

LETTER TO THE EDITOR

Agroinoculation of *Carica papaya* with infectious clones of papaya mosaic virusW. SHEN[†], Y. WANG[‡], D. TUO, P. YAN, Y. YANG, X. LI, P. ZHOU^{*}

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Papaya (*Carica papaya* L.) production in China is threatened mostly by two known viral diseases caused by papaya ringspot virus (PRSV; the family *Potyviridae*, the genus *Potyvirus*) and papaya leaf distortion mosaic virus (PLDMV; the family *Potyviridae*, the genus *Potyvirus*) (1, 2). However, another virus, papaya mosaic virus (PapMV; the family *Flexiviridae*, the genus *Potexvirus*), was detected recently on Hainan Island, China, and designated PapMV-HN isolate (3). PapMV was initially identified in 1962 in Florida, USA and spread mainly on American continent (4). It is transmitted by mechanical inoculation rather than by seeding (5). Symptoms caused by PapMV infection in papaya include stunted plants and systemic vein-clearing, mottle, or mosaic on leaves (6). PapMV is a positive-sense RNA plant virus and its genome comprises 6,656 nucleotides [excluding the poly(A) tail] with five open reading frames encoding a 176-kDa RNA-dependent RNA polymerase (RdRp), three triple gene block (TGB) proteins named TGB1 (26 kDa), TGB2 (12 kDa) and TGB3 (7 kDa), and a 23-kDa capsid protein (CP) (Fig. 1a) (3, 7). In a previous study, a full-length infectious clone of PapMV was constructed for *in vitro* transcription; it was shown to have infectivity in *Gomphrena globosa* and *C.*

papaya (8). However, *in vitro* transcription is a challenging and expensive process, and inoculation of *in vitro* transcripts into host plants is inefficient. In contrast, agroinoculation is a simple and efficient approach for systemic infection of plants with viruses, which is performed by infiltrating leaves (or injecting stems) with *Agrobacterium tumefaciens* cells carrying binary plasmids containing full-length cDNAs of virus genome components (9). In the current study, an agroinoculation system for PapMV was established, which should lay the foundation for further study of the molecular mechanisms of PapMV infection and for the developing a viral vector (10).

Total RNA was extracted from the diseased leaves identified to be infected with PapMV using TRIzol reagent (Life Technologies, USA) (3). A primer pair (*PaMV-1*: 5'-GAAAAGAAACACAAAGCAAAGCAAAGC-3', *PaMV-2*: 5'-GGAAAGGAAA TTTGCAAAACCAAAC-3') was designed to produce the full-length cDNA of PapMV based on the previously published PapMV-HN sequence (Acc. No. JX524226) using the RNA PCR Kit (AMV) (Takara, Japan). Subsequently, the full-length cDNA of PapMV was cloned into the pGEM-T easy vector (Promega, USA) to generate pGEM-T-PapMV. Sequence analysis showed that the cloned sequence shares 100 % nucleotide identity with the PapMV-HN isolate, but 99.6% nucleotide sequence identity with the PapMV reported by Sit *et al.* (Acc. No. NC_001748) (7). Based on the restriction enzymes sites in the full-length sequence of PapMV, the plant transient expression vector pHBT (11) and T-DNA binary vector pGreenII0000 (12)

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Abbreviations: C4PPDK = maize C4 pyruvate orthophosphate dikinase gene; CP = capsid protein; PapMV = papaya mosaic virus; TGB = triple gene block

were selected to construct the full-length infectious clone of PapMV mediated by agroinoculation. First, a primer pair *PaMV-F/R* (*PaMV-F*: 5'-AGAGGATCCGAAAAGAAACACA AAG-3'; italics indicates the *Bam*HI site, *PaMV-R*: 5'-GAGCT GCAGT₂₇GGAAAGG AAATTTGC-3'; italics indicates the *Pst*I site) were designed, two restriction sites and a poly (A) tail with 27 nucleotides in length were added to the primers. The full-length PapMV-HN cDNA fragment with *Bam*HI and *Pst*I sites, and the poly(A) tail was PCR-amplified using *Pyrobest*[™] DNA Polymerase (TaKaRa, Japan) with the pGEM-T-PapMV as the template and the primers *PaMV-F/R*. Amplified products were then digested with *Bam*HI and *Pst*I and ligated into the vector pHBT digested previously with the same restriction enzymes, resulting in the clone p35SPapMV (Fig. 1b), which made the full-length copy of PapMV cDNA under the control of a chimeric promoter 35SC4PPDK and a 3' nopaline synthase (NOS) transcription terminator. The 35SC4PPDK is a strong constitutive promoter consisting of the cauliflower mosaic virus (CaMV) 35S enhancer fused to the basal promoter and the 5' untranslated region of maize C4 pyruvate orthophosphate dikinase gene (*C4PPDK*) (13). Then, p35SPapMV was cleaved with *Xho*I-*Eco*RI, which produced a cassette with 35SC4PPDK-*PapMV*-NOS, and this was inserted into an *Xho*I-*Eco*RI-digested pGreenII0000 to form the recombinant plasmid pGreen35SPapMV (Fig. 1c).

To determine whether *agrobacterium*-mediated delivery of the viral cDNA could be an efficient method to initiate replication of PapMV, the pGreen35SPapMV was transformed

into *A. tumefaciens* strain GV3101, and the transformed strain was used for agroinoculation. The abaxial surface of papaya plants (4–6 weeks old) was inoculated with the recombinant *A. tumefaciens* suspension [in a buffer consisting of 10 mmol/l MgCl₂, 10 μmol/l MES (pH 5.5), and 100 μmol/l acetosyringone] using a 1-ml needleless syringe. At 25 days post-inoculation, the papaya plants showed systemic infection and developed vein-clearing and mosaic on leaves, similar to the symptoms caused by the wild-type control virus. Total RNA extracted from the systemic non-agroinoculated leaves was analyzed for PapMV by reverse-transcription polymerase chain reaction (RT-PCR) using the RNA PCR kit (TaKaRa, Japan) with PapMV CP-specific primers *CP-F* (5'-ATGTCTAAGTCAAGTatgtccacacc-3') and *CP-R* (5'-TTATTCGG GGGGTGGAAGGAATTGG-3'). RT-PCR analysis detected the PapMV CP gene (648 bp) in systemic leaves. Furthermore, to test PapMV-encoded proteins in the systemic non-agroinoculated leaves, polyclonal antiserum against bacterial-expressed PapMV CP was prepared by GenScript Corporation (NanJing, China). Western blot analysis using the anti-CP serum and HRP-conjugated goat anti-rabbit IgG secondary antibody (Tiangen, China) detected a specific band of predicted molecular weight (approximately 23 kDa) in the systemic non-agroinoculated leaves, but not in leaves of mock-inoculated plants.

To our knowledge, this work presents the first report on the development of a full-length infectious cDNA clone of PapMV that is able to infect papaya efficiently by

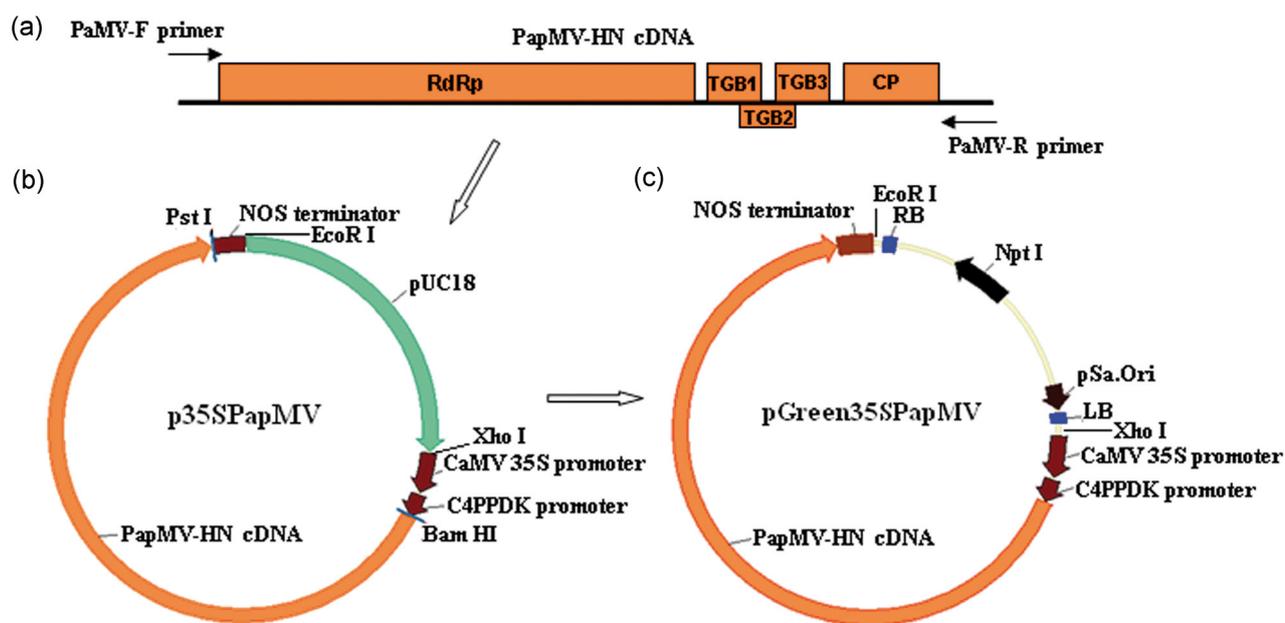


Fig. 1
A schematic of the binary plasmid construct pGreen35SPapMV containing full-length cDNA clones of PapMV-HN
(a) PapMV-HN cDNA (b) p35SPapMV (c) pGreen35SPapMV.

agroinoculation, which provides opportunities to develop PapMV-derived vectors for expressing foreign genes or for investigating gene function in papaya plants using virus-induced gene silencing (VIGS) (10).

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