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Rapid generation of inoculum of a plant RNA virus using overlap PCR

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ABSTRACT

We have developed an efficient method to rapidly generate infectious inoculum of a plant RNA virus and confirmed its infectivity by mechanical inoculation. The method takes advantage of overlap PCR to bypass the cloning steps, which makes it relatively simple, rapid, and inexpensive compared to the traditional methods. Using this approach, inoculum of a tobamovirus, *Turnip vein clearing virus* (TVCV), was generated. PCR products specific for the 35S promoter and TVCV genome were used as templates for overlap PCR to form a single product containing the full-length TVCV cDNA under the control of the double 35S promoter, and the entire process took only 8 h. This inoculum was infectious in *Nicotiana benthamiana*, and its infectivity was ca. 67% compared to 0% and 100% with negative and positive controls, respectively. Thus, this rapid method generates efficient infectious inoculum for a plant RNA virus.

1. Introduction

One of the basic tasks in plant virology laboratories is creating and maintaining virus sources that are essential for all experiments that involve virus infection. Inocula can be prepared directly from infected plants in a form of sap extract or purified virions. However, it is difficult to maintain stable virus in plants due to rapid mutations through multiple passages in the host. To solve this problem, viral cDNA clones have been constructed in bacterial plasmids; these clones are stable and allow *in vitro* or *in vivo* generation of RNA transcripts that preserve the original characteristics of virus isolates (Chaturvedi et al., 2012). Transcription of viral cDNAs by phage RNA polymerases requires expensive materials and stringent conditions for RNA preservation. *In vivo* infectious clones, which are based on recombinant bacterial vectors, provide a cheaper procedure for delivery of the viral inocula into plants by *Agrobacterium*-mediated transient transformation (Annamalai and Rao, 2006) or by rub-inoculation of plants recalcitrant to *Agrobacterium* infection (Seo et al., 2009). In both approaches, however, the initial construction of recombinant infectious clones requires numerous cloning steps which are time-consuming; furthermore, these clones often necessitate the presence of plant introns to mitigate cytotoxic effects of viral sequences during their maintenance in the host cells or to avoid unwanted recombination (Tran et al., 2019). Therefore, it would be useful to develop a simple, rapid, and inexpensive method for generation of virus inoculum. We addressed this need using overlap-extension polymerase chain reaction (overlap PCR).

Overlap PCR is a variation of PCR known to allow assembly of multiple DNA fragments into one construct in a single reaction (Horton et al., 1989; Liu et al., 2017). Here, we took advantage of this ability of overlap PCR to bypass cloning steps and rapidly produce an infectious inoculum of a tobamovirus, *Turnip clearing vein virus* (TVCV), which are directly delivered into plants though a simple mechanical inoculation. This cheap and rapid approach was then applied to generate a TVCV-based recombinant virus expressing a green fluorescent protein (GFP) reporter.

2. Results and discussion

2.1. Generation of virus inoculum by overlap PCR

The 35S promoters and TVCV were first amplified by PCR (Fig. 1A). The PCR products were then purified and used in an overlap PCR reaction to generate the 2 × 35S-TVCV construct, in which the 2 × 35S sequence was inserted upstream of the TVCV genome (Fig. 1B). The whole process took less than 8 h.

The infectivity of the virus inoculum generated by overlap PCR was accessed by inoculation onto *N. benthamiana* plants using the experimental design summarized in Fig. 1C. The inoculations were performed under three different conditions: mock (buffer) inoculation (negative control), infected plant sap inoculation (positive control), and the 2 × 35S-TVCV overlap PCR inoculation. The infectivity was monitored using digital imaging to observe the appearance of the hallmark viral disease

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symptoms, i.e., necrotic local lesions or systemic vein yellowing and mosaic, and stunted growth. Fig. 2A shows that the development of these symptoms at 4 days after inoculation (dpi) and 7 dpi. Symptom scoring at 7 dpi revealed that none of the 9 plants mock-inoculated with buffer became symptomatic (0% infection), all 9 plants inoculated with the virus-containing sap developed symptoms (100% infection), and 6 out of 9 plants inoculated with 2 × 35S-TVCV developed symptoms (ca. 67% infection). The lower infection efficiency of the 2 × 35S-TVCV overlap PCR product compared to that of sap still allowed easy detection of infectivity of the construct. The presence of TVCV in the infected tissues was confirmed by RT-PCR and DNA sequencing (Fig. 2B). Collectively, these observations indicate that the overlap PCR can generate an infectious virus inoculum which can be used for simple and rapid detection of viral infectivity.

2.2. Application of overlap PCR to generate a recombinant TVCV

Depending on individual study goals, modifications of tobamoviral genomes to express foreign proteins involve duplications of sub-genomic promoters or introduction of a heterologous promoter from a related virus to drive expression of a transgene (Rhee et al., 2016; Shivprasad et al., 1999). Alternatively, a transgene is fused to one of the native viral genes (Kawakami et al., 2004; Levy et al., 2015). Both strategies are based on multiple and labor-intensive and time-consuming molecular cloning steps. Our overlap PCR strategy circumvents this difficulty.

We used overlap PCR to generate a recombinant TVCV ($TVCV_{MP::GFP}$) which expresses GFP fused to the C-terminus of the viral movement protein (MP) and carries a heterologous coat protein (CP) expression cistron from *Tobacco mild green mosaic virus* (TMGMV). As illustrated in Fig. 3A, $TVCV_{MP::GFP}$ was amplified from four separated templates, PCR1 (containing the 2 × 35S promoter and the TVCV 5′ fragment from nucleotide 1 to the codon that precedes the MP stop codon, from template 2 × 35S-TVCV), PCR2 (GFP), PCR3 (TVCV 3′-UTR, nucleotides 6077 to 6273), and PCR4 [containing the CP cistron and 3′-UTR of TMGMV from p30B (Shivprasad et al., 1999)]. These fragments were amplified using primers with additional nucleotides to produce amplicons with 20 overlapping nucleotides. The infectivity of the $TVCV_{MP::GFP}$ inoculum was then tested by mechanical inoculation onto *N. benthamiana* leaves. The expression of MP::GFP in whole plants was observed as GFP fluorescence under UV irradiation; this signal was observed in the inoculated leaves at 3 dpi and in systemic leaves at 10 dpi (Fig. 3B). No fluorescent signal was detected in mock-inoculated, negative control plants (Fig. 3B). Expression of the GFP reporter in the $TVCV_{MP::GFP}$ inoculated plants was confirmed on the cellular and subcellular levels, using epifluorescence and confocal fluorescence microscopy. Fig. 3C shows that a large number of cells surrounding the inoculation site expressed GFP (upper panel) whereas, in the individual expressing cells, the GFP signal accumulated as fluorescent puncta, diagnostic of plasmodesmata, where the GFP-tagged MP is expected to accumulate. Sap obtained from the $TVCV_{MP::GFP}$-infected plants stably maintained the ability to infect plants and produce GFP expression in inoculated leaves through several passages (data not shown). These data indicate that the overlap PCR method can generate infectious recombinant TVCV carrying and expressing a foreign transgene.

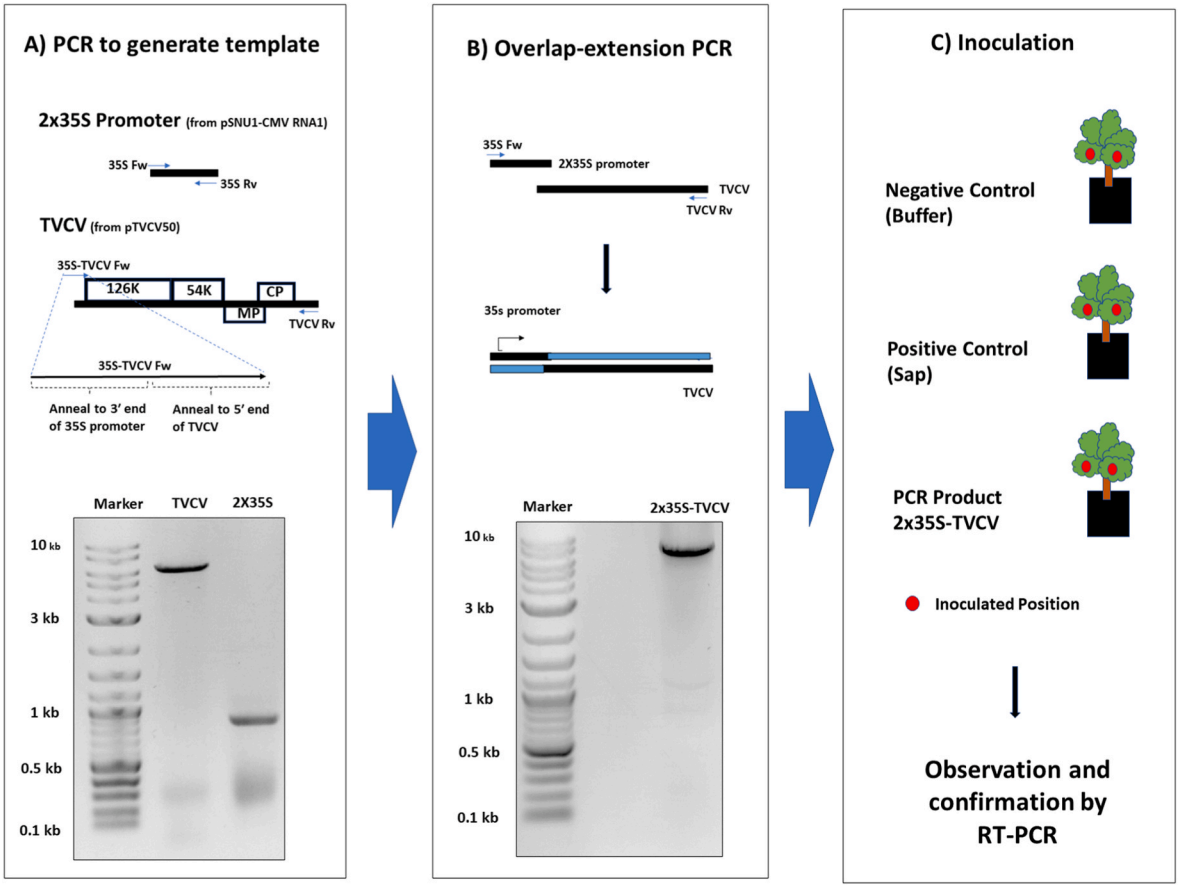

Fig. 1. Schematic presentation of overlap PCR to generate infectious TVCV inoculum. (A) PCR to generate templates: 2 × 35 promotor and the full-length TVCV sequences were amplified from pSNU1-CMV RNA1 and pTVCV50 respectively. (B) Overlap-extension using the templates 2 × 35S promoter and TVCV. The PCR products were analyzed by agarose gel electrophoresis (bottom panels in A and B). (C) Schematic design of inoculations with three groups of samples: negative control (buffer), positive control (sap of TVCV-infected plants), and the 2 × 35S-TVCV PCR product. The infection was confirmed by symptom observations and RT-PCR.

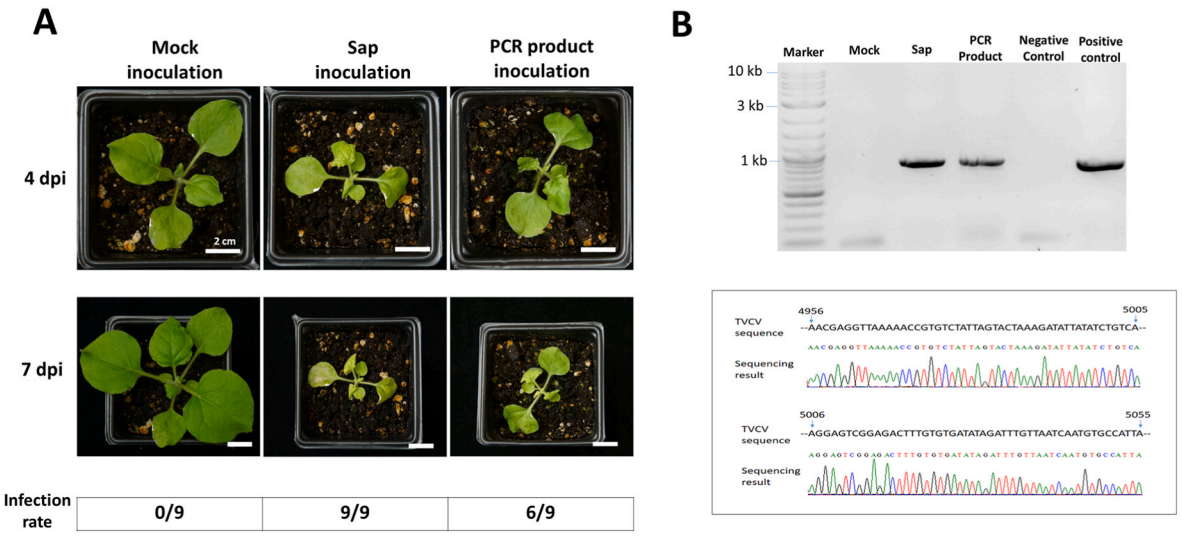

Fig. 2. Infectivity of the TVCV inoculum generated by overlap PCR. (A) Images of plants inoculated with the indicated inocula at 4 dpi and 7 dpi. Infection efficiency is indicated as number of symptomatic plants out of the total number of the inoculated plants. (B) The presence of TVCV genomes in the inoculated tissues. TVCV sequences were detected by RT-PCR with TVCV MP-specific primers (top panel); the PCR products were confirmed by DNA sequencing (bottom panel).

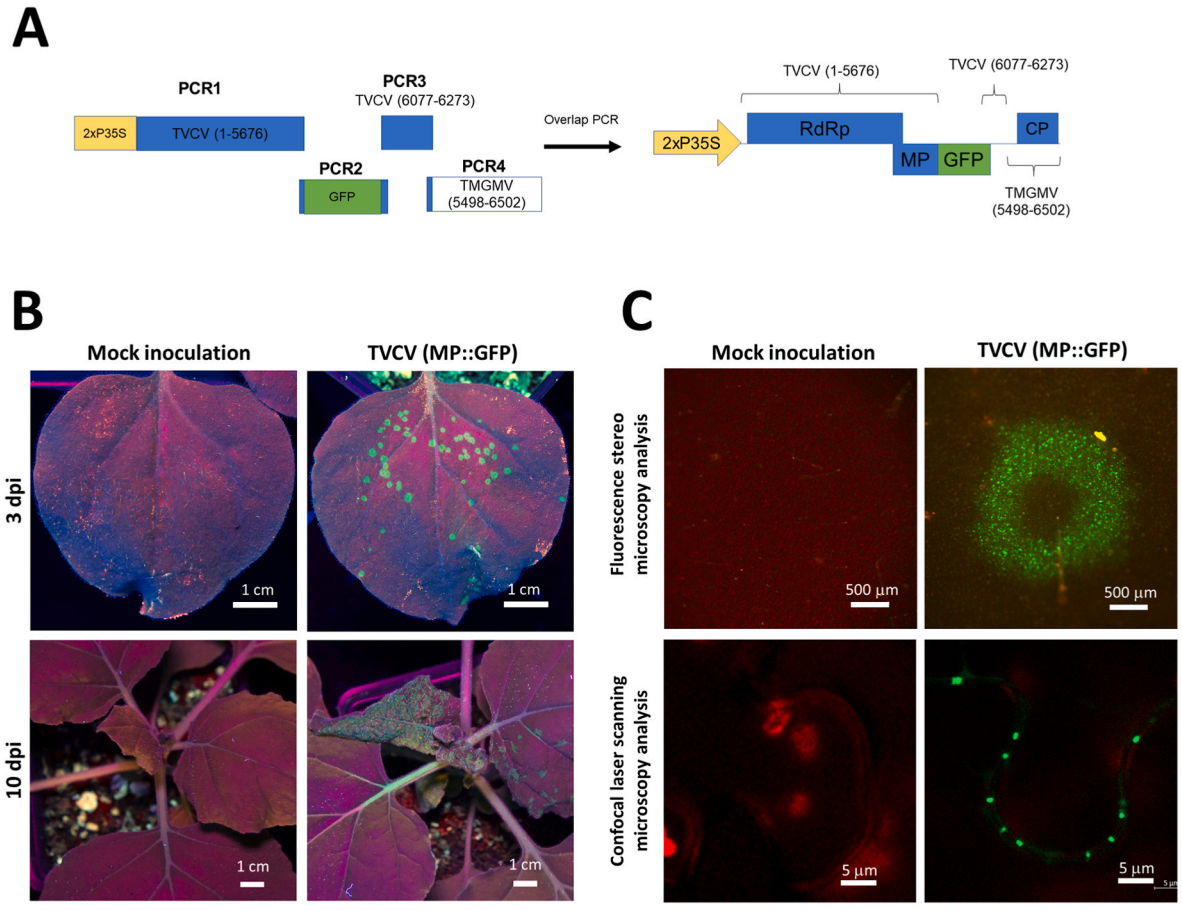

Fig. 3. Recombinant TVCV express MP::GFP locally and systemically. (A) Generation of $TVCV_{MP::GFP}$. $TVCV_{MP::GFP}$ was assembled by overlap-PCR of the following PCR-amplified templates: 35S-TVCV (PCR1)::GFP(PCR2)::3′-TVCV(PCR3)::TMGMV CP cistron (PCR4). (B) GFP expression in leaf tissues of plants inoculated with $TVCV_{MP::GFP}$. *N. benthamiana* plants were mock-inoculated with buffer or with $TVCV_{MP::GFP}$, and the Inoculated and uninoculated, upper leaves were analyzed for the presence of the GFP fluorescence at 3 dpi and 10 dpi, respectively. (C) GFP expression in leaf cells of plants inoculated with $TVCV_{MP::GFP}$. Plants were inoculated as described in (B) and GFP expression was analyzed at 3 dpi using the fluorescence stereo microscope (lower magnification) and the confocal laser scanning microscope (higher magnification).

One possible limitation of our technique is the DNA amplification errors, e.g., base substitutions, that could occur during long PCR. Thus, in terms of genetic homogeneity, the inocula generated by these PCRs are more diverse than the ones from the plasmid-based infectious clones driven by 35S promoter. This limitation, however, has its own advantages. While *in vivo* transcription, mediated by the host RNA polymerase II with the error rate of 3.9×10^{-6} (Gout et al., 2017), from a single plasmid-based infectious clone generates a near single-genotype inoculum, our PCR strategy creates a population of virus inocula that is closer to the native and genetically variable virus population in the infected plants. This variation could be unacceptably increased by the use of low-fidelity DNA polymerases or poor PCR conditions; however, with high-fidelity DNA polymerase and optimized PCR conditions, the viral populations generated by the PCR inoculum would be comparable to, or even less variable than, the well-established *in vitro* transcripts produced by bacteriophage RNA polymerases. Mutation rates of those enzymes, such as T7 (0.5×10^{-4}) or SP6 RNA polymerase (1.34×10^{-4}) (Pugachev et al., 2004), are substantially higher than those of the *taq* polymerase (1.3×10^{-5}) or the *pfu* fusion DNA polymerase (1.3×10^{-6}) (Potapov and Ong, 2017). Therefore, the expected purity of the recombinant viruses generated by the well-controlled PCR inocula should be acceptable for the general applications in plant virology.

Overall, our study demonstrated a simple, rapid, and inexpensive way to generate a native and a recombinant form of a plant RNA virus, TVCV. This method could be further applied for other positive-sense single-stranded plant RNA viruses which only require the viral transcript for initiation of infection.

3. Materials and methods

3.1. Overlap-extension polymerase chain reaction

PCR was performed using the PfuUltra II Fusion HS DNA polymerase (Agilent, CA, USA) in the C1000 thermal cycler (Bio-Rad, CA, USA). Then, the products were analyzed by electrophoresis through a 1.0% agarose gel in 0.5× TAE buffer and stained by the Safeview DNA stain (Applied Biological Materials, British Columbia, Canada). As a size marker, the 10 kb plus DNA ladder (New England Biolabs, MA, USA) was used. In detail, to generate the templates for the overlap PCR, the double 35S promoter and the full-length TVCV were amplified from plasmids pSNU1-CMV RNA1 (Vo Phan et al., 2014) and pTVCV50 (Zhang et al., 1999) using primers 1 and 2 and primers 3 and 4, respectively (Table 1). The amplification was performed according to the manufacturer's instructions with the following minor modifications: initial denaturation was done at 95 °C for 2 min; number of cycles and extension time varied, depending on constructs, i.e., 30 cycles with the promoter (95 °C for 20 s, 55 °C for 20 s, 72 °C for 15 s, and 35 cycles with the TVCV (55 °C for 20 s, 55 °C for 20 s, 72 °C for 1 min 15 s); the final extension was done at 72 °C for 5 min. The resultant PCR products were gel-purified using the DNA Gel Extraction Kit (Bioneer, Daejeon, South Korea). The purified PCR products were used as templates in an overlap PCR mixture (50 μl) with 5 ng of the promoter template and 35 ng of the TVCV template (1:1 in molar ratio). The reaction was performed with same DNA polymerase using primer 1 and 4 (Table 1) in an experimentally optimized conditions: 95 °C for 2 min for initial denaturation, 30 cycles of amplification (95 °C for 20 s, 55 °C for 5 min, 72 °C for 2 min) for extension, and final extension at 72 °C for 5 min. The product of the overlap PCR, designated 2 × 35S-TVCV, was separated from unincorporated primers and dNTPs using the PCR clean up kit (Bioneer, South Korea). The recombinant 2 × 35S-TVCV expressing GFP was generated by the same strategy using primers 7 and 8 (Table 1). All junctions of the fused fragments were confirmed by DNA sequencing using primers 14, 15, and 16 (Table 1).

3.2. Virus inoculation and confirmation of infectivity

Nicotiana benthamiana plants were maintained in growth chamber at 23 °C with relative humidity of 50%–60% under a 16:8-h ratio of light to dark photoperiod with the light intensity of 100 μmol photons m^{-2} s^{-1}. Two weeks after sowing, the plants were used for virus inoculation.

TVCV infectious sap and the phosphate buffer (0.05 M, pH 7.4) was used as positive and negative controls, respectively. The overlap PCR product was diluted in the phosphate buffer to the concentration of 100 ng/μl. Two fully expanded leaves per plant were dusted with carborundum and inoculated with 1 μg of the DNA inoculum by gentle rubbing with gloved fingers. Thirty seconds after inoculation, the leaves were rinsed with water to remove excess inocula and carborundum dust. The plants were placed in growth chambers and observed daily for signs of infection, i.e., necrotic local lesions or systemic vein yellowing and mosaic, and their images were recorded every few days to capture the progression of the virus symptoms. Each inoculation was conducted in three independent plants and repeated three times.

To confirm TVCV infection, the systemic leaves from the plants with typical symptoms were harvested at 4 dpi. Their total RNA was extracted using Triazol reagent (Invitrogen, CA, USA). The cDNA was generated by the RevertAid first strand cDNA synthesis kit (Thermo Fisher Scientific, MA, USA) using random hexamers. Detection of TVCV sequences was carried out using EX *tag* polymerase (Takara, Shiga, Japan) with primers specific for the TVCV movement protein (MP) (primers 5 and 6, Table 1). The PCR product was purified and its DNA sequence was determined (Genscript, NJ, USA).

To visualize infection of the recombinant 2 × 35S-TVCV expressing GFP, the inoculated (3 dpi) and upper (10 dpi) leaves from infected

Table 1
Primers used for PCR.

No.	Primer name	Sequence (5′ to 3′)	Purpose
1	pCAMBIA LB Fw	gctggctggtggcaggata	Amplification of 2 × 35S promoter
2	35S Rv	cctctccaaatgaaatgaacttcc	Amplification of 2 × 35S promoter
3	35S TVCV Fw	ggaagttcatttcatttggagagggtttagttttattgcaacaacaacaacaaaattac	Amplification of TVCV
4	TVCV Rv	tgggcccctacccggggtta	Amplification of TVCV
5	TVCV MP Fw	atgtcgatagtctcgtacgaac	Detection of TVCV
6	TVCV MP Rv	agcattggtatgggctctgc	Detection of TVCV
7	TVCV MP-GFP Rv	atccctccaccgcctccagcattggtatgggctctgc	Amplification of 2 × 35S-TVCV (1-5676)
8	TVCV MP-GFP Fw	atgctggaggcggtggagggatggctagcaaaggagaagaac	Amplification of GFP
9	GFP-TVCV 3UTR Rv	cgcactacgcttatttgtagagctcatccatgccatg	Amplification of GFP
10	GFP-TVCV 3UTR Fw	agctctacaaataagcgtagtgcgcacgatagcg	Amplification of 3′-UTR of TVCV (6077-6273)
11	TVCV 3UTR-TMGMV Rv	gagtttcacagccacttttacttattcccactgtaatacacgt	Amplification of 3′-UTR of TVCV (6077-6273)
12	TVCV 3UTR-TMGMV Fw	ggaataagtaaaagtggctgtgaaactcgaaaaggttcc	Amplification of CP cistron and 3′-UTR of TMGMV (5498-6502)
13	TMGMV Rv	tgggccgctacccgcggttag	Amplification of CP cistron and 3′-UTR of TMGMV (5498-6502)
14	2 × 35S 52[a] Fw	ctatccttcgcaagacccttc	Sequencing to confirm junction of 3 × 35S and TVCV
15	TVCV MP 578 Fw	gagagtggctagtgccagac	Sequencing to confirm junction of MP and GFP
16	TMGMV CP 410 Rv	tcaacggatcaagcgtcgaa	Sequencing to confirm junction of GFP, TVCV 3′-UTR, and TMGMV CP cistron

[a] The numbers indicate the distance of the primer from the first nucleotide of the indicated junction.

plants were irradiated by the long-wave UV light (366 nm) using the UVGL-25 lamps (UVP, CA, USA). The images of infected plants were recorded using a digital camera (Sony, Japan) with the following settings: focal length 30 mm, ISO 100, aperture f/4, and shutter speed 15 s. To obtain images of infected cells, the inoculated leaves were analyzed using the MZ FLIII fluorescence stereo microscope with GFP filters (Leica, Switzerland) or the LSM 900 confocal microscope (Zeiss, Germany).

CRediT authorship contribution statement

Phu-Tri Tran: Conceptualization, Methodology, Investigation, Visualization, Writing - original draft. **Chao Feng Zhang:** Investigation, Validation, Writing - original draft. **Vitaly Citovsky:** Supervision, Funding acquisition, Project administration, Writing - review & editing.

Declaration of competing interest

The authors declare no conflict of interest.

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