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Viral sequences required for efficient viral infection differ between two Chinese pepper mild mottle virus isolates

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ABSTRACT

Pepper mild mottle virus (PMMoV) causes mosaic symptoms and malformation on both leaf and fruit of pepper, reduces considerable economical yields and poses threats to human health. In this study, infectious clone of PMMoV Huludao (HLD) isolate (pCB-PMMoV-HLD) was constructed and its infectious ablility in *Nicotiana benthamiana* was confirmed by virions observation and Northern blot analysis. The mutant PMMoV (HLD-fsCP) that cannot express coat protein (CP) showed reduced viral accumulation but can systemically infect *N. benthamiana*. We constructed several chimeric mutant viruses (ZA-HB-HC, HA-ZB-HC, HA-HB-ZC and HA-ZB-ZC) by sequences substitution between PMMoV-HLD and PMMoV Zhejiang isolates (PMMoV-ZJ) and analyzed their infectious abilities in *N. benthamiana* and *Capsicum annuum*. The results showed that the chimera virus expressed by pCB-ZA-HB-HC, pCB-HA-HB-ZC and pCB-HA-ZB-ZC, but not by pCB-HA-ZB-HC, exhibited reduced infectious ability compared with wild-type PMMoV-ZJ and PMMoV-HLD, which indicated that RNA sequences required for efficient infection of PMMoV differ between the two virus isolates. The differential requirement of viral RNA sequences for efficient PMMoV infection provided theoretical value to further understand the infection and pathogenesis of PMMoV.

1. Introduction

Hot pepper (Capsicum spp.) was the third largest vegetable crop in the world (Kim et al., 2018). Pepper mild mottle virus (PMMoV) infecting pepper causes severe mosaic mottling and shriveled symptoms on leaves and fruits malformation, which results in considerable economic losses in pepper production and poses threats to human health, worldwide (Peng et al., 2015; Shirasaki et al., 2018; Colson et al., 2010; Haramoto et al., 2013). The virions of PMMoV have been found to be abundant in pepper sauce (Peng et al., 2015), drinking water (Haramoto et al., 2013) and is proposed as an indicator of fecal pollution in surface water (Shirasaki et al., 2018; Symonds et al., 2016; Shrestha et al., 2018). Studies have also shown an association between the presence of PMMoV in feces and specific immune response, suggesting that PMMoV may have pathogenic effect on human health (Colson et al., 2010). The impact of PMMoV on the ecological environment and food safety is increasing as the virions of PMMoV are considerably stable in contaminated seeds, plant debris, soil and surface water (Ikegashira et al., 2004; Shrestha et al., 2018; Genda et al., 2005).

The species *Pepper mild mottle virus* belongs to the genus *Tobamovirus* in the family *Virgaviridae* and has rod-shaped virions (Adams et al., 2009). The genomic RNA of PMMoV is composed of 6356–6357 nucleotides (nts) and encodes at least four open reading frames (ORFs), including the 126 kDa and 183 kDa proteins required for genome replication that are translated via genomic RNA. The 126 kDa protein possesses methyltransferase and helicase activities and functions as a suppressor of RNA silencing (Tsuda et al., 2007; Souza et al., 2013). PMMoV also encodes a 30 kDa movement protein (MP) and a 17 kDa coat protein (CP) that are translated via subgenomic RNAs (Alonso et al., 1991; Mizumoto et al., 2014; Han et al., 2017).

We previously sequenced the complete genome nucleic acids of PMMoV-HLD (accession number MG515725) collected from Liaoning province in the northeast China (Yu et al., 2018). It should be emphasized that the outburst of viral disease caused by PMMoV-HLD resulted in almost one-third yield losses on sweet pepper in greenhouses and fields in Huludao, Liaoning province (Li et al., 2016) and this significant incidence promoted us to conduct further experiments to reveal the infection and multiplication mechanisms of the virus.

Plant RNA viruses use elaborate ways to efficiently replicate their genomes in the hosts. The development of plant virus infectious clone

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provides important tools to reverse genetically study the infection mechanisms and pathogenicity of the virus by introducing sequences deletion, exogenous gene and chimeric mutation into the infectious clone (Brewer et al., 2018; Junqueira et al., 2014). Construction of chimera viruses containing heterologous sequences can be used for identifying the RNA sequences necessary for infection. In addition, development of virus infectious clones and introduction of the mutations using homologous recombination technology greatly shortens the construction procedure, which is more efficient compared with the traditional restriction enzyme-mediated subcloning methods (Tuo et al., 2015).

In this study, we constructed infectious clones for PMMoV-HLD and various PMMoV mutants using homologous recombination technique to reverse genetically identify the viral sequences and proteins that play crucial roles in the virus infection. These results indicated that RNA sequences required for efficient viral infection differ between PMMoV-HLD and PMMoV-ZJ according to the difference in symptomology and RNA accumulation between wild-type and the chimeric mutants of PMMoV.

2. Materials and methods

2.1. Viral sampling and growth of test plants

Peppers (*Capsicum. annuum* L.) infected by PMMoV-HLD were collected from Huludao city, Liaoning, China. Seeds of *N. benthamiana*, pepper cv. Zunla-1 (Qin et al., 2014) were sown in separate pots and were grown in a chamber set at 25 °C and 16 h/8 h (light/dark) conditions.

2.2. Sequence comparison and phylogenetic analysis

The complete sequence of PMMoV-HLD was determined as described previously (Yu et al., 2018). The BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to compare the homology of the PMMoV-HLD whole gene sequence with 18 other PMMoV isolates and two other tobamoviruses including tobacco mosaic virus (TMV, NC_001367) and cucumber green mottle mosaic virus (CGMMV, NC_001801). The phylogenetic tree was constructed by MEGA 7.0.14 according to the sequences of the above virus isolates to identify its phylogenetic relationship.

2.3. Construction of infectious clone for PMMoV-HLD and the mutants

pCB301 is a streamlined vector that is less than 1/2 the size of the parent vector pBIN19 and completely functional in transforming various model plants (Xiang et al., 1999). In this study, a pCB301 based plasmid pCB301-PMMoV-ZJ (MH574770) was used as a template and amplified with P-NOS + /PMV35S- using 2 × TransStart FastPfu PCR SuperMix (TransGen Biotech, Beijing, China) to generate approximate 4900 bp vector fragments. Thereafter, DMT enzymes (TransGen, Biotech, China) were used to digest the non-mutated parental plasmid. The cDNA of PMMoV-HLD was used as a template and amplified by primer pairs 35S-P + /2463- and 2481 + /NOS-P- by KOD Plus DNA polymerase (Toyobo, Osaka, Japan) to generate two DNA fragments that covered the complete genome of PMMoV-HLD. The amplified vector and the two PCR fragments were ligated to construct the infectious clone of pCB-PMMoV-HLD using a ClonExpressTM MultiS One Step Cloning Kit (Vazyme, Nanjing, China). The pCB-PMMoV-HLD contained 2 × 35S promoter at the upstream and a downstream hepatitis delta virus ribozyme together with a 35S terminator as well as that of the parental plasmid pCB-PMMoV-ZJ. To generate pCB-PMMoV-fsCP, pCB301-PMMoV-HLD was used as a template and amplified with primer pairs of PMMoVfsCP + /PMMoVfsCP- and the amplified PCR products were treated by DMT enzymes and subjected to self-ligation using ClonExpressTM MultiS One Step Cloning Kit (Vazyme, Nanjing, China). To construct chimeric PMMoV mutants pCB-ZA-HB-HC, pCB-HA-ZB-HC and pCB-HA-HB-ZC, the plasmid pCB-PMMoV-ZJ was used as a template and amplified by three primer pairs 35S-P + /2463-, 2481 + /4446- and 4415 + /NOS-P-, respectively, to generate three DNA fragments PA, PB and PC as inserts (Fig. 3). Thereafter, three vector DNA fragments were amplified with three primer pairs 2481 + /P-35S-, 4415 + /2463- and P-NOS + /4446-, respectively, using pCB301-PMMoV-HLD as a template (Fig. 3). Subsequently, ligation of the respective inserts and vectors were conducted using the ClonExpressTM MultiS One Step Cloning Kit (Vazyme, Nanjing, China). The same amplification and ligation strategy was used to construct pCB-HA-ZB-ZC (Fig. 3). The constructs were transformed into *A. tumefaciens* GV3101 and infiltrated into leaves of 25 °C cultivated 30 days old *N. benthamiana* for the subsequent analysis. Primers used for PCR to generate constructs were listed in Table 1.

Table 1
Nucleic acid sequences of oligonucleotide primers used to generate constructs.

Primer name	Sequence of primer
35S-P +	CATTTGGAGAGGGGTAAATTTTTCACAATTTAAC
P-35S-	TGAAAAATTTACCCCTCTCCAAATGAAATGAAC
2481 +	TGAGCTCCGAATCTTTGGTG
2463-	ACACCAAAGATTCGGAGCTC
4415 +	TGCATGTCTGTCCTCTATGC
4446-	TAGCATAGAGGACAGACATGC
P-NOS +	GTAGCGGCCCAGGGTCGGCATGGCATCTCC
NOS-P-	CATGCCGACCCTGGGCCGCTACCCGCGGTTC
FsCP +	TTTTAACTACGGCTTACACAGTTTCCAG
FsCP-	GTGTAAGCCGTAGTTAAAACGAAGAAGAC

2.4. Northern blot and statistical analysis

In Northern blot analysis, total RNAs extracted from *N. benthamiana* leaves inoculated with *A. tumefaciens* carrying above constructs together with upper un-inoculated leaves were separated in a 1.5% agarose gel containing formaldehyde and MOPS and subsequently transferred to Nylon membrane Amersham Hybond-N + using siphon method. The digoxigenin-labelled positive sense RNA detection probe corresponding to the CP and 3′ untranslated region (UTR) of PMMoV was establishment (data not shown). The RNA probe and a DIG Northern Starter Kit (Roche Mannheim, Germany) were used following manufacturer's instructions in the Northern blot analysis. Specific bands of positive-strand genome RNAs and subgenome RNAs of PMMoV were visualized by using Chemical luminous imaging system Tanon 5200 (Tanon, Shanghai, China). Northern blot analysis was performed at least three times and relative accumulation of PMMoV gRNA in inoculated leaves and upper leaves of *N. benthamiana* was shown. The statistical analysis was carried out with SPSS 13.0 (SPSS Inc. Chicago. IL) and different letters indicate significant differences in viral RNA accumulation among the wild-type PMMoV and the mutants ($P < 0.05$). The data comparison was tested with Duncan's new multiple range, one-way ANOVAs (n = 3).

2.5. Virions purification and electron microscopy observation

Virions extraction and purification from infectious clones inoculated *N. benthamiana* leaves were performed as described (Gooding et al., 1967). The purified virions concentration were measured by using a Nano-Drop TM 1000 spectrophotometer (Fisher Scientific, Pittsburgh, USA) and adjusted to the final concentration of 2 mg/ml. The purified PMMoV virions were treated by using phosphotungstic acid stain method (Quintarelli et al., 1971) and observed using a transmission electron microscopy HT7700 Exalens (Hitachi, Ltd, Tokyo, Japan).

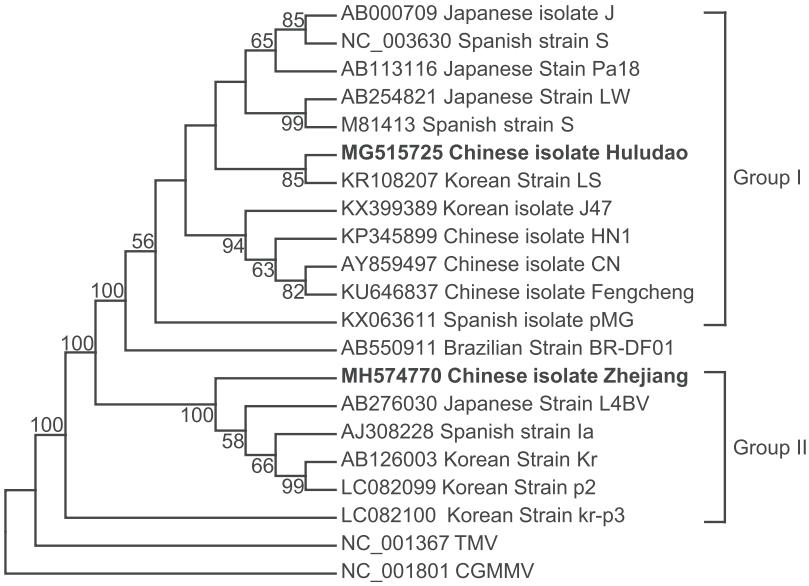

Fig. 1. Phylogenetic trees produced by MEGA7 based on the nucleotide sequences of PMMoV Huludao (HLD) isolate, PMMoV Zhejiang (ZJ) isolate and multiple PMMoV isolates together with TMV and CGMMV using the Maximum Likelihood method. Numbers at the nodes of the branches represent percentage bootstrap values (1000 replicates).

3. Results

3.1. Sequencing and phylogenetic analysis of PMMoV-HLD

In this study, the complete sequence of PMMoV-HLD (MG515725) and PMMoV-ZJ (MH574770) together with other 16 PMMoV isolates and other two tobamovirus isolates were subjected to sequence alignment and phylogenetic analysis (Fig. 1). The results of phylogenetic analysis indicated that all of the tested PMMoV isolates can be divided into two major groups, group I and group II. The results indicated that PMMoV-HLD was most closely related with PMMoV Korean isolate (KR108207) with 99.7% sequence identity. Results of phylogenetic analysis showed that PMMoV-HLD closely clustered with PMMoV Korean isolate (KR108207), Spanish isolate (M81413), Japanese isolate (AB254821, AB113116), Spanish isolate (NC_003630), Hunan isolate (KP345899), Fengcheng isolate (KU646837), Korean isolate (KX399389), Spanish isolate (KX063611) and Brazilian isolate (AB550911) in group I. It should be noted that PMMoV-HLD is comparatively distantly related to PMMoV-ZJ (MH574770) that clustered with Japanese isolate (AB276030), Spanish isolate (AJ308228) and Korean isolate (AB126003, LC082099, LC082100) in group II. Sequences of other tobamoviruses including TMV (NC_001367) and CGMMV (NC_001801) were used as outgroups (Fig. 1).

Comparison of sequences between PMMoV-HLD (MG515725) and PMMoV-ZJ (MH574770) indicated 97.4% overall nucleotide identity. The differences in nucleotide sequences resulted in 99.46%, 99.38%, 98.45% and 100% amino acid identities in the 126 kDa, 183 kDa, MP, and CP respectively. Fourteen amino acid differences were detected between PMMoV-HLD and PMMoV-ZJ on the full-length amino acid sequences, with ten differences in the 126 kDa and 183 kDa protein, including C166 V, T491 A, T627 A, N663S, L759Q, R868 K, S1173 N, V1231I, L1252 F, T1350S. Four amino acid residues A19 T, K136 N, L231 V and G235R differed in the MP coding region, but amino acids were identical between the two PMMoV isolates in the CP coding region (Fig. S1).

3.2. Construction of infectious clone for PMMoV-HLD

pCB-PMMoV-HLD was constructed by integrating complete nucleotide sequences of PMMoV-HLD into a binary vector pCB301 and confirmed by sequencing analysis. To test the infectivity, *A. tumefaciens* strain GV3101 containing pCB-PMMoV-HLD, pCB-PMMoV-ZJ and an empty vector were infiltrated into 30 days old *N. benthamiana* leaves. Typical chlorosis and shriveled symptoms caused by PMMoV appeared on the upper leaves of *N. benthamiana* inoculated by pCB-PMMoV-HLD and pCB-PMMoV-ZJ at 7 days post inoculation (dpi) (Fig. 4 A and B). Results of Northern blot analysis showed that the viral accumulation level of the PMMoV-HLD from the un-inoculated upper leaf was as high as that of the PMMoV-ZJ (Fig. 2D). The results demonstrated that PMMoV-HLD expressed by the infectious clone is capable to systemically infect *N. benthamiana* (Fig. 2D). Thereafter, transmission electron microscopy observation was performed to confirm the presence of the PMMoV virions from pCB-PMMoV-HLD and pCB-PMMoV-ZJ inoculated *N. benthamiana* leaves (Fig. 5A and B). In addition, *A. tumefaciens* harboring the pCB-PMMoV-HLD and pCB-PMMoV-ZJ were also infiltrated into leaves of 20 *C. annuum* plants, respectively. The incidence of the PMMoV-HLD and PMMoV-ZJ were 8/20 and 10/20, respectively, and part of the detection results using dot-blot were shown (Fig. S2). Intriguingly, no visual symptoms were observed in all inoculated plants at 10 dpi (data not shown). Such results suggested that symptomology and the disease incidence caused by agro-infiltration of PMMoV on *C. annum* was much lower and unstable compared with mechanical inoculation of the virus extracted from PMMoV infected *N. benthamiana* leaves.

3.3. Analysis of the wild-type and mutant PMMoV clones in system infection

The genome sequences and their roles were assessed in the pathogenesis between PMMoV-ZJ and PMMoV-HLD. After the successful construction of infectious clone pCB-PMMoV-HLD, site mutation was introduced into pCB-PMMoV-HLD to construct pCB-HLD-fsCP that cannot express CP. We also used the two PMMoV isolates to form chimeras between PMMoV-ZJ and PMMoV-HLD. Based on the infectious clones of pCB-PMMoV-ZJ and pCB-PMMoV-HLD, we divided the complete PMMoV genome sequences into three parts (PA nt 1–2482, PB nt 2450–4466, PC nt 4395–6356) and constructed a series of chimeric virus that have sequences substitution between PMMoV-ZJ and PMMoV-HLD.

N. benthamiana plants inoculated with *Agrobacteriums* harboring pCB-HA-ZB－HC, as well as with pCB-PMMoV-HLD and pCB-PMMoV-ZJ demonstrated visible shriveled symptoms in upper non-inoculated leaves at 5 dpi (Fig. 4), thereafter the inoculated plants developed significant stunting after 6 dpi, whereas *N. benthamiana* inoculated with pCB-HLD-fsCP, pCB-HA-ZB-ZC, pCB-ZA-HB－HC, pCB-HA-HB-ZC and empty vector did not develop any observable symptoms (Fig. 4). Sap of

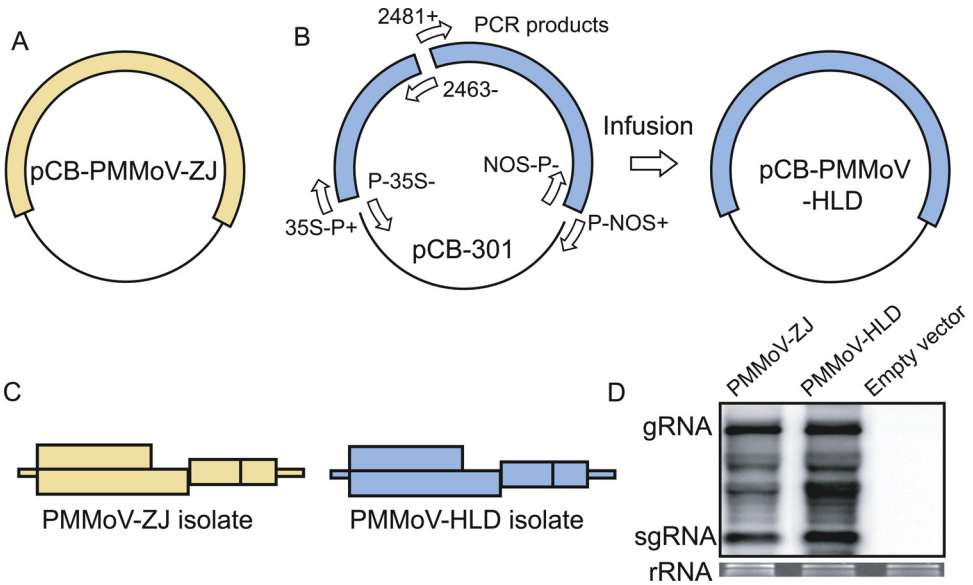

Fig. 2. Construction of PMMoV-HLD full-length infectious cDNA clone. (A) Schematic representation of the infectious clone pCB-PMMoV-ZJ. (B) Two DNA fragments overlapping the complete genome of PMMoV-HLD were amplified by primer pairs 35SPMV + /2463, 2481 + /Nos-P- and integrated into P-NOS + /P-35S- amplified pCB-301 vector to generate the pCB-PMMoV-HLD using homologous recombination technique. (C) Schematic representations of the genomic structure of PMMoV-ZJ and PMMoV-HLD; (D) Northern blot analysis of the viral genomic RNA (gRNA) and subgenomic RNA (sgRNA) accumulation from *N. benthamiana* inoculated with PMMoV-HLD, PMMoV-ZJ and an empty vector. Agarose gel electrophoresis of the ribosomal RNA was shown as a loading control in each lane.

the *N. benthamiana* leaves infected with the wild-type and the mutants of PMMoV were extracted and inoculated into leaves of *C. annuum*. The symptoms severity on the upper leaves developed by the wild-type and the mutants PMMoV on *C. annuum* (Fig. S4) was parallel with that on *N. benthamiana*.

Total RNA extracted from inoculated leaves and upper leaves of *N. benthamiana* were analyzed by Northern blot. The accumulation level of the viral genomic and subgenomic RNAs of HA-ZB – HC was as high as that of the wild-type PMMoV (Fig. 6). Thus the results suggested that the substitution of heterologous PB (nt 2450–4446) from PMMoV-ZJ did not affect the infection of mutant virus HA-ZB – HC in *N. benthamiana* plants. In contrast, the RNA accumulation levels of PMMoV mutants HLD-fsCP, HA-ZB-ZC, ZA-HB – HC and HA-HB-ZC were much lower compared with that of wild-type PMMoV (Fig. 6). The accumulation of PMMoV mutant ZA-HB – HC was barely detectable in Northern blot analysis (Fig. 6), but can be amplified in both of the inoculated leaves and upper leaves using RT-PCR (data not shown), which indicated that the mutant virus can induce systemic infection of the host plant, but differed in infectious ability.

To demonstrate if the chimeric mutation affected virions formation of PMMoV, transmission electron microscope was used to observe the virions morphology of the wild-type and mutants of PMMoV (Fig. 5). Rod shaped virions of PMMoV with approximately 300 nm in length can be observed from the leaf samples inoculated with wild-type PMMoV and the mutants except for HLD-fsCP and the empty vector control (Fig. 5). The results showed that there were no significant differences in the morphology of PMMoV virions formed by the wild-type and other mutant viruses, which indicated that the chimeric mutation do not affect the formation of the virions. It should be emphasized that the concentration of virions formed by PMMoV mutant HA-ZB-ZC and ZA-HB – HC were much lower compared with that of PMMoV-HLD and HA-ZB – HC. (data not shown).

4. Discussion

Nowadays, the liberalization of trade in agricultural products make it much easier for the spread of PMMoV through diseased pepper seeds and pepper sauce (Peng et al., 2015; Genda et al., 2005). In recent years, PMMoV has attracted more attention of researchers in the fields of plant pathology, food security, environmental science and clinical medicine (Shirasaki et al., 2018; Colson et al., 2010). Therefore, it is necessary to conduct further studies on the infection mechanism and

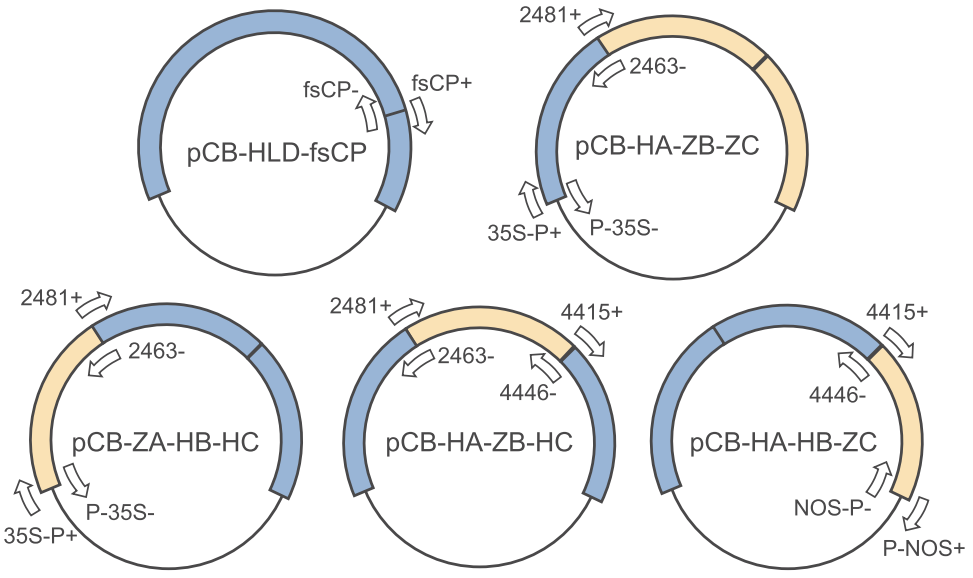

Fig. 3. Strategy for constructing PMMoV Chimeric mutants between PMMOV-HLD. pCB-HLD-fsCP was contructed by a nucleotide substitution at the start codon of CP ORF using primer pairs fsCP + /fsCP-. PCR amplification were performed using primers shown in arrows to construct pCB-HA-ZB-ZC, pCB-ZA-HB – HC, pCB-HA-ZB – HC and pCB-HA-HB-ZC. For others, refer to the legend of Fig. 2.

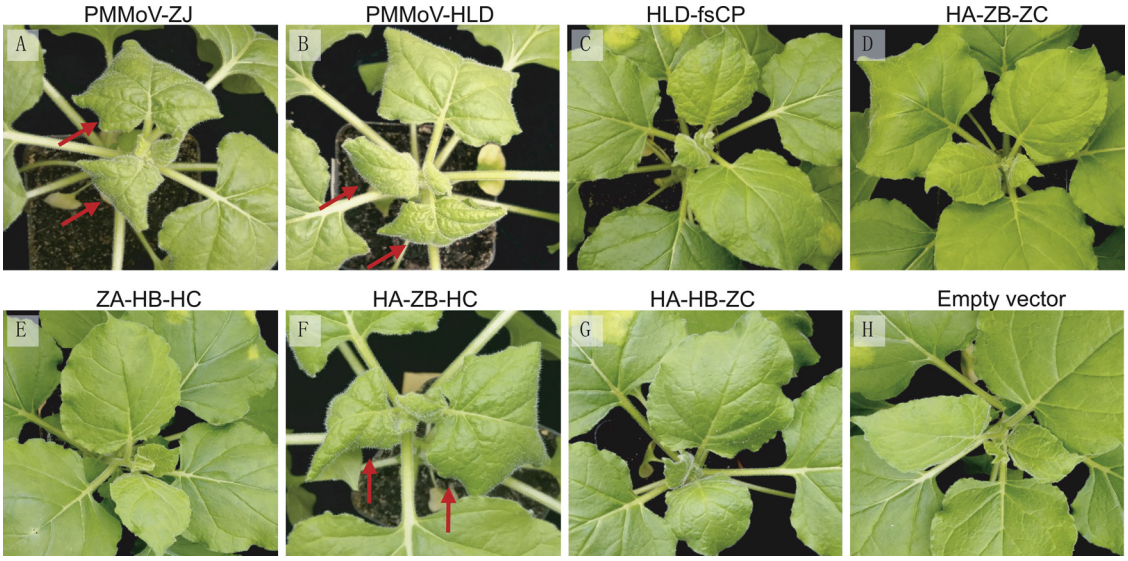

Fig. 4. Symptoms of *Nicotiana benthamiana* inoculated with *Agrobacteriums* haboring pCB-PMMoV-ZJ (A), pCB-PMMoV-HLD (B), pCB-HLD-fsCP (C), pCB-HA-ZB-ZC (D), pCB-ZA-HB－HC (E), pCB-HA-ZB－HC (F), pCB-HA-HB-ZC (G) and empty vector (H). Typical chlorosis and crinkle symptoms shown in upper un-inoculated leaves were annotated with red arrows.

pathogenicity of PMMoV.

In this study, we developed complete infectious clone of PMMoV Huludao isolate (pCB-PMMoV-HLD) using a streamlined vector based on homologous recombination technology. The virus clone expressed by *Agrobacteriums* harboring pCB-PMMoV-HLD was highly infectious to *N. benthamiana* as well as that of the PMMoV-ZJ. The successful construction of the infectious clone will allow the synthesis of mutagenized PMMoV RNAs by genetic manipulation on cDNA clones of pCB-PMMoV-HLD to reverse genetically study the interaction between PMMoV and the host plant. Moreover, the virus vector is expected to be modified to express efficient exotic protein in the hosts since the expressed viral RNA can move systemically. Modification of the PMMoV infectious clone into virus induced gene silencing (VIGS) vectors are also expected for their application in *C. annuum* and *N. benthamiana*.

The mutant virus expressed from pCB-HLD-fsCP cannot form virions (Fig. 5C) but can systemically infect *N. benthamiana*. To exclude the possibility for mutation recovery of HLD-fsCP, RNA extracted from upper un-inoculated leaves of 6 pCB-HLD-fsCP infiltrated *N. benthamiana* plants were reverse transcribed and subjected to sequencing analysis (Fig. S3). The results indicated that the introduced site mutation in the start codon of CP-ORF were well maintained and no other sequence mutation occurred in the virus after systemic infection (data not shown). We also confirmed systemic infection of HLD-fsCP in *C. annuum* by Northern blot (data not shown). Previous studies have shown that the CP is required for the systemic movement of tobamoviruses (Donson et al., 1991; Lindbo, 2007). CP is crucial for viral RNA encapsidation to form virions, which can protect the virus from nuclease degradation and RNA silencing of the host plants (Ivanov and Makinen, 2012). A previous study showed that CP of TMV plays an important role in the phloem dependent accumulation of the virus (Ding et al., 1996). In addition, it was reported that CP of brome mosaic virus (BMV) co-localizes with sites of viral replication in the cell, which allows encapsidation of newly synthesized viral RNA and its protection from nuclease degradation immediately after synthesis (Bamunusinghe

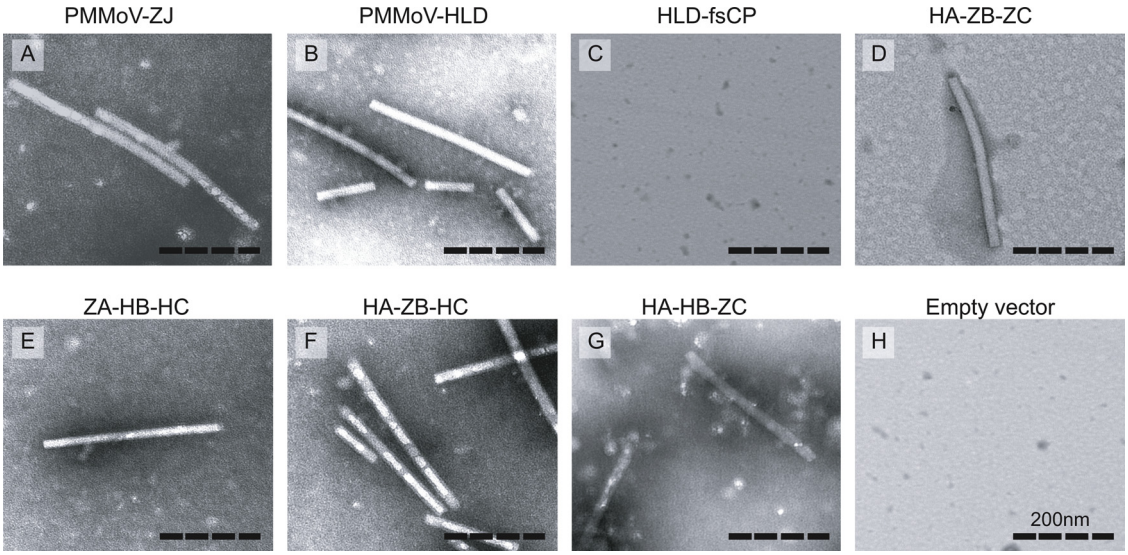

Fig. 5. Electron micrograph of viral particles in leaf homogenates *N. benthamiana* inoculated with wild-type PMMoV-HLD, PMMoV-ZJ and the mutants. Rod-shaped particles about 200 nm long were observed in PMMoV-ZJ (A) and PMMoV-HLD (B) and chimeric mutants (D, E, F and G) by transmission electron microscopy. No virions can be observed from samples infected with HLD-fsCP (C) and empty vector (J).

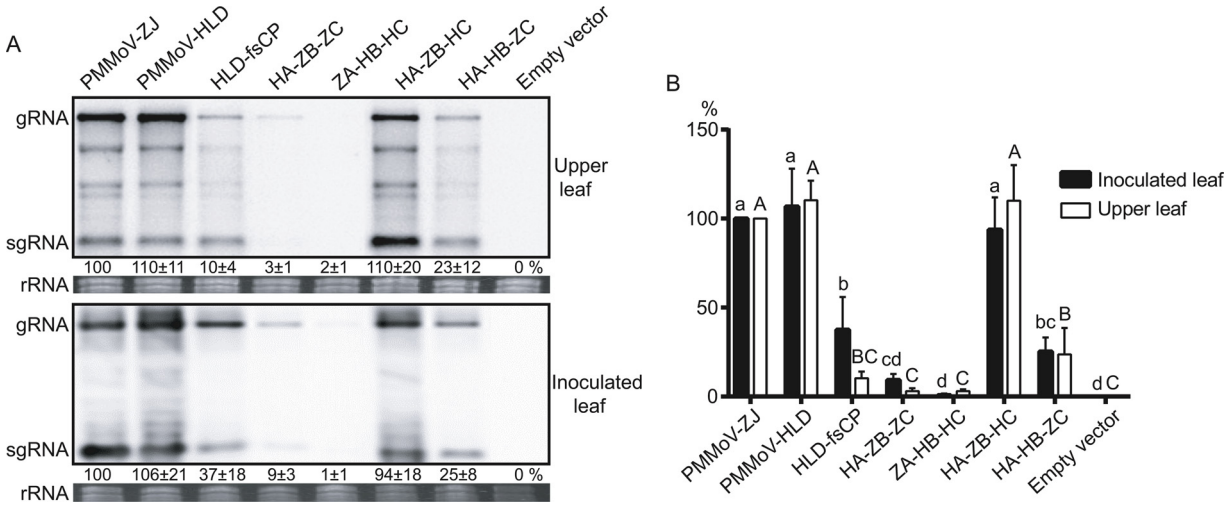

Fig. 6. Viral RNA accumulation in *N. benthamiana* inoculated with PMMoV-HLD, PMMoV-ZJ, PMMoV mutants (HLD-fsCP, HA-ZB-ZC, ZA-HB－HC, HA-ZB－HC and HA-HB-ZC) and empty vector in 6 dpi. (A) PMMoV gRNA and sgRNA were detected by Northern blot analysis from inoculated leaves and un-inoculated upper leaves of *N. benthamiana* and the ribosomal RNA is shown as a loading control in each lane. (B) Northern blot analysis was performed at least three times and relative accumulation of PMMoV gRNA in inoculated leaves (closed bar) and upper leaves (open bar) of *N. benthamiana* were shown. Different letters on column indicates the significant differences in viral RNA accumulation among the wild-type PMMoV and the mutants using SPASS with ANOVA procedure.

et al., 2011). Results of this study showed that CP is dispensable for systemic infection, as the PMMoV mutant virus HLD-fsCP can be detected in the non-inoculated upper leaves (Fig. 6A; Fig. S3), but played important roles in disease symptomology and viral RNA accumulation (Figs. 4C and 6 ; S4 C).

To accomplish efficient infection, plant viruses can switch between variable RNA conformations to efficiently translate viral proteins, replicate their genome RNA and accomplish virions assembly (Zhang et al., 2006). Nucleotides changes in viral RNA can result in changes in RNA secondary structure as well as changes in amino acid sequences, which causes changes in conformation and function of the viral protein (Zhang et al., 2006). In order to identify the sequences that play important roles in effective infection of PMMoV, we generated chimeric mutants (HA-ZB-ZC, ZA-HB－HC, HA-ZB－HC, HA-HB-ZC) of PMMoV containing various substitutions in the genome RNA to examine their effects on chimeric virus accumulation on inoculated leaves and upper leaves of *N. benthamiana*. The chimeric mutants HA-ZB-ZC and ZA-HB－HC were constructed by substitution of heterologous PA between PMMoV-ZJ and PMMoV-HLD, respectively (Fig. 3). The PA region composed of the Met domain and the most part of the Hel domain of p126. A previous study demonstrated that a variation in the PMMoV Hel domain has also been shown to have a significant effect on viral accumulation in peppers and in *N. benthamiana* (Tena et al., 2012). The considerably low viral accumulation of HA-ZB-ZC (3–9%) and ZA-HB－HC (1–2%) in *N. benthamiana* (Fig. 6) suggested that the heterologous substitution PA region between PMMoV-ZJ and PMMoV-HLD have deleterious effects on symptomology and viral RNA accumulation of the chimeric PMMoV mutants (Fig. 4D and E; Fig. 6). The infectivity of mutant HA-ZB－HC was as high as that of PMMoV-HLD (Fig. 6), which indicated that the substitution of PMMoV-HLD PB with heterologous PMMoV-ZJ PB do not influence the infectivity of the chimera virus HA-ZB－HC (Fig. 6). The infectivity of HA-HB-ZC was lower (23–25%) compared with wild-type PMMoV and HA-ZB－HC, but still comparatively higher than that of HA-ZB-ZC and ZA-HB－HC (Fig. 6).

We summarized the infectivity of PMMoV-HLD and several chimeric mutants PMMoV according to the differences in the accumulation of viral RNAs (Fig. S5). The extremely low infectivity of HA-ZB-ZC and ZA-HB－HC may be due to the changes in the viral genome RNA secondary structure, which resulted in significant replication deficiency. It is also possible that amino acid differences in the replicase (ZA-HB-ZC) or MP (HA-HB-ZC) induced conformational change of the viral proteins that resulted in low infectious activity. As there were no differences in symptomology and viral RNAs accumulation between HA-ZB－HC and PMMoV-HLD, it is also possible that long-distance RNA-RNA interactions exist between PA and PC regions of PMMoV-HLD, which contributed to efficient virus infection. To verify the above possibilities, more detailed PMMoV mutants with nucleotides substitution or point mutations are required to further study the relationship of viral RNA secondary structures and infection mechanisms of PMMoV-HLD.

In summary, the development of the infectious clone for PMMoV-HLD (pCB-PMMoV-HLD) provides effective tool to further study the gene function and pathogenesis of the virus. In addition, the infectious clone is expected to be applied in multiple research purposes including exogenous gene expression in the plants and as a VIGS vector. In this study, we constructed mutants by introducing point mutation and sequences substitution into pCB-PMMoV-HLD and pCB-PMMoV-ZJ and test their infectious abilities in the plants. The results indicated that PMMoV-HLD and PMMoV-ZJ require different RNA sequences for efficient infection. The results also provided important theoretical value to further understand the infection mechanism and pathogenicity of PMMoV.

Conflict of interest

No conflict of interest exits in the submission of this manuscript, and the manuscript is approved by all authors for publication.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.virusres.2019.04.007.

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