Virology 526 (2019) 13–21

Contents lists available at ScienceDirect

Virology

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

Effects of deletion at the TTTSTTT motif of *Hibiscus latent Singapore virus* coat protein on viral replication and long-distance movement

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ARTICLE INFO

Keywords:
Hibiscus latent Singapore virus
TTTSTTT motif
Virus replication
Virus systemic movement

ABSTRACT

Hibiscus latent Singapore virus (HLSV) mutant HLSV-22A could not express coat protein (CP) nor infect plants systemically (Niu et al., 2015). In this study, a serine- and threonine-rich motif TTTSTTT at the C-terminus of HLSV CP was found to be involved in virus replication and systemic movement. Deletion the last amino acid residue in HLSV-22A led to a more rapid virus replication, but with delayed systemic movement. When the RNA structure in TTTSTTT motif was altered, while keeping its amino acids unchanged, mutants HLSV-87A-mmSL and HLSV-22A-mmSL showed no change in viral replication. These results indicated that the unique TTTSTTT motif is associated with virus replication and systemic movement. Deletion but not substitution of amino acid(s) at the C-terminus of TTTSTTT motif of HLSV CP with short internal poly(A) track enhanced virus replication, whereas the virus with a longer internal poly(A) tract of 87 A showed delayed systemic movement (147 words).

1. Introduction

Hibiscus latent Singapore virus (HLSV) is a member of the genus *Tobamoviruses*. Its genome structure is similar to other tobamoviruses with a 5’ untranslated region (UTR), four open reading frames (ORFs) and a 3’ UTR (Zaitlin, 1999). In most other tobamoviruses, their 3’ UTRs consist of two parts: an upstream pseudoknot domain (UPD), followed by a tRNA-like structure (TLS) (van Belkum et al., 1985; Gallie and Walbot, 1990; García-Arenal, 1988; Isomura et al., 1991). A study on the function of *Tobacco mosaic virus* (TMV) 3’UTR showed that the UPD function in enhancing gene expression is similar to that of a poly(A) tail (Gallie and Walbot, 1990). In TMV, deletion of the UPD reduced replication and symptom development, and the last pseudoknot in the UPD which is adjacent to the TLS is most important for replication (Takamatsu et al., 1990). However, in HLSV, it does not have an UPD but possesses a unique internal poly(A) tract (IPAT) of variable lengths, normally ranging from 77 to 96 nucleotides (nt) upstream of the TLS in the 3’ UTR of wild-type HLSV (Srinivasan et al., 2005). This IPAT has been previously studied for its role in virus replication (Niu et al., 2015). The IPAT also exists in another tobamoviruses *Hibiscus latent Fort Pierce virus* (HLFPV) (Adkins et al., 2003; Yoshida et al., 2014). In the immediate upstream of the IPAT of the HLSV genome is an open reading frame (ORF) encoding a coat protein (CP) of 163 amino acids. The multi-functions of the CP of RNA plant viruses relevant to virus replication and movement have been reviewed (Callaway et al., 2001; Ivanov and Mäkinen, 2012; Ni and Cheng Kao, 2013). Functional CP or formation of stable virions have been shown to be essential for long-distance movement in many RNA plant viruses such as *Alfamoviruses* (Spitsin et al., 1999), *Cucumoviruses* (Suzuki et al., 1991), *Dianthoviruses* (Vaewhongs and Lommel, 1995), *Potyviruses* (Dolja et al., 1995), *Sobemoviruses* (Brugidou et al., 1995) and *Tobamoviruses* (Fuentes and Hamilton, 1993; Saito et al., 1990), with the exception of a few other viruses (Ryabov et al., 1999; Scholthof et al., 1993). In *Turnip crinkle virus* (TCV), its CP plays a host-dependent role in long-distance virus movement (Cao et al., 2010; Cohen et al., 2000; Hacker et al., 1992; Heaton et al., 1991). Proper virion assembly in *Beet black scorch virus* is needed for virus systemic movement (Zhang et al., 2013). In *Olive latent virus 1*, CP C-terminal deletion mutants could form intact virions but were unable to move systemically, indicating that virion formation is necessary but not sufficient for long-distance movement (Pantaleo et al., 2006). *Wheat streak mosaic virus* can tolerate deletion up to C-terminal 17 amino acids of its CP without affecting long-distance movement (Tatineni et al., 2014).

In addition to the function of C-terminal amino acids of CP in virus long-distance movement, we investigated possible involvement of nucleotide sequence or its RNA structure in the phenomenon observed. Up to now, many studies have focused on the functions of RNA structures of 5’ and 3’ UTRs in virus replication or viral protein translation.

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https://doi.org/10.1016/j.virol.2018.09.027
Received 5 July 2018; Received in revised form 29 September 2018; Accepted 29 September 2018

Translation enhancement by interaction of 5' and 3' UTRs has been reported in *Barley yellow dwarf virus*, *Tomato bushy stunt virus* and *Tobacco necrosis virus* (Fabian and White, 2004; Guo et al., 2001; Rakotondrafara et al., 2006; Shen and Miller, 2004). In *Dengue virus*, the balance between linear and circular forms of the genome, which is critical for replication but not translation, has been reported to be controlled by several sets of overlapping 5' and 3' interacting sequences (Villordo et al., 2010). In addition, an RNA element in the CP coding region has been shown to form an interactive network with its 3' UTR of TCV to support viral replication and translation (Yuan et al., 2012).

In this study, a serine- and threonine-rich motif TTTSTTT was found at the C-terminal of each HLSV CP subunit. Deletion, but not substitution, of HLSV with 22 IPAT resulted in enhanced viral RNA replication. This effect was attributed by amino acid(s) deletion but not by amino acid substitution or RNA structure alteration. Additionally, deletion of amino acids in the TTTSTTT motif of HLSV with 87 A IPAT delayed viruses to move to upper leaves and delayed virus systemic movement. Taken together, depending on the short or long length of HLSV IPAT, the TTTSTTT motif at the C-terminus of viral CP is involved in viral replication and systemic movement during its infection in host.

2. Results

2.1. Deletion of the C-terminal amino acid(s) from CP of HLSV with 22A IPAT facilitates its viral RNA replication in protoplasts and plants

Our previous study has shown that CP expression of HLSV is influenced by the length of IPAT. When the IPAT was shortened to 22 A, viral RNA and CP expression of HLSV-22A was undetected in inoculated plants (Niu et al., 2015). From the HLSV virion structure (Tewary et al., 2011), a serine- and threonine-rich motif TTTSTTT was found at the C-terminal of HLSV CP (Fig. 1A, boxed). To test whether this motif is involved in HLSV replication, a mutant HLSV-22A-ΔT was constructed, which has a 22 nt IPAT, with its last amino acid T at the C-terminus of HLSV CP deleted (Fig. 1).

HLSV-22A was unable to replicate as the IPAT was too short (Niu et al., 2015). However, to our surprise, we observed that mutant HLSV-22A-ΔT was able to replicate in *N. benthamiana* protoplasts. The accumulation of viral RNA increased in protoplasts over time (Fig. 2A). Additionally, viral genomic RNA and subgenomic RNAs were detectable in inoculated *N. benthamiana* leaves (Fig. 2B), and viral CP was also expressed in HLSV-22A-ΔT inoculated leaves (Fig. 2C). All these results indicated that deletion of one amino acid in the TTTSTTT motif of HLSV CP rendered HLSV-22A-ΔT to replicate in *N. benthamiana* protoplasts and plants.

This phenomenon led us to generate two additional deletion mutants, with the last four or last seven amino acids deleted, giving rise to constructs HLSV-22A-ΔTSTTT and HLSV-22A-ΔTTTSTTT, respectively (Fig. 1). To find out the putative function of TTTSTTT for HLSV replication, viral RNA accumulation of wild-type HLSV-87A, HLSV-22A and their TTTSTTT motif deletion mutants were analyzed in *N. benthamiana* protoplasts after transfection of respective *in vitro* transcripts of the constructs. Our previous study showed that longer IPAT facilitates HLSV replication. HLSV-87A infected *N. benthamiana* plants showed a higher viral RNA accumulation than those HLSV with shorter IPAT. HLSV-87A was used as a positive control when compared to the amount of viral RNA accumulation in plants infected by TTTSTTT motif deletion mutants of HLSV-22A.

HLSV-22A-ΔT showed a higher viral RNA accumulation, with no significant difference to that of HLSV-87A at 46 h post transfection (hpt). (Fig. 2A). HLSV-22A-ΔTSTTT and HLSV-22A-ΔTTTSTTT also demonstrated an increased viral RNA accumulation after transfection over time (Fig. 2A).

In the inoculated *N. benthamiana* plants, Northern blot results showed that HLSV-22A-ΔT, HLSV-22A-ΔTSTTT and HLSV-22A-ΔTTTSTTT all could replicate in inoculated plants, as viral genomic RNA and subgenomic RNAs were detected in inoculated leaves at 10 days post inoculation (dpi) (Fig. 2B). As the IPAT in HLSV-22A was much shorter than that of HLSV-87A, it was not surprising that the viral RNA levels of HLSV-22A TTTSTTT deletion mutants were lower than that of the wild-type HLSV-87A. Consistent with our previously published results, viral RNA accumulation was not detected in HLSV-22A inoculated leaves (Fig. 2B).

In view of the increased viral RNA accumulation in leaves inoculated with the CP C-terminal deletion mutants of HLSV-22A, western blot was performed to determine CP accumulation in inoculated leaves. Coat protein expression was detected in all three TTTSTTT deletion mutants, although the expression level was lower than that of HLSV-87A inoculated leaves (Fig. 2C).

2.2. Deletion of amino acids in TTTSTTT motif of HLSV-CP disrupts virus systemic movement to the upper leaves of plants

Genomic RNAs and subgenomic RNAs of HLSV-22A-ΔT, HLSV-22A-ΔTSTTT and HLSV-22A-ΔTTTSTTT were detected in the inoculated leaves, which indicated those mutants could replicate in plant leaves. However, viral RNAs of those HLSV-22A TTTSTTT deletion mutants were not detected in the upper leaves at 28 dpi (Fig. 2D). There was no viral CP expression in the upper leaves (Fig. 2E). Rod-shaped virions were observed in crude saps of inoculated leaves with HLSV-22A deletion mutants (data not shown). It indicated that mutant viruses could assemble into virions in the inoculated leaves after replication. However, no viral RNAs nor viral coat proteins of these three HLSV-22A TTTSTTT deletion mutants was detected in the upper leaves of infected plants. We hypothesize that deletion of the amino acids in TTTSTTT motif of HLSV CP disrupts virus systemic movement, preventing the virus to move into the upper leaves.

As HLSV possessing a longer IPAT replicates faster than HLSV-22A CP deletion mutants with shorter IPATs, we tested our hypothesis using HLSV-87A and its deletion mutant HLSV-87A-ΔT. Both viral RNA and CP were detectable in the upper leaves of HLSV-87A-infected plants at 9 dpi, but not in that of HLSV-87A-ΔT-infected plants (Fig. 3A). Viral RNA and CP of HLSV-87A-ΔT were only detected in the upper leaves at 14 dpi (Fig. 3B). These results indicated that deletion of the last amino acid T in the TTTSTTT motif of HLSV CP delayed virus systemic movement.

2.3. Deletion of the C-terminal amino acid(s) of HLSV CP facilitates extension of IPAT in HLSV

Our previous results showed that viral RNAs of HLSV-22A was not detected in inoculated plants by Northern blot, but it can be detected by RT-PCR (Niu et al., 2015). The viral RNA of HLSV-22A in inoculated plants were obtained and sequenced to analyze whether the length of IPAT has changed over time after inoculation. Also, the progeny virus sequences of three HLSV-22A TTTSTTT mutants were amplified and compared using RNA samples isolated from the inoculated *N. benthamiana* leaves. DNA sequencing results showed that the IPAT length from the three deletion mutants (HLSV-22A-ΔT, HLSV-22A-ΔTSTTT, HLSV-22A-ΔTTTSTTT) was extended ∼3- to 4-fold longer than the length of IPAT in HLSV-22A (Fig. 4A). It indicated that deletion of the amino acid(s) in the TTTSTTT motif of HLSV CP facilitates the extension of IPAT in viral genome. In addition, DNA sequencing results showed that the CP sequences had no changes and no further mutations had occurred in the progeny viruses (Fig. 4B).

2.4. C-terminal of HLSV CP amino acid(s) regulates RNA replication

In view of the rapid viral RNA accumulation of the deletion mutant HLSV-22A-ΔT, an additional mutant HLSV-22A-TTTSTTA with the last amino acid T of the CP substituted with an alanine (Fig. 1A) was constructed to investigate whether the amino acid threonine specifically regulates RNA replication. The RNA accumulation in protoplasts

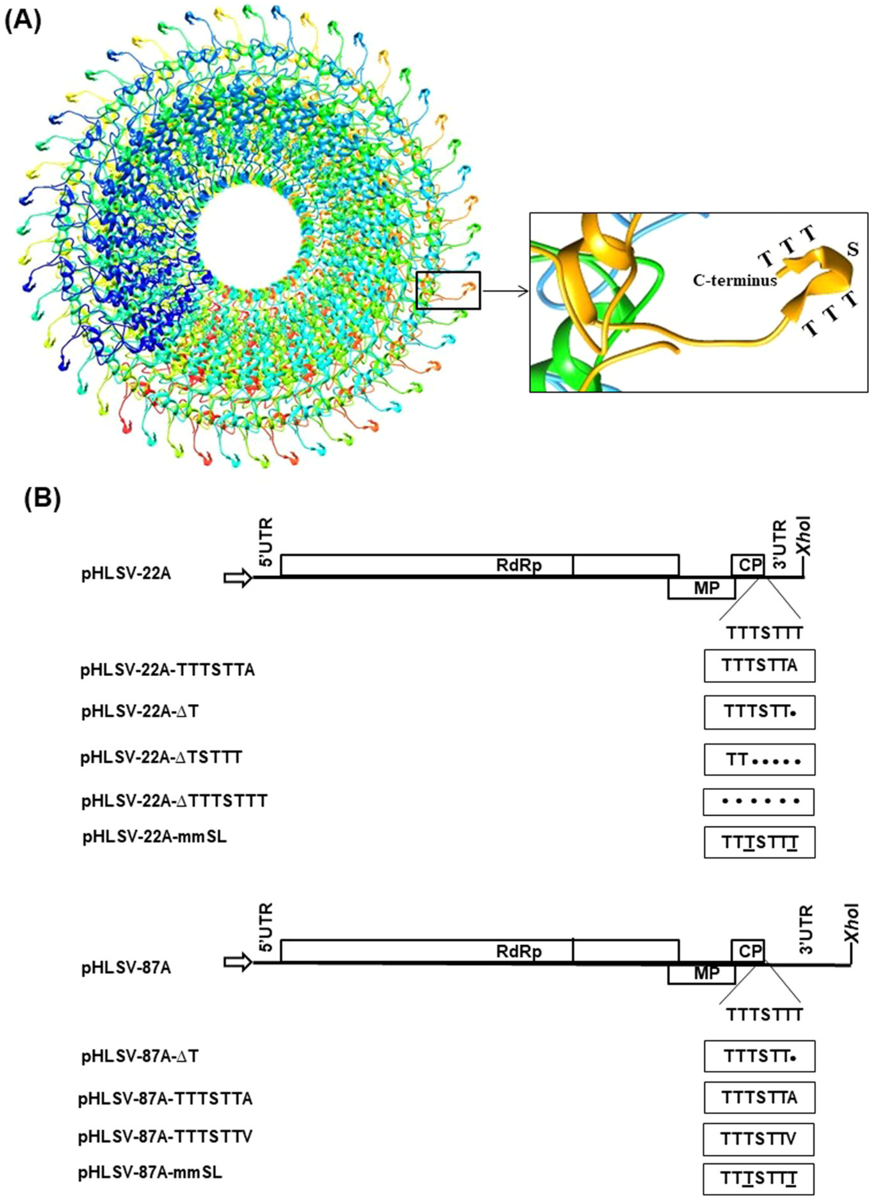

Fig. 1. (A) Biological assembly of HLSV based on its fiber diffraction structure. The software UCSF Chimera (http://www.cgl.ucsf.edu/chimera) was used for HLSV biological assembly. The left panel shows the assembled structure (3 turns, 49 subunits of the virus repeating units) showing the free N and C termini of the virus projected away from the virus helix axis. The right panel is the close up of part of the virion surface showing a typical hook-shaped TTTSTTT motif of CP. (B) Schematic representation of HLSV full-length cDNA clone and its mutants. Untranslated region (UTR) and tRNA-like structure (TLS) are indicated accordingly. TTTSTTT are the last 7 amino acids in the C-terminal of HLSV CP and the last T, TSTTT and TTTSTTT are deleted in the corresponding constructs and the black dots (•) in the rectangles represent the deleted amino acids. The last amino acid T in TTTSTTT were substituted into alanine (A) and valine (V). The original codon ACG of two underlined amino acids **T** was changed to ACA to alter the RNA structure, without changing the original amino acid.

transfected with HLSV-22A-TTTSTTA reached a similar level as that of HLSV-22A (Fig. 2A) and viral RNA was not detected in leaves inoculated with HLSV-22A and HLSV-22A-TTTSTTA at 10 dpi (Figs. 2B, 2C), which indicated that substitution of amino acids in TTTSTTT motif did not increase virus replication.

To further test this hypothesis in HLSV with a longer internal poly (A) tract, HLSV-87A-TTTSTTT with its last amino acid T of the CP substituted with an alanine (A) or a valine (V), to become HLSV-87A-TTTSTTA and HLSV-87A-TTTSTTV, respectively, were constructed. Substitution with a Val would have been a more informative comparison, as structurally it is closer to Thr - only having a methyl substituted for a hydroxyl in its R-group (Fig. 5A). After inoculation in *N. benthamiana*, the viral RNA accumulation of HLSV-87A-TTTSTTA and HLSV-87A-TTTSTTV were tested to be similar to that of wild type HLSV-87A (Fig. 5B). It implied that substitution of amino acids in TTTSTTT motif would not enhance virus replication.

Deletion but not substitution of the C-terminal amino acid resulted in a higher replication level of HLSV RNA and detectable level of CP accumulation. It is possible that deletion of the C-terminal amino acids leads to RNA structural changes in their coding sequence which results in faster RNA replication. To test this hypothesis, online software was used to predict the RNA structure in HLSV-CP TTTSTTT motif area (Mathews et al., 2004). Fragment of HLSV from 6218 nt to 6296 nt showed a hairpin with an internal loop close to the internal poly(A) tract (Fig. 5C). The internal loop comprised of 8 nucleotides (boxed) which was not changed in HLSV-TTTSTTA (Fig. 5C).

To further investigate whether the RNA structure or the C-terminal amino acid(s) of CP regulates HLSV RNA replication, a silent mutant, termed as HLSV-22A-mmSL with its coding nucleotide sequence ACG for the third and the last amino acids changed to ACA, was constructed (Fig. 1). According to RNA structure prediction, the internal loop composed of 8 nucleotides (boxed) in HLSV-22A is unable to form in HLSV-22A-mmSL, due to nucleotide sequence substitution (Fig. 5C). The synonymous nucleotide substitution does not alter the amino acid sequence. Results showed that viral RNA and CP of HLSV-22A-mmSL were not detected in inoculated leaves, which was the same as that of HLSV-22A (Fig. 5D). HLSV-22A-mmSL presented a different predicted RNA structure from that of HLSV-22A, while keeping the same amino acids in the TTTSTTT motif of HLSV-CP. There was no viral RNA accumulation in leaves inoculated with HLSV-22A-mmSL. Moreover, a silent mutant HLSV-87A-mmSL was constructed, which the third and the last amino acid (T) in TTTSTTT motif were kept unchanged but its

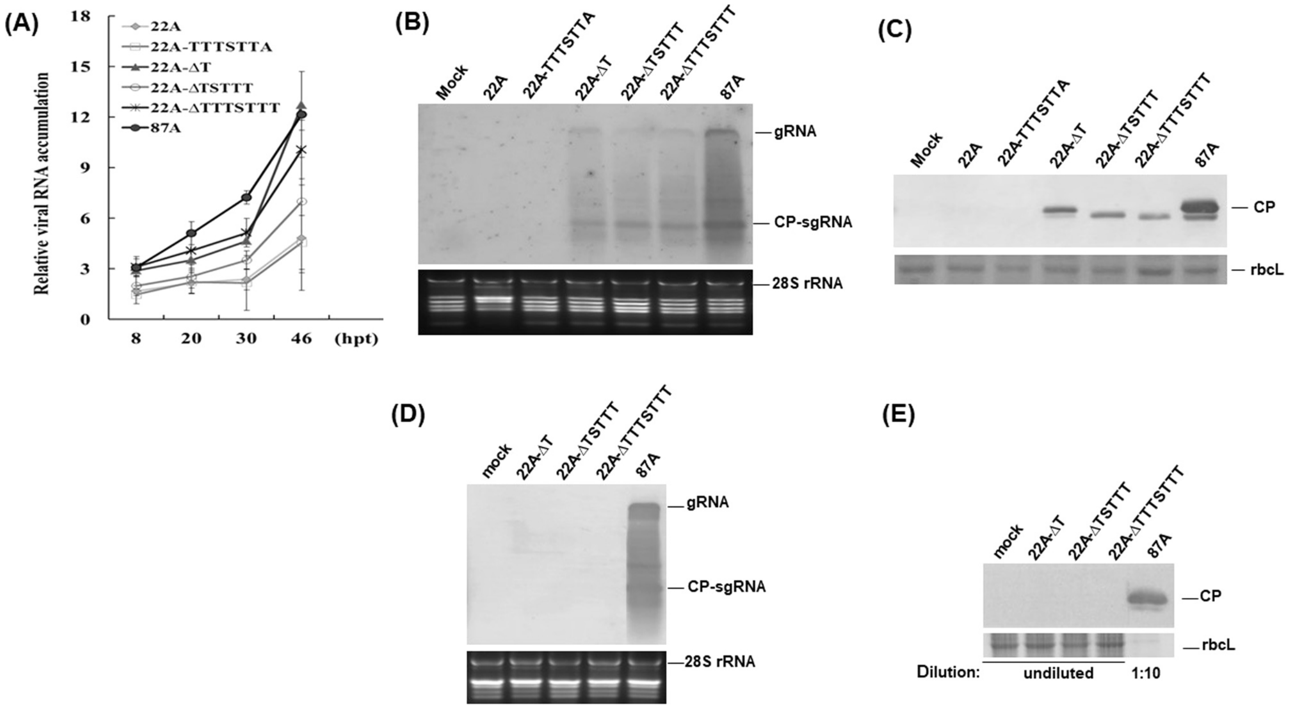

Fig. 2. Detection of HLSV RNA, CP accumulation in *N. benthamiana* infected with HLSV-22A CP mutants. (A) Quantification of viral RNA accumulation in transfected protoplasts at 8, 20, 30 and 46 h post transfection (hpt). *In vitro* transcripts (5 μg for each transfection) derived from pHLSV-22A, -22A-TTTSTTA, -22A-ΔT, -22A-ΔTSTTT, -22A-ΔTTTSTTT and -87A were transfected into protoplasts (8×10^5) in triplicates. Protoplasts were collected at each of the time points, followed by qRT-PCR. (B) Northern blot of HLSV RNA accumulation in inoculated leaves. *In vitro* transcripts (2.5 μg in 10 μl inoculation buffer for each leaf) derived from the above-mentioned constructs were inoculated onto *N. benthamiana* leaves. At 10 dpi, the inoculated leaves were collected for total RNA extraction and viral RNA detection. (C) Leaf samples collected on the same days were used for HLSV CP detection by western blot. (D) Northern blot of HLSV RNA accumulation in upper leaves. At 28 dpi, upper leaves were collected for total RNA extraction and viral RNA detection. (E) Leaf samples collected on the same days were used for HLSV CP detection.

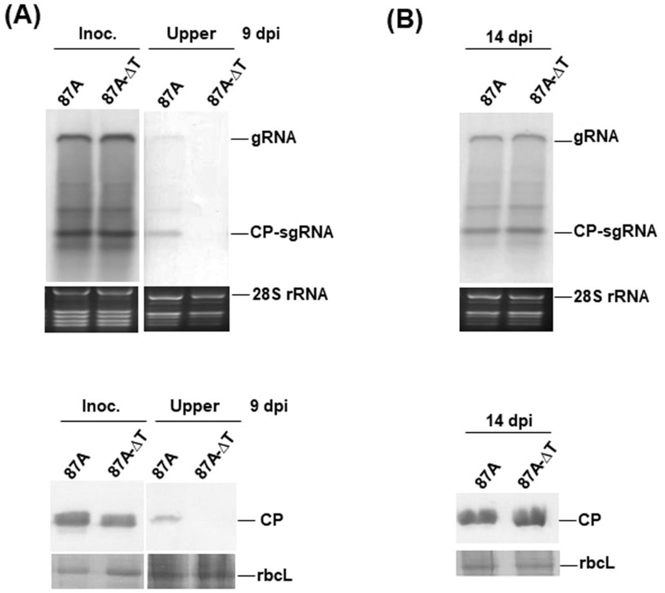

Fig. 3. (A) Viral RNA accumulation and CP expression of HLSV-87A and HLSV-87A-ΔT in inoculated and upper leaves at 9 dpi, respectively. (B) Viral RNA accumulation and CP expression of HLSV-87A and HLSV-87A-ΔT in upper leaves at 14 dpi.

coding nucleotide sequence ACG was changed to ACA, which was the same as that of HLSV-22A-mmSL, the predicted RNA structure of HLSV-87A-mmSL was different from that of HLSV-87A. However, after inoculation in *N. benthamiana*, the viral RNA accumulation of HLSV-87A-mmSL was found to be similar to that of HLSV-87A (Fig. 5B). Although the predicted viral RNA structure was changed, the viral RNAs of HLSV-22A-mmSL and HLSV-87A-mmSL showed no differences with that of wild type. These results indicated that variation of RNA structure in TTTSTTT motif area did not affect virus replication, but amino acid(s) deletion in TTTSTTT motif could increase virus replication.

3. Discussion

3.1. Deletion of amino acid(s) in TTTSTTT motif of HLSV CP facilitates virus replication and lengthens IPAT

Positive-sense single-stranded plant RNA viruses use diverse strategies for their replication and movement. In this study, we discovered that deletion, but not substitution of the C-terminal amino acid(s) in the TTTSTTT motif of HLSV CP facilitates virus replication and CP expression.

Increasing evidence shows that viral CPs are involved in a variety of interactions with host proteins to modify the cellular environment so that selectively benefits viral infection. *Potato virus X* CP interacts with the ER-associated protein NbPCIP1, as a susceptible factor for viral infection in *N. benthamiana* plants (Park et al., 2009). PVX CP also interacts with host protein plastocyanin. Downregulation of plastocyanin decreased PVX accumulation and reduced symptoms severity in infected plants (Qiao et al., 2009). BMV CP binding to oxidoreductase HCP1 controls BMV infection in barley (Okinaka et al., 2003). TMV CP is dispensable for virus replication, but CP facilitates the formation of viral replication complex and increases its size, which promotes virus replication (Asurmendi et al., 2004). All these studies show that CP is important for virus replication in host plants.

For HLSV, if there were interactions between host factors and viral CP, deletion of CP would negatively affect such interaction. However, in HLSV-22A, CP-deletion mutants HLSV-22A-ΔT, HLSV-22A-ΔTSTTT

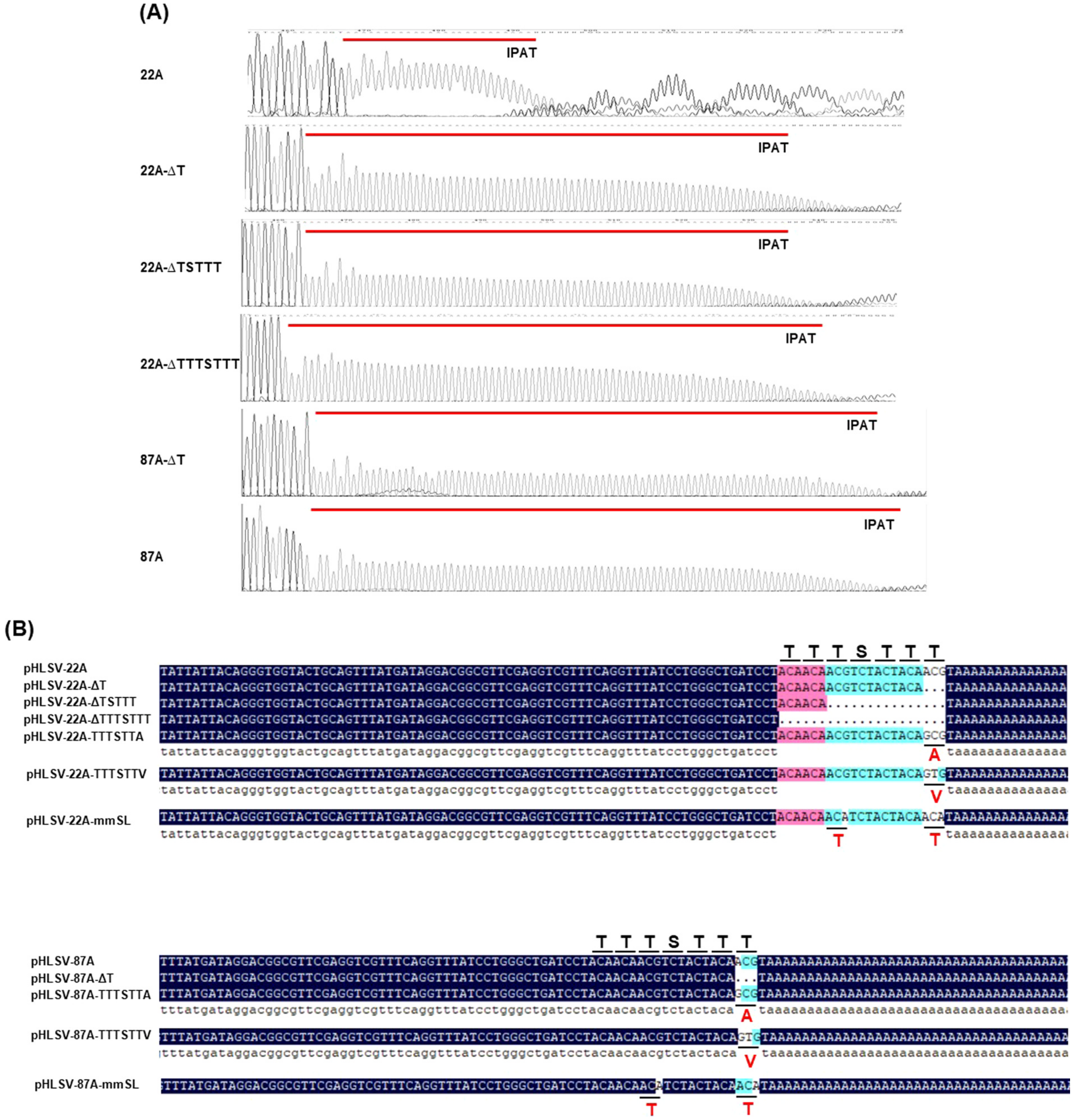

Fig. 4. (A) DNA sequences of the internal poly(A) tract (IPAT) extension in leaves inoculated with HLSV-22A CP deletion mutants. (B) Analysis of HLSV viral sequences to show that there was no CP sequence changes and recovery in progeny viruses of those mutants.

and HLSV-22A-ΔTTTSTTT showed higher viral RNA accumulation and enabled virus CP expression, as compared with that of HLSV-22A which has no CP expression due to its short IPAT. This indicated that for HLSV-22A, deletion of amino acid(s) in the TTTSTTT motif of CP can enhance viral RNA replication due to its short IPAT. However, the efficiency of enhanced viral RNA replication diminishes with the lengthening of the IPAT in its viral genome. As compared with viral RNA and CP expression of HLSV-87A and HLSV-87A-ΔT in inoculated leaves, there was no significant difference at 9 dpi.

HLSV-22A-mmSL and HLSV-87A-mmSL, these two silence mutants were predicted to have altered viral RNA structure, while maintaining the same amino acids in the TTTSTTT motif, did not exhibit any enhanced viral RNA replication. It is therefore concluded that deletion of the amino acid(s) in the TTTSTTT motif, but not alteration in RNA structure, led to enhanced HLSV replication.

Viral RNA structures, particularly the 5' and 3' UTR structures and their long-range RNA-RNA interaction, are important to virus replication (Archer et al., 2013; Chen et al., 2012; Fabian and White, 2004; Guo et al., 2001; Niu et al., 2015; Rakotondrafara et al., 2006; Shen and Miller, 2004). PVX CP was reported to bind to a host protein NbDnaJ, which specifically recognizes a stem-loop structure in the 5' UTR of PVX RNA, is essential for viral replication (Cho et al., 2012). Same as these silence mutants, HLSV-22A-TTTSTTA, HLSV-87A-TTTSTTA and HLSV-87A-TTTSTTV, these amino acids substitution mutants showed similar viral RNA accumulation as that of wild type virus, which indicated that the substitution in TTTSTTT motif did not enhance virus replication. Deletion but not substitution of amino acids in the TTTSTTT motif of HLSV-CP enhanced virus replication. The dimension of CP structure would be altered after one or more amino acids are deleted. The truncated HLSV CP in these deletion mutants might contribute to enhanced virus replication. The truncated HLSV CP may allow it to recruit host factors that can fit into the space of virus replication complex

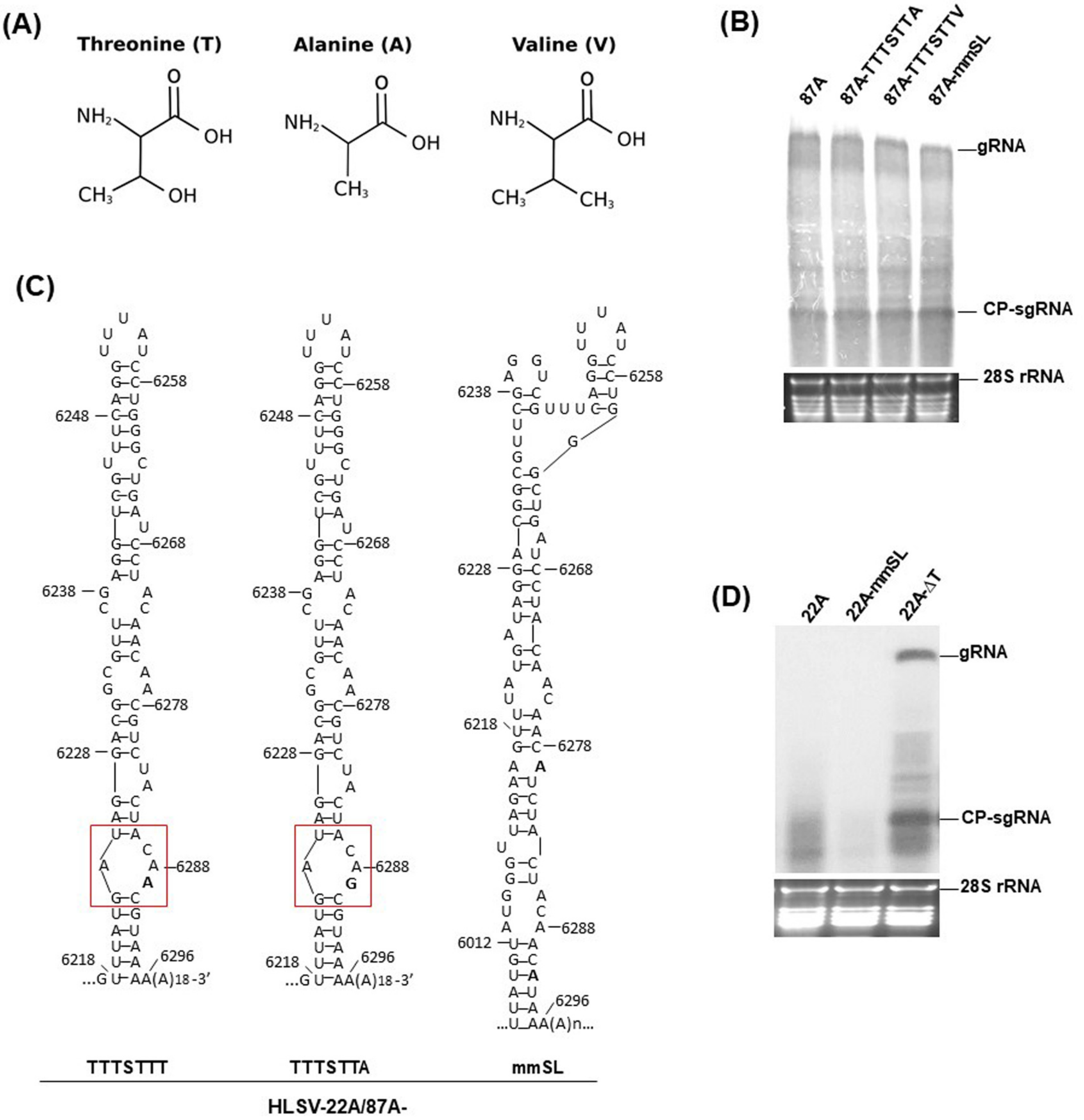

Fig. 5. (A) Amino acid structures of Threonine, Alanine and Valine. (B) Viral RNA accumulation of wild type HLSV-87A and mutants HLSV-87A-TTTSTTA, HLSV-87A-TTTSTTV and HLSV-87A-mmSL in inoculated *N. benthamiana*. (C) Computer predicted RNA structures. Sequences of CP gene and 22 nt IPAT in HLSV and its mutants were used for RNA secondary structure prediction by using software RNAstructure, (Version 5.7). Only partial structure related to the C-terminus coding sequences is shown. Bold G represents the mutated nucleotide in pHLSV-22A/87A-TTTSTTA, in which the last amino acid threonine was replaced with alanine by mutating the codon ACG to GCG. Symbol (…) represents 5' HLSV CP coding sequence. The third and the last amino acid codon, ACG, in the TTTSTTT motif was changed to ACA in pHLSV-22A/87A-mmSL, resulting in disappearance of the last loop structure (boxed). (D) Viral RNA accumulation of pHLSV-22A, -22A-mmSL and -22A-ΔT in inoculated leaves at 13 dpi.

(VRC). In addition, after deletion of the amino acids in the TTTSTTT motif of HLSV CP, the length of IPAT in the mutant virus extended significantly faster than that of HLSV-22A. This is consistent with our earlier results which showed that IPAT is important to HLSV replication and longer IPAT replicates faster than that with shorter IPAT. Therefore, deletion of amino acids in the TTTSTTT motif probably enhanced HLSV replication through an unknown mechanism. The modification of CP increased virus replication, this in turn allowed for increased opportunity for recovery of longer internal poly(A) tract. The resulting progeny would then be more efficient at translation which in turn would further enhance genome replication efficiency.

3.2. Deletion of amino acids in the TTTSTTT motif of HLSV CP hinders virus systemic movement

For systemic infection, plant viruses move from *meso*phyll cells to the vasculature and then move to distant sites. There are several cellular barriers between *meso*phyll layers and vasculatures such as bundle sheath, vascular parenchyma and companion cells, and viruses must cross these barriers into sieve elements (Hipper et al., 2013). This crossing requires compatible interactions between virus and plant host factor(s). Proper unloading is also important for virus systemic infection as virus loading into sieve elements occurs in all vein classes, while unloading only occurs in main veins, indicating that different mechanisms exist in virus loading and unloading from the phloem (Cheng et al., 2000; Roberts et al., 1997; Silva et al., 2002). Host protein pectin methylesterases have been reported to facilitate TMV long-distance movement, possibly through enhancing unloading of the virus to sink tissue (Chen and Citovsky, 2003). Another host protein CP-Interaction Protein-L has been reported to assist ToMV long-distance movement through interacting with its CP (Li et al., 2005). In our study, deletion of the last C-terminal amino acid in TTTSTTT motif of HLSV CP delayed its systemic movement. The C-terminal amino acids of HLSV CP are located at the exterior of the virion (Fig. 1A). Therefore, it is reasonable to believe that it does not affect virus assembly. However, it is noted that the C-terminus of HLSV CP has a beta sheet fold, which is different from other rod-shaped viruses including TMV, *Cucumber green mottle mosaic virus* (CGMMV) and *Ribgrass mosaic virus* (RMV). The C-terminus of their CPs is all unstructured loops (Supplementary Fig. 1). Structure of a protein plays a major role in protein-protein interactions. We hypothesize that deletion of the last amino acid of HLSV CP results in weakening of the interaction between CP and host factor(s) which is important for virus loading and/or unloading, resulting in delayed long-distance virus movement. Identification of host factor(s) interacting with the C-terminal amino acids of HLSV CP will be carried out in further studies.

Deletion of amino acid in TTTSTTT motif increased replication of HLSV-22A in inoculation leaves, as HLSV-22A-ΔT was detected but HLSV-22A was not. However, HLSV-22A-ΔT, HLSV-22A-ΔTSTTT and HLSV-22A-ΔTTTSTTT were not detectable in upper leaves at 28 dpi. In the upper leaves of HLSV-87A and HLSV-87A-ΔT-inoculated plants, HLSV-87A-ΔT was detected later than the wild type virus. The TTTSTTT motif in HLSV CP is shown to be correlated with virus replication. We also showed that deletion of amino acid in TTTSTTT delayed virus systemic movement. Deletion of amino acid(s) in TTTSTTT motif facilitates extension of internal poly(A) tract which enhanced virus replication. However, how the TTTSTTT motif influences viral systemic movement is unclear and needs further investigation.

Alignment of a total of 31 CP amino acids sequences of Tobamoviruses available online showed that only HLSV possesses the TTTSTTT motif (Table S1). It is located at the C-terminus of the CP which protrudes out from its virion as a symmetrical hook (Fig. 1A, boxed). Threonine and serine residues are potential glycosylation and phosphorylation sites. In *Plum pox virus*, glycosylation of the N-terminal amino acids of its CP facilitates virus infection slightly (Chen et al., 2005). The TTTSTTT motif is also found in the *Simian immunodeficiency virus* envelope in macaques during infection and is thought to create new sites for *o*-linked glycosylation to escape host immunity (Overbaugh and Rudensey, 1992). However, the motif is not found in *Human immunodeficiency virus* envelope, although both threonine- and serine-rich sequences have been reported (Wang et al., 1995). It is possible that glycosylation of the amino acids in the TTTSTTT motif of HLSV CP enhances its interaction with host factor(s) which in turn increases virus loading and/or unloading. The functional role of symmetry of the threonine residues in the TTTSTTT motif of HLSV CP needs further investigation. A related tobamovirus, HLFPV, which also possesses an IPAT in its genome, does not possess the TTTSTTT motif in its CP. *Obuda pepper virus* possesses a similar WTTTTTT sequence at the C-terminal of its CP but its function is unknown (Table S1). Recently the Namba Group in Japan reported a HLSV-J isolate which has only 86.7% nucleotide identity to HLSV Singapore isolate, but the amino acids identity is more than 95% (Yoshida et al., 2018). Interestingly, the C-terminal amino acids sequence of HLSV-J is SATSTTT. This sequence motif resemblance suggests that the last few threonine-rich amino acids that are present in HLSV and OPV may serve similar functions in virus-host interactions. During co-evolution of plant viruses and their hosts, both pathogens and hosts develop strategies to attack or defend each other to survive. Virus fitness in plants does not always correlate with its enhanced ability to replicate (Zhang et al., 2004). Perhaps the length of IPAT of HLSV regulates replication, while the C-terminal amino acids of its CP facilitates long-distance movement through some unknown mechanism(s).

In conclusion, deletion but not substitution of amino acids in TTTSTTT motif of HLSV-CP enhanced virus replication in virus, but with delayed systemic movement.

4. Materials and methods

4.1. Construction of HLSV mutants

All primers and HLSV constructs used in this study are listed in Table S1 and Fig. 1, respectively. Quick Change mutagenesis (Stratagene, USA) strategy was used for all plasmid construction. Using pHLSV-22A as template, primer pairs HL-F24M and HL-R19M were used for construction of pHLSV-22A-ΔT, in which the last amino acid of CP was deleted. The same primers were used for construction of pHLSV-87A-ΔT by using pHLSV-87A as the template. Similarly, pHLSV-22A-ΔTSTTT, pHLSV-22A-TTTSTTA and pHLSV-22A-ΔTTTSTTT and pHLSV-22A-mmSL were constructed with relevant primers using pHLSV-22A as template and verified by DNA sequencing. pHLSV-87A-TTTSTTA, pHLSV-87A-TTTSTTV and pHLSV-87A-mmSL were constructed using pHLSV-87A as template with relevant primers (Table S2).

4.2. Transfection of protoplasts and inoculation of plants with HLSV and its mutants

Full-length cDNA clones pHLSV-87A and its mutants were linearized with *Xho*I, followed by phenol/chloroform extraction and ethanol precipitation. Purified DNA was used for *in vitro* transcription using the T7mMessagemMachine kit (Life Technologies, Ambion). *In vitro* transcripts were purified by LiCl precipitation and used for transfection of *N. benthamiana* protoplasts as previously described (Niu et al., 2015) or for mechanical inoculation onto plants. Protoplasts were incubated and collected at different time points according to different experiments, followed by total RNA extraction using Trizol reagent (Life Technologies, Invitrogen). Five-leaf-stage *N. benthamiana* plants were inoculated with the respective *in vitro* transcripts and total RNA was extracted from leaves of test plants.

4.3. Detection of viral RNA accumulation by Northern blot and qRT-PCR

Total RNA extracted from *N. benthamiana* protoplasts or leaves was resolved on a 1.2% TAE agarose gel and transferred onto a positively charged nylon membrane (Roche) using alkaline buffer (3 M NaCl, 0.1 N NaOH) for 90 min at room temperature by capillary action. The viral RNA was fixed using a crosslinker UVC500 (Hoefer), followed by hybridization with a DIG-labeled HLSV CP gene probe, according to the manufacturer's instructions (Roche). To quantify viral RNA accumulation in inoculated plants, total RNA from each of the triplicate samples of each treatment was extracted and reverse transcribed by using Superscript III Reverse TranscrIPATse (Life Technologies, Invitrogen) using primers HL-R8 and actin-2. Quantitative RT-PCR was performed in triplicates with KAPA SYBR on the CFX384 Real-Time PCR system (Bio-Rad) with primers HL-F9 and HL-R9, using *actin* as an internal control.

4.4. Detection of HLSV CP by Western blot

N. benthamiana leaves (0.1 g) of test plants were ground in 100 µl chilled protein extraction buffer (220 mM Tris-HCl, pH 7.4, 1 mM $MgCl_2$, 250 mM sucrose, 50 mMKCl), followed by centrifugation to remove tissue debris. Total proteins were separated on 15% SDS-PAGE gels, and transferred onto a nitrocellulose membrane (Bio-Rad), using a mini trans-blot cell apparatus (Bio-Rad). Western blot was performed using a polyclonal antiserum specific to HLSV (Srinivasan et al., 2002) and a secondary antibody with goat anti-rabbit IgG conjugated with alkaline phosphatase (Sigma-Aldrich). Bands were visualized by colorimetric detection using NBT/BCIP as substrates (Thermo Scientific Fermentas).

4.5. Analysis of HLSV viral sequences

For analysis of viral genomic sequence after replication, total RNA isolated from upper leaves of infected *N. benthamiana* at 9 dpi was used for reverse transcription with Primer HL-R using SuperScript III Reverse Transcriptase kit (Invitrogen). DNA fragment were amplified with Primer HL-F and HL-R for DNA sequencing using Applied Biosystems 3100 xl DNA analyzer. The sequence was aligned using DNAMAN software. Three independent experiments were performed to determine that the mutant sequence was not reverted or altered.

Acknowledgements

This work was supported by Ministry of Education Academic Research grant R-154-000-A34-114 through National University of Singapore. We thank Emeritus Professor Robert J. McGovern, University of Florida, USA for editing the manuscript. Molecular graphics and analyses were performed with the UCSF Chimera package. Chimera was developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California at San Francisco, USA.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.virol.2018.09.027.

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