad et al., 2006). Data presented in Fig. 5G show that co-inoculation with SMV-N/G7dP3 increased the sizes of GUS foci, but not when SMV-G7d/NP3 served as the helper virus (Fig. 5, compare H with G). In all of the co-inoculated plants, GUS expression was detected only in the inoculated leaves (Fig. 5) with no sign of expression in the non-inoculated leaves (data not shown).

Discussion

All the SMV *pipo* stop codon mutants, irrespective of harboring one or three simultaneous stop codon mutations, or of virus strain, were replication competent in the biolistically inoculated primary leaves of Williams82. However, all lost long-distance movement function and remained confined to small clusters of cells within the inoculated leaves. Two lines of observations provide convincing evidence in support of this conclusion. First, the visual observation of GUS expression by the *pipo* stop codon mutants in the biolistically inoculated primary leaves of Williams82, but not by SMV-NGUS or a SMV-NGUS-derived polyprotein frameshift mutant (SMV-$N_{\Delta 2341A}$GUS) in biolistically inoculated primary leaves of L78-379 (*Rsv1*) or Williams82 (*rsv1*), respectively. Soybean line L78-379 (*Rsv1*) is functionally immune to SMV-N and the virus cannot be recovered from the inoculated leaves (Hajimorad and Hill, 2001; Zhang et al., 2009). Hence, any GUS expression by the stop codon mutants in the inoculated Williams82 leaves is not simply a consequence of expression of the transcripts derived from the delivered plasmids containing the infectious SMV cDNAs, but rather it is indicative of active accumulations of GUS protein due to replication. Second, nested-PCR detection of SMV-N-derived *pipo* stop codon mutant SMV-N_{A2894T} from biolistically inoculated primary leaves of Williams82 combined with verification of its identity by sequencing. The data in Fig. 3 show that the mutant was detected in DNase I-treated total RNA from biolistically inoculated Williams82 only in the presence of reverse transcriptase. This suggests that the delivered plasmid DNA containing the infectious cDNA of the SMV did not serve as the template for PCR. Furthermore, the detection of the mutant sequences at 8 dpi by nested-PCR suggests that viral RNA derived from replication, and not transcript-derived RNA from the inoculated plasmid, must have served as the template for PCR.

The finding that disruption of SMV *pipo* is not lethal to the virus is in contrast to the observations made on TuMV where the introduction of stop codon mutation was found to be lethal to the virus in *N. benthamiana* (Chung et al., 2008). The authors acknowledged that under their experimental conditions, any local infection in the inoculated leaves by TuMV-derived *pipo* stop codon mutants, could have gone undetected. It is possible that the GFP expression system that was utilized was not as sensitive as GUS to reveal any small infection foci. The authors did not search for the presence of TuMV in the inoculated *N. benthamiana* leaves by nested-PCR either. Nevertheless, the possibility that the SMV/soybean pathosystem is different from that of TuMV/*N. benthamiana* cannot be excluded.

Two out of five nucleotide substitutions in the GA_6 motif of SMV-NGUS also resulted in impairment of the movement of the resultant mutants, and the phenotype of GUS expression resembled those of *pipo* stop codon mutants. Similar observations were made when a

similar substitution was introduced into the motif of SMV-G7d. This demonstrates that the contribution of the GA_6 motif to movement of SMV *in planta* is not strain-specific. The new codons in the context of SMV polypeptide occurred with the same frequencies as the codons that were replaced (data not shown). It is worth to note that one of the synonymous mutations, A2761G, introduced by Choi et al. (2005) into the P3 cistron of WSMV, to generate G217 mutant, also restricted the virus to a small cluster of cells within the inoculated wheat leaves. We re-examined the position of this substitution in relation to the putative *pipo* of WSMV and found that A2761G substitution in WSMV corresponds to A2882G (Fig. 4E) within the SMV-N motif. Thus, A2761G is in fact a substitution within the conserved motif (G_2A_6) of the putative *pipo* of WSMV that changes lysine to glutamic acid.

The role of the GA_6 motif in expression of PIPO protein is unknown at the present as it is not well clear how PIPO protein is expressed *in vivo*. The *pipo* motif (U GAA AAA AUC) is similar to the reported frameshifting signal sequence "X XXY YYN" (where X = A, G or U, Y = A or U and N is A, C or U) that allows translation to proceed in the −1 (+2) frameshift (Brierley et al., 1992; Farabaugh, 2000). This type of frameshifting expression strategy has been documented for many other viral systems (Atkins et al., 1990). Chung et al. (2008) stated that PIPO protein is in the +2 frame relative to P3. However, based on failure to detect the product of only the *pipo* ORF, free of fusion to another protein in TuMV infected tissues, they proposed that PIPO protein is not expressed via an internal ribosome entry site, shunting or ribosome slippage strategies. Interestingly in SMV, amino acid substitutions that resulted in loss of long-distance movement functions of two mutants, altered two conserved amino acids (lysine to glutamic acid and asparagine to aspartic acid) encoded by the two codons within the conserved motif. Both substitutions likely affect the charge of the PIPO protein as lysine is positively charged while glutamic acid is a negatively charged residue. Also, asparagine is a polar residue while aspartic acid is negatively charged. It is known that change in the charge of a protein influences protein–protein interactions. Modifications of the in-frame stop codon adjacent to the GA_6 motif to code for arginine or serine, with or without alteration in P3 amino acids, had no impact on the phenotype of the resultant mutants. This observation suggests that the most likely impact of mutations in SMV-N_{A2882G} and SMV-N_{A2885G} is on the PIPO protein rather than the secondary structure of the corresponding RNA as little conservation or correlation of RNA structure with these mutations was observed (data not shown). However, one needs to examine the impact of these substitutions on the level of expression of PIPO protein *in planta*.

Our attempt to complement the movement-defective *pipo* mutants with movement-competent SMV strains was partially successful. Choi et al. (2005) were unsuccessful in complementing a movement-defective WSMV mutant with the homologous wild type virus. Interestingly in our study some complementation of cell-to-cell movement in the biolistically inoculated leaves did occur, but only in the presence of heterologous virus strains. SMV-N was not capable of complementing local movement of its *pipo* stop codon derivative mutants, but surprisingly both SMV-G7 and SMV-G7d did and the ability was mapped to the P3 cistron. The reason for failure of SMV-N to complement local movement of its *pipo* derivative movement-defective mutants remains unknown; however, it is unlikely to be as a result of cross-protection. SMV-N was able to establish systemic infection in all co-infected plants, which was irrespective of simultaneous or sequential inoculation. It is more likely that the abilities of SMV-G7 and SMV-G7d to complement local movement of SMV-N-derived *pipo*-mutants reside on P3, PIPO protein or both. The P3 of SMV-N differs from those of SMV-G7 and SMV-G7d by 23 and 27 amino acids, respectively (Hajimorad et al., 2006) and there are four amino acids differences between P3 of SMV-G7 and SMV-G7d (Hajimorad et al., 2003). As both SMV-G7 and SMV-G7d were capable of providing limited complementation of SMV-N-derived movement-defective *pipo* mutants, it is likely that the four residue differences between P3 of the two viruses have no impact on this capability.

The most logical interpretation of our data is that the putative PIPO protein plays a critical role in movement of SMV, which indicates that *pipo* is essential in the life cycle of the virus. However, currently there is no physical evidence for the presence of non-fused PIPO protein *in vivo*. Chung et al. (2008) were unable to detect a ~7-kDa protein corresponding to that of TuMV PIPO in the infected plants. Instead, they detected a larger protein of ~25-kDa, and proposed that the PIPO protein is expressed as a fusion protein with the N-terminus P3 (P3N + PIPO) via ribosomal frameshifting or transcriptional slippage facilitated by the conserved motif. Nevertheless, one cannot exclude the possibility that PIPO protein free of other viral proteins is also expressed *in vivo*, but only in a very small quantity. Slippery sequence AAAAAAT has shown to have 10.1% frameshift efficiency in an *in vitro* translation system (Brierley et al., 1992). If this happens to be the case, its detection *in planta* can be a challenging task.

Materials and methods

Viruses, soybean genotypes, inoculation, and SMV detection

The previously described infectious cDNA clones of pSMV-N (SMV-N), pSMV-G7 (SMV-G7), pSMV-G7d (SMV-G7d), pSMV-N/G7dP3 (N/G7dP3), pSMV-G7d/NP3 (G7d/NP3) and pSMV-N-GUS (NGUS) served as the sources of viruses (Hajimorad et al., 2003, 2006; Wang et al., 2006). The pSMV-G7d tagged with GUS (G7dGUS), was synthesized essentially as described by Wang et al. (2006) with some modifications as detailed in Supplementary Materials and methods. All plasmids were propagated in ElectroMax DH5α-E (Invitrogen, Carlsbad, CA) and purified by using a QiaPrep Spin MiniPrep Kit (Qiagen, Valencia, CA). To establish infection with plasmid DNA, fully expanded primary leaves of soybean seedlings were biolistically inoculated as described previously (Hajimorad et al., 2003, 2008). The soybean (*Glycine max*) cultivar Williams82 (Bernard and Cremeens, 1988), susceptible to all strains of SMV, and line L78-379 (*Rsv1*) (Buzzell and Tu, 1984), which contains the *Rsv1* resistance gene conferring extreme resistance against SMV-N (Hajimorad and Hill, 2001), were used in this study. Soybean seeds were obtained from greenhouse grown plants shown to be free of SMV by indexing. The inoculated plants were maintained in a growth chamber operating at 22 °C with a photoperiod of 16 h. Detection of SMV in the inoculated plants was done by either histochemical assay of GUS expression (Jefferson, 1987) or RT-PCR (Hajimorad et al., 2008) at different time points following inoculations (7–21 days). To detect SMV in biolistically inoculated leaves by RT-PCR, total RNA extract was subjected to DNase I treatment (New England BioLabs, MA) prior to cDNA synthesis.

Site-directed mutagenesis

The megaprimer PCR-based mutagenesis method, as described by Sambrook and Russell (2001), was used for synthesis of site-directed mutants. The oligonucleotides used are listed in Supplementary Table 1 (Table S1) and details of syntheses are presented in Supplementary Materials and methods.

RT-PCR and sequencing

To verify the stability of the introduced mutations *in vivo*, total RNA was isolated from the infected leaves by using an RNeasy Plant Mini Kit (Qiagen). The genomic regions of the progeny viruses were reverse transcribed and subsequently subjected to RT-PCR. The amplified products were purified and sequenced. Sequencing was done at The University of Tennessee DNA sequencing facility. The

sequences were edited and analyzed using Vector NTI (Invitrogen). The oligonucleotides used for RT-PCR and sequencing of the progeny viruses are listed in Supplementary Table S1 and the detail of RT-PCR and nested PCR are presented in Supplementary Materials and methods.

Acknowledgments

The authors are grateful to Drs. A.E. Eggenberger and J.H. Hill (Iowa State University) for a copy of the plasmid pSMV-NGUS, M. Mazarei and B.H. Ownley (The University of Tennessee) for comments on the manuscript. This project was supported by The University of Tennessee, College of Agricultural Sciences and Natural Resources and The Tennessee Agricultural Experimental Station.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2010.01.022.

References

Adams, M.J., Antoniw, J.F., Fauquet, C.M., 2005. Molecular criteria for genus and species discrimination within the family *Potyviridae*. Arch. Virol. 150, 459–479.

Atkins, J.F., Weiss, R.B., Gesteland, R.F., 1990. Ribosome gymnastics—degree of difficulty 9.5, style 10.0. Cell 62, 413–423.

Bernard, R.L., Cremeens, C.R., 1988. Registration of "Williams82" soybean. Crop Sci. 28, 1027–1028.

Brierley, I., Jenner, A.J., Inglis, S.C., 1992. Mutational analysis of the "slippery-sequence" component of a coronavirus ribosomal frameshifting signal. J. Mol. Biol. 227, 463–479.

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

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

Choi, Il.-R., Horken, K.M., Stenger, D.C., French, R., 2005. An internal RNA element in the P3 cistron of *Wheat streak mosaic virus* revealed by synonymous mutations that affect both movement and replication. J. Gen. Virol. 86, 2605–2614.

Chung, B.Y.-W., Miller, W.A., Atkins, J.F., Firth, A.E., 2008. An overlapping essential gene in the potyviridae. Proc. Natl. Acad. Sci. USA 105, 5897–5902.

Farabaugh, P.J., 2000. Translational frameshifting: implications for the mechanism of translational frame maintenance. Prog. Nucleic Acid Res. Mol. Biol. 64, 131–170.

Hajimorad, M.R., Hill, J.H., 2001. *Rsv1*-mediated resistance against *Soybean mosaic virus*-N is hypersensitive response-independent at inoculation site, but has the potential to initiate a hypersensitive response-like mechanism. Mol. Plant-Microbe Interact. 14, 587–598.

Hajimorad, M.R., Eggenberger, A.L., Hill, J.H., 2003. Evolution of *Soybean mosaic virus*-G7 molecularly cloned genome in *Rsv1*-genotype soybean results in emergence of a mutant capable of evading *Rsv1*-mediated recognition. Virology 314, 497–509.

Hajimorad, M.R., Eggenberger, A.L., Hill, J.H., 2006. 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. Virology 345, 156–166.

Hajimorad, M.R., Eggenberger, A.L., Hill, J.H., 2008. Adaptation of *Soybean mosaic virus* avirulent chimeras containing P3 sequences from virulent strains to *Rsv1*-genotype soybeans is mediated by mutations in HC-Pro. Mol. Plant Microbe Interact 21, 937–946.

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

Jefferson, R.A., 1987. Assaying chimeric genes in plants: the GUS gene fusion system. Plant Mol. Biol. Rep. 5, 387–405.

McCaughan, K.K., Brown, C.M., Dalphin, M.E., Berry, M.J., Tate, W.P., 1995. Translational termination efficiency in mammals is influenced by the base following the stop codon. Proc. Natl. Acad. Sci. USA 92, 5431–5435.

Poole, E.S., Brown, C.M., Tate, W.P., 1995. The identity of the base following the stop codon determines the efficiency of *in vivo* translational termination in *Escherichia coli*. EMBO J. 14, 151–158.

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

Wang, L., Eggenberger, A., Hill, J., Bogdanove, A.J., 2006. *Pseudomonas syringae* effector *avrB* confers soybean cultivar-specific avirulence on *Soybean mosaic virus* adapted for transgene expression but effector *avrPto* does not. Mol. Plant-Microbe Interact. 19, 304–312.

Zhang, C., Hajimorad, M.R., Eggenberger, A.L., Tsang, S., Whitham, S.A., Hill, J.H., 2009. Cytoplasmic inclusion cistron of *Soybean mosaic virus* serves as avirulence determinant on *Rsv3*-genotype soybean and a symptom determinant. Virology 391, 240–248.