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Short communication

The 5′ untranslated region of *Bean pod mottle virus* RNA2 tolerates unusually large deletions or insertions

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ABSTRACT

Bean pod mottle virus (BPMV) is a bipartite, positive-sense (+) RNA virus of *Secoviridae*. We recently reported that a 137 nucleotide (nt) stretch (#263–399) of the 466 nt 5′ untranslated region (5′ UTR) of BPMV RNA2 can be deleted without compromising BPMV propagation in host plants [Lin et al., J. Gen. Virol. 94 (2013) 1415–1420]. Here we demonstrate that nonviral insertions of up to 625 nt is tolerated by the same region. Furthermore, one insertion mutant underwent recombination in infected plants, leading to the truncation of nt #250–361, thus extending the dispensable sequence to 150 nt (nt #250–399). We are unaware of any other (+) RNA virus that tolerates insertion/deletion of these sizes (625 nt/150 nt) within its 5′ UTR. Importantly, tolerance of large insertions within the RNA2 5′ UTR offers a novel, more convenient site for incorporating host gene fragments, making BPMV a more versatile vector of virus-induced gene silencing.

The untranslated regions (UTRs) at both 5′ and 3′ termini of positive sense (+) RNA virus genomes commonly contain critical *cis*-acting elements required for genome replication and/or translation of virus-encoded proteins, hence are thought to be intolerant to large deletions or insertions. Indeed, the entire 5′ and 3′ UTRs are frequently retained in defective interfering (DI) RNAs of some viruses, and must be included in minimal replicons of others (Wu et al., 2009; Sztuba-Solinska et al., 2013). While deletion mutants of human rhinovirus type 14 (HRV14) and poliovirus 1 (PV1) lacking the entire 3′ UTR could replicate in cell cultures at reduced rates, it was unclear whether they retained infectivity in human hosts of these viruses (Todd et al., 1997). By comparison, the 5′ UTRs of (+) RNA virus genomes have been found to be notoriously sensitive to alterations of even a few nucleotides (nt), hence were rarely subjected to deletions or insertions of large sizes (Andino et al., 1990; Niesters and Strauss, 1990; Turner and Buck, 1999; Annamalai et al., 2003; Filomatori et al., 2006). Notable exceptions are the internal ribosomal entry site (IRES) elements of poliovirus and several flaviviruses, which can be replaced by functionally analogous elements of related viruses (Lu and Wimmer, 1996; Frolov et al., 1998). Nevertheless, these examples involved exchange of elements from similar locations of related viruses, with similar functions. Therefore, 5′ UTRs of (+) RNA virus genomes are not known to permit large deletions (more than 100 nt) or nonviral insertions (more than 600 nt).

In this report, we show that RNA2 of the bipartite, (+) RNA genome of bean pod mottle virus (BPMV) tolerates deletions of up to 150 nt, and nonviral insertion of up to 625 nt within its 5′ UTR. BPMV is a member of the *Comovirus* genus in the *Comovirinae* subfamily of *Secoviridae* (Sanfaçon et al., 2009). Like other comoviruses, BPMV partitions its coding capacity to two genomic RNA segments, with RNA1 encoding viral proteins required for genome replication, and RNA2 encoding proteins needed for viral cell-to-cell movement and particle assembly (Gu and Ghabrial, 2005 and Fig. 1). In a previous study, we have found that as many as 137 nt of the 466 nt long 5′ UTR of BPMV RNA2 could be deleted through two separate deletions (ΔSLA, 45 nt; and ΔSLB, 92 nt; Fig. 1) without compromising the infectivity of BPMV in its host plants lima bean and soybean (Lin et al., 2013; also see Fig. 1, ΔSLA/B). That observation led us to ask whether the same 5′ UTR would also permit large sized insertions of nonviral sequences. To this end, we have replaced nt #263–309 of BPMV RNA2 with two restriction enzyme (RE) sites – a BamHI and an Acc65I site separated by a 6-nt (TTAATT) spacer – that permit convenient insertion of foreign sequences (Figs. 1 and 3).

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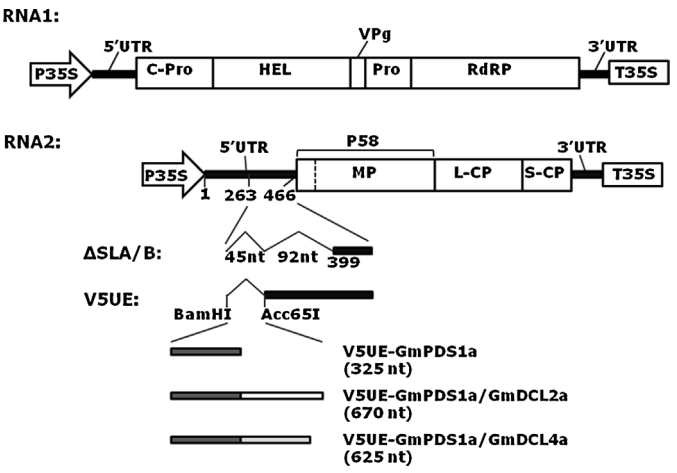

Fig. 1. Schematic representations of BPMV RNA1 and RNA2 constructs used in this study. The top diagram depicts the full length cDNA of RNA1 flanked by CaMV 35S promoter and terminator (P35S and T35S), used to support RNA2 replication in the particle bombardment experiments. The long open box denotes the single open reading frame (ORF) encoded by RNA1, with vertical lines depicting the boundaries of five mature proteins released by protease (Pro)-mediated processing. C-Pro: putative protease cofactor; HEL: putative helicase; VPg: viral protein-genome-linked; RdRP: RNA-dependent RNA polymerase. Similarly, the RNA2-encoded polyprotein is processed into P58, movement protein (MP), large and small capsid protein subunits (L-CP and S-CP). The 5′ UTR of RNA2 is 466 nt long, with the two deletion mutants (ΔSLA/B) between nt #263 and 399 reported in an earlier study (Lin et al., 2013). The V5UE mutant replaces nt #263–310 with BamHI and Acc65I sites separated by a 6-nt spacer (see Fig. 3 for detailed sequences). The three insertion mutants derived from V5UE are depicted at the bottom and described in detail in the main text. The *GmPDS1a*, *GmDCL2a*, and *GmDCL4a* inserts are depicted as thin boxes with dark gray fill, no fill, and light gray fill, respectively.

Additionally, a new Eco72I site (CACGTG) was also created to permit convenient screening of mutant constructs. The resulting RNA2 mutant was designated V5UE (Figs. 1 and 3). As in the previous report (Lin et al., 2013), the RNA2 cDNA was derived from an Iowa isolate (IA-D35) kindly provided by Dr. Steve Whitham (Zhang et al., 2010).

Three different sized fragments of soybean origin were inserted into V5UE to assess whether they can be tolerated by BPMV RNA2 (Fig. 1). The first insert, GmPDS1a, was a 325 nt fragment of the soybean (*Glycine max*; Gm) phytoene desaturase 1a cDNA used previously by Zhang and colleagues (2010) to induce robust *GmPDS1a* silencing. Since insertion of this fragment elsewhere in BPMV RNA2 was known to cause virus-induced silencing (VIGS) of *GmPDS1a* mRNA and photobleaching of soybean leaves. Its inclusion in V5UE should provide easily trackable visual indicator of virus viability (Zhang et al., 2010). The second and third inserts, GmPDS1a/GmDCL2a and GmPDS1a/GmDCL4a, were chimeric inserts with cDNA fragments of *GmDCL2a* (345 nt) and *GmDCL4a* (300 nt), respectively, fused to the aforementioned GmPDS1a insert. The reason for choosing *GmDCL2a* and *GmDCL4a* as additional VIGS targets is because their Arabidopsis orthologs, *Dicer-Like 2* and *4*, were implicated in antiviral RNA silencing by previous studies (Qu et al., 2008; Zhang et al., 2012). We hence wanted to assess whether silencing of these two genes could affect the effectiveness of VIGS targeting other genes (*e.g. GmPDS1a*). The *GmDCL2a* fragment selected corresponds to nt #1811–2154 of the full length *GmDCL2a* cDNA (XM_003534726.1), that of *GmDCL4a* corresponds to nt #2720–3019 of full length *GmDCL4a* cDNA (XM_003541423.1). Together the sizes of the three nonviral inserts were 325, 670, and 625 nt, respectively (Fig. 1). All inserts are in antisense orientation relative to their corresponding mRNAs to permit more efficient silencing (Zhang et al., 2010). Additionally, in order to avoid unintended translational initiation from the inserts, the sequences were modified to remove AUG start codons in all three reading frames, and the modified fragments were custom-synthesized (Eurofins, Huntsville, AL) and incorporated into V5UE with routine recombinant DNA techniques.

Three constructs containing the cDNAs of three V5UE derivatives, all flanked by the 35S promoter and terminator (P35S and T35S) of cauliflower mosaic virus, were first brought into lima bean (*Phaseolus lunatus*; variety Hendersen Bush) cotyledons together with an RNA1 construct (R1M in Zhang et al., 2010 and Fig. 1) with particle bombardment in order to amplify the viral inocula (Lin et al., 2013). At four days after bombardment, the treated cotyledons were homogenized and used to rub-inoculate young soybean (variety Williams 82) plants at the two-leaf stage. The infected soybean plants were maintained in a greenhouse room and monitored on a daily basis for symptom development. As shown in Fig. 2B, plants infected with the V5UE mutant without any insert were mildly symptomatic with slightly bumpy and deformed, yet completely green leaves. By contrast, plants infected with V5UE-GmPDS1a mutant developed extensive photobleaching on systemically infected leaves starting as early as eight days post inoculation (8 dpi), indicating efficient multiplication and spread of the mutant, as well as the retention of the GmPDS1a insert in the mutated viral RNA2 (Fig. 2C and K).

Interestingly, plants infected with the V5UE-GmPDS1a/GmDCL2a mutant, although showing symptoms similar to V5UE-infected plants, developed only sporadic white spots on leaf margins or along the veins, suggesting that while this construct was able to cause systemic infections in soybean, the GmPDS1a insert might not have been stably maintained during the infection process (Fig. 2D). Indeed this was confirmed with reverse transcription-polymerase chain reaction (RT-PCR; see later). Notably, the V5UE-GmPDS1a/GmDCL4a mutant, whose insert was only 45 nt shorter than that of V5UE-GmPDS1a/GmDCL2a, caused the infected plants to develop largely photobleached systemic leaves which are similar to those infected with V5UE-GmPDS1a (Fig. 2E). These results suggest that unlike the GmPDS1a/GmDCL2a insert, the GmPDS1a/GmDCL4a insert was probably relatively stable.

Indeed, the effective silencing of GmPDS1a by V5UE-GmPDS1a and V5UE-GmPDS1a/GmDCL4a was confirmed by semi-quantitative RT-PCR with total RNA extracts from systemic leaves collected at 15 dpi. As shown in Fig. 2F, top panel, the level of GmPDS1a-specific RT-PCR (30 PCR cycles) product amplified from leaves infected with these two mutants (lanes 4 and 6) was visibly lower than mock-inoculated control leaves (lane 2) or those infected with the other two constructs (lanes 3 and 5). Furthermore, this product was undetectable when reverse transcriptase (RT) was omitted, hence, confirming it as mRNA-derived (Fig. 2F, bottom panel). Additionally, the comparable level of a 345 nt RT-PCR (30 PCR cycles) product of a soybean actin mRNA among samples showed that the amount of total RNA used for each reaction was similar. Together these data confirmed that both V5UE-GmPDS1a and V5UE-GmPDS1a/GmDCL4a induced efficient silencing of GmPDS1a, hence further suggesting that the inserts in both constructs (325 and 625 nts, respectively) were probably stable (see later).

By contrast, the GmDCL2a-specific RT-PCR (42 PCR cycles) product was at similar levels in all infected plants, including those infected with V5UE-GmPDS1a/GmDCL2a (Fig. 2H, lane 5). This is consistent with symptoms of the infected plants, and RT-PCR results of GmPDS1a, both suggesting the instability of the GmPDS1a/GmDCL2a insert. However, it is worth noting that the GmDCL4a-specific RT-PCR (42 PCR cycles) product was likewise not reduced by infections with the V5UE-GmPDS1a/GmDCL4a mutant (Fig. 2I, lane 6). The inability to silence GmDCL4a with this construct could be due to multiple reasons. Zhang et al. (2010) reported that fragments derived from different regions of the full length *GmPDS1a*