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Short communication

Construction of an infectious cDNA clone and gene expression vector of *Tobacco vein banding mosaic virus* (genus *Potyvirus*)

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ABSTRACT

Tobacco vein banding mosaic virus (TVBMV, genus *Potyvirus*) mainly infects solanaceous plants and is of increasing economic importance in China. Here, we report sequence determination of the full-length 5′-untranslated region of TVBMV isolate HN39 and construction of an infectious clone. The resultant clone, pTVBMV, which was stabilized by introducing three introns in the P3 and CI-encoding regions, induced similar disease symptoms and accumulated similar titers of virus in plants of *Nicotiana benthamiana*, *Nicotiana tabacum* and *N. rustica* as the wild type HN39 isolate. Mutation of arginine to isoleucine (R182I) or aspartic acid to lysine (D198K) in HC-Pro alleviated the symptoms of pTVBMV significantly, indicating a role of the two amino acids in regulating virulence of TVBMV. The *Aequoria victoriae* gene for green fluorescent protein was inserted between the NIb and CP encoding regions of pTVBMV and expressed stably in the systemically infected *N. benthamiana* leaves, indicating suitability of pTVBMV for expression of foreign proteins in plants.

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Construction of full-length infectious cDNA clones is critical for the functional analysis of RNA viruses. Infectious cDNA clones will allow mapping the viral determinants involved in virus replication, local and systemic movement and symptom development, and interactions between viral and host factors during virus infection in plants (Nagyová and Subr, 2007). Infectious cDNA clones of viruses can also be developed into vectors for foreign gene expression or virus induced gene silencing (Holzberg et al., 2002; Kelloniemi et al., 2008; Lacomme et al., 2003; Lindbo, 2007; Ratcliff et al., 2001; Sainsbury et al., 2010).

There are two major strategies for constructing infectious cDNA clones of plant viruses. The first one is to place the viral cDNA under the promoter SP6, T3 or T7 to produce infectious viral RNA *via in vitro* transcription (Nagyová and Subr, 2007). These promoters are small and can be synthesized as part of the primers used for cloning. However, the costs of use of infectious viral clones made by this method are high because viral RNA needs to be synthesized and, in some viruses, viral RNAs need to be capped before use for inoculation. The second strategy is to use the *Cauliflower mosaic virus* (CaMV) 35S promoter, by which infectious RNA can be produced *via in vivo* transcription. This latter method is less expensive and more practical, but may suffer from low infectivity levels when mechanical rather than biolistic inoculation is carried out (Jakab et al., 1997; Nagyová and Subr, 2007).

The viral genome may contain promoter-like elements resulting in production of toxic products in *Escherichia coli* cells during cloning, and spontaneous mutations, deletions and rearrangements may occur during propagation of the plasmids in *E. coli* cells (Jakab et al., 1997; Johansen, 1996; Johansen and Lund, 2008). To alleviate such problems, AU-rich plant introns can be inserted into viral genomes to terminate expression of undesired toxic proteins in *E. coli* cells. Following introduction of the transcripts into plant cells, the intron sequences are removed precisely to produce the full length infectious RNA of the virus (González et al., 2002; Johansen, 1996; Johansen and Lund, 2008; López-Moya and García, 2000; Olsen and Johansen, 2001; Ülper et al., 2008; Yang et al., 1998). However, the number and/or locations of intron insertions needed to stabilize individual infectious clones vary, even for clones representing closely related viruses (Johansen, 1996).

Tobacco vein banding mosaic virus (TVBMV) is a potyvirus of increasing economic importance in China due to the yield losses it causes in tobacco production (Chen et al., 2009; Tian et al., 2007). Like other potyviruses, the TVBMV genome contains a single large open reading frame (ORF) that encodes a polyprotein, which is cleaved into ten mature proteins by three self-encoded proteinases. Additionally, a small protein is translated from an ORF residing within the P3-encoding sequences by a frame-shifting strategy

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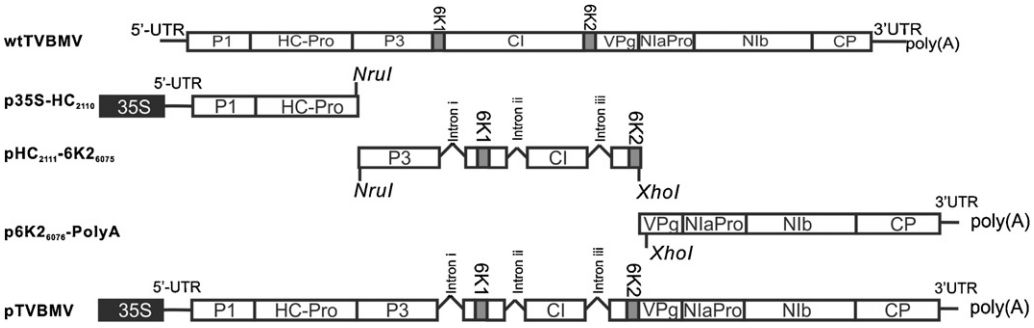

Fig. 1. Construction of the infectious clone pTVBMV. wtTVBMV shows the genome organization of TVBMV. p35S-HC_{2110}, pHC_{2111}-$6K2_{6075}$ and $p6K2_{6076}$-PolyA are the three intermediate plasmids used to construct the full length cDNA clone of TVBMV. pTVBMV shows the structure of the infectious clone of TVBMV and the positions of three introns inserted to stabilize the clone. The mature viral proteins: P1, the first protein; HC-Pro, helper component proteinase; P3, third protein; 6K1 and 6K2, 6 kDa proteins; CI, cylindrical inclusion protein; VPg, viral genome-linked protein; NIa-Pro, the main viral proteinase; NIb, replicase; and CP, coat protein. $A_{(18)}$ indicates the poly(A) tail. The 5′ and 3′ untranslated regions (UTR) are depicted.

(Chung et al., 2008; Zhang et al., 2011). TVBMV isolates are clustered phylogenetically to three groups which correlate with the geographic origin (Tian et al., 2007). The genome sequences of TVBMV isolates HN39 and YND, which differ in virulence and belong to different strains, have been determined (Wang et al., 2010; Yu et al., 2007). Selection, gene flow and recombination are important factors driving the evolution of TVBMV in nature (Zhang et al., 2011).

In a previous study, presence of only box 'b' was revealed in the 5′-untranslated region (5′-UTR) of TVBMV-HN39 genome (NCBI sequence accession number EU734432; Wang et al., 2010). In the present study, the 5′-UTR of HN39 was determined using the 5′-RACE method (Scotto-Lavino et al., 2007). HN39 was maintained in *Nicotiana tabacum* cv. Samsun plants grown in a growth chamber under 16 h photoperiod (110 $\mu E\,m^{-2}\,s^{-1}$; illumination of fluorescent lamps alternately with tubes of 58 W/830 and 36 W/77) at 23 °C under 40% relative humidity. Plants were watered when needed and fertilized weekly with 1% N:P:K = 16:9:22 fertilizer (Yara, Espoo, Finland). TVBMV particles were purified from infected Samsun leaves as previously reported (Fribourg and Nakashima, 1984). Viral RNA was extracted from purified particles as described by Monger et al. (2001). The amplification products were gel-purified and ligated into the pGEM-T vector, followed by transformation into the *DH5α* competent cells. Plasmids were isolated from individual colonies using GenElute plasmid purification kit as instructed (Sigma–Aldrich, Steinheim, Germany). Two independent 5′-RACE reactions were made, and ten clones were sequenced to determine the first nucleotides of the TVBMV HN39 5′-UTR. The length of 5′-UTR varied in different clones. Among the ten clones sequenced, three had the longest 5′-UTR of 171 nucleotides (nt; accession number JQ407081), which is 25 nt longer than that reported previously and contained both boxes 'a' (ACAACAU) and 'b' (UCAAGCA) sequences that are highly conserved in the *Potyviridae* family (Simón-Buela et al., 1997).

To construct a full-length cDNA clone of TVBMV HN39, three fragments covering the full length genomic sequence were prepared (Fig. 1 and Suppl. Table 1). p35S-HC_{2110} contained the 35S promoter and the first 2110 nt of the 5′-part of TVBMV genome. pHC_{2111}-$6K2_{6075}$ contained TVBMV nt 2111–6075 and three introns. $p6K2_{6076}$-*Sma*I covered the 3′-part of TVMBV genome from nt 6076 to the 3′-end poly(A) tail and contained a *Sma*I restriction site at the end. The three fragments were used to assemble the full-length TVBMV clone pTVBMV. To stabilize the clone, three introns (one in the P3-encoding region and two in the CI-encoding region) amplified from the infectious clone for *Potato virus Y* (PVY) (Bukovinszki et al., 2007) were inserted at nt positions 3141, 3981 and 5380, respectively (Fig. 1 and Suppl. Table 1). Removal of one or two introns from pTVBMV resulted in deletions or rearrangements of the viral sequence, as tested by restriction analysis of the plasmid, which further emphasized the importance of the introns in stabilizing the viral genome in the plasmid.

To test infectivity, pTVBMV was inoculated to fully developed leaves of 6-week-old *Nicotiana benthamiana* plants by particle bombardment (Sikorskaite et al., 2010). Nine plants were inoculated with each plasmid and experiments were repeated for three times. The inoculated plants were grown in growth chambers at 22 °C under a 16-h photoperiod (relative humidity 75%). Leaf discs from systemically infected leaves of four inoculated plants for each treatment were collected at 5, 8, 11, 14, 17 and 20 days post-inoculation (dpi). Virus accumulation levels were determined by indirect plate-trapped antigen enzyme-linked immunosorbent assay (PTA-ELISA) using TVBMV coat protein (CP) specific antibodies (Lan et al., 2007). Absorbance values (A_{405}) were recorded for each sample using a Benchmark plate reader (Bio-Rad). Total RNA was extracted from infected leaves using a homemade TRIzol as described (Caldo et al., 2004). First-strand cDNA synthesis was done using an oligo(dT) primer and M-MLV reverse transcriptase (Promega). Viral RNA accumulation in leaves was monitored by quantitative real time reverse transcription PCR (qRT-PCR) using specific primers TVBMV CP-F and TVBMV CP-R (Suppl. Table 1).

Systemically infected leaves in all plants inoculated with pTVBMV showed vein clearing symptoms similar to the original TVBMV-HN39 by 8 dpi (Fig. 2A), followed by development of severe mosaic symptoms in the youngest leaves and stunting of the plants by 14 dpi. Sequencing of progeny viruses from the pTVBMV-inoculated plants showed that all three introns had been removed completely and precisely.

Crude extracts were prepared from the leaves of *N. benthamiana* systemically infected with either the pTVBMV-derived virus or wild type HN39 and used for mechanical inoculation of seedlings of *N. benthamiana*, *N. tabacum* and *N. rustica*. By 5 dpi all the inoculated plants showed similar vein clearing and mosaic symptoms in the systemically infected leaves, indicating that the progeny viruses were spreading systemically in these host plants at the same rate. Analysis of the plants by PTA-ELISA showed that both viruses accumulated to similar levels in the host plants (Fig. 2B).

To test importance of the conserved motifs $FR_{182}NK$ and $CD_{198}N$ in HC-Pro on virulence of TVBMV, substitutions of isoleucine for arginine (R182I) and lysine for aspartic acid (D198K) were introduced to modify the motifs, respectively, using primers (Suppl. Table 1) designed as described by Liu and Naismith (2008). The mutated clones were verified by sequencing and no additional mutations were detected. Inoculation of *N. benthamiana* plants showed that the pTVBMV-derived virus caused mosaic and distortion in the systemically infected leaves and stunting of the plants as before. In contrast, the viruses derived from $pTVBMV_{R182I}$ and $pTVBMV_{D198K}$ carrying a mutation in the motifs FRNK and CDN,

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