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Recombination-based generation of the agroinfectious clones of *Peanut stunt virus*

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A B S T R A C T

Full-length cDNA clones of *Peanut stunt virus* strain P (PSV-P) were constructed and introduced into *Nicotiana benthamiana* plants *via Agrobacterium tumefaciens*. The cDNA fragments corresponding to three PSV genomic RNAs and satellite RNA were cloned into pGreen binary vector between *Cauliflower mosaic virus* (CaMV) 35S promoter and nopaline synthase (NOS) terminator employing seamless recombinational cloning system. The plasmids were delivered into *A. tumefaciens*, followed by infiltration of hosts plants. The typical symptoms on systemic leaves of infected plants similar to those of wild-type PSV-P were observed. The presence of the virus was confirmed by means of RT-PCR and Western blotting. Re-inoculation to *N. benthamiana*, *Phaseolus vulgaris*, and *Pisum sativum* resulted in analogous results. Generation of infectious clones of PSV-P enables studies on virus-host interaction as well as revealing viral genes functions.

1. Introduction

Peanut stunt virus (PSV) is an important pathogen mainly infecting legume plants, such as peanut (*Arachis hypogaea* L.), soybean (*Glycine max* (L.) Merr.), bean (*Phaseolus vulgaris* L.), pea (*Pisum sativum* L.), yellow lupine (*Lupinus luteus* L.), alfalfa (*Medicago sativa* L.), celery (*Apium graveolens* L.), black locust trees (*Robinia pseudoacacia* L.), etc. Soybean and lupine are mostly affected by PSV infection. The yield loss of about 33% in soybean infected by PSV was reported due to reduction of number and size of seeds as well as decrease in seed quality due to mottling of the seeds (Saruta et al., 2012). Together with *Cucumber mosaic virus* (CMV), *Tomato aspermy virus* (TAV), and *Gayfeather mild mottle virus* (GMMV), PSV belongs to *Cucumovirus* genus in *Bromoviridae* family. It is transmitted by aphids in non-persistent manner. PSV infection symptoms and their severity might vary, depending on virus strain and plant host analyzed. The local infection symptoms are mostly chlorotic lesions, whereas the symptoms of systemic infection are mosaics, malformations, chlorotic lesions, necrotic lesions, stunting, vein clearing as well as the infection might be latent (Obrępalska-Stęplowska et al., 2008a). PSV contains a positive-sense (+) single-stranded RNA genome, consisting of three genomic and two subgenomic strands. Each genomic strand has a 3′ tRNA-like structure and a cap at 5′ end. RNA1 and RNA2 serve as templates for translation for the viral components of the replicase complex. RNA2 also encodes 2b protein known for the participation in viral movement and suppression of postranscriptional gene silencing. 2b protein is synthesized from the subgenomic RNA4A. RNA3 is dicistronic and encodes for two proteins, movement protein (3a) and coat protein (CP); the latter translated from subgenomic RNA4. Some strains may be associated with satellite RNA (satRNA). PSV has been reported in many countries worldwide. It was first identified in the United States of America (Miller and Troutman, 1966). Since then, many strains have been characterized. PSV strain P (PSV-P) was first reported in Poland in 1983 (Pospieszny, 1988; Twardowicz-Jakusz and Pospieszny, 1983), followed by its genome sequence description (Obrępalska-Stęplowska et al., 2008b).

Construction of infectious clones of RNA viruses (*in vitro* transcripts as well as cDNA clones) has raised the possibility of functional investigation of viruses. It facilitates analysis of their nature and pathogenesis, including their genes function and involvement in host-virus interaction, especially for those present in low titers in infected cells or whose isolation is problematic. Generation of *in vitro* transcripts usually involves cloning of the virus cDNA sequence under T7, T3, or SP6 promoter followed by *in vitro*

Abbreviations: CaMV, *Cauliflower mosaic virus*; NOS, nopaline synthase; PSV-P, *Peanut stunt virus* strain P; RT-PCR, reverse transcriptase PCR.

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transcription. It is essential that the cDNA sequence for *in vitro* transcription corresponds to the whole virus sequence. The sequence at 5′ or 3′ ends may influence the infectivity of the viral transcripts (number and sequence of nonviral, additional nucleotides introduced during cloning, presence of a cap structure at 5′ end or a poly(A) tail at the 3′ end) (Boyer and Haenni, 1994). Previous studies indicated that the 5′ extensions decrease or abolish infectivity. Plasmid constructs of CMV strain Q with an additional one or two G residues at 5′ end of each RNA resulted in higher yield of *in vitro* transcription, whereas the infectivity was lower than that of those transcripts with no additional residues at the 5′ end (Hayes and Buck, 1990).

Generally, the presence of the cap structure at 5′ end is required for optimal infectivity of the transcripts. The lack of cap structure contributed to generation of transcripts with no or reduced level of infectivity (Beck et al., 1990; Burgyán et al., 1990; Chiang and Yeh, 1997; Hayes and Buck, 1990; Jakab et al., 1997; Meshi et al., 1986; Puurand et al., 1996; Sit and AbouHaidar, 1993). However, in some cases uncapped RNAs were as highly infectious as capped transcripts (Gulati-Sakhuja and Liu, 2012; Hearne et al., 1990; Rochon and Johnston, 1991; Scheets et al., 1993). Also, members of the families *Secoviridae* and *Potyviridae*, some members of the family *Luteoviridae*, and members of the genus *Sobemovirus* instead of cap structure possess small protein known as VPg (*virus protein*, *genome linked*). In reference to the *in vitro* transcribed virus infectious clones, VPg is not absolutely required for their infectivity as it was shown when at the 5′ end of *Tobacco vein mottling virus* (TVMV) RNA VPg was replaced by the cap structure (Domier et al., 1989).

For viruses possessing poly(A) at their 3′ end, a meaningful factor is the length of poly(A) tail. According to the studies with *Clover yellow mosaic virus* (CYMV) and *Papaya mosaic virus* (PMV) the increasing length of poly(A) enhanced the infectivity of viruses. Moreover, when the number of A residues was shortened the infectivity decreased (Holy and AbouHaidar, 1993; Sit and AbouHaidar, 1993).

The second possibility of expression of infectious viral RNAs is through *in vivo* transcription (in host cells) of cDNA-containing vectors from 35S promoter of *Cauliflower mosaic virus* (CaMV). This approach has several advantages as it does not require *in vitro* transcription, the infectivity is independent on viral replication process, and is less susceptible to RNA degradation (Boyer and Haenni, 1994). cDNA clones in the form of isolated plasmid DNA are stable *in vitro* for a long time. On the other hand, there might be a problem with low efficiency of introducing the cDNA viral sequence into nucleus as well as in transport of newly transcribed complex RNA molecules to cytoplasm. Additionally, AU-rich sequences may be exposed to nuclear RNA-processing machinery while being recognized as introns (Gleba et al., 2004; Nagyová and Šubr, 2007).

Infectious cDNA clones are mostly used for DNA viruses, however, the cloned viral genomes were developed for many RNA plant viruses as well (Crutzen et al., 2009; Kang et al., 2015; Orílio et al., 2014; Wieczorek et al., 2015). Infectious clones of viruses can be easily modified, therefore, there are many researches applying this system to elucidate viral gene functions in host cells (Obrępalska-Stęplowska et al., 2013; Salvador et al., 2008; Wieczorek and Obrępalska-Stęplowska, 2016) or to use them as vectors (Fujiki et al., 2008; Rhee et al., 2016; Zhao et al., 2016).

Here we describe the construction of infectious cDNA clones of RNA virus based on the seamless recombinational cloning, which allows insertion of one or more DNA fragments into a plasmid vector without extra sequences, and without the need of restriction endonuclease digestion and ligation. The reaction is very straightforward and carried out in isothermal conditions. The correctly assembled plasmid constructs are delivered to plant host cells throughout *Agrobacterium tumefaciens* infection, where the infectious virus copies are generated with exact 5′ and 3′ ends. In this work, we developed full-length infectious cDNA clones for PSV-P RNA1, RNA2, RNA3, and satellite RNA (satRNA). Agroinoculation with RNA1, RNA2, RNA3, with or without satRNA constructs under CaMV 35S promoter resulted in systemic infection of *Nicotiana benthamiana*, *P. vulgaris*, and *P. sativum* plants.

2. Materials and methods

2.1. Plants and virus materials

N. benthamiana, *P. vulgaris*, and *P. sativum* plants were grown in controlled conditions in separate greenhouse chambers with 14 h light/10 h dark cycle in 26 °C day/21 °C night. Infectious copies of PSV-P alone or in combination with satRNA-P in *A. tumefaciens* were infiltrated into 5–6 week-old *N. benthamiana* seedlings. Mock-treated *N. benthamiana* plants infiltrated only with infiltrating buffer were grown as negative controls. The plants were harvested at 21 days post inoculation (dpi). Some of the infected plant material was used for sap inoculation into 5–6 week-old *N. benthamiana*, *P. vulgaris*, and *P. sativum* plants. Negative controls were mock-inoculated (treated only with 0.05 M phosphate buffer, pH 7.5) plants of *P. vulgaris*, *P. sativum*, and *N. benthamiana*, whereas wild-type PSV-P infected plants served as positive controls. Then, plants were harvested at 23th dpi.

2.2. Cloning full-length PSV-P genomic strands

Full-length copies of viral RNAs were amplified by means of PCR using previously described templates: infectious clones of PSV-P cloned under T7 RNA polymerase promoter (Obrępalska-Stęplowska et al., 2013). Briefly, the PCR mixture (50 μl) contained 1× reaction buffer, 200 μM deoxyribonucleotides, forward and reverse primers (125 ng each, Table 1), 1 μl (up to 20 ng) of plasmid DNA, and 1 μl of *Pfu*Ultra II Fusion HS DNA Polymerase (Agilent Technologies, Santa Clara, CA, USA). The reaction mixture was initially incubated at 95 °C for 2 min followed by eight cycles of: 20 s at 95 °C, 30 s at 53 °C, and 3 min at 68 °C. Next round of 17 cycles of PCR was done, however, with annealing temperature of 55 °C. Finally, the mixture was incubated for 10 min at 68 °C. The PCR was performed in Biometra TProfessional Basic Thermocycler, followed by products' electrophoresis in 1% agarose-TBE gel. The PCR products of expected lengths were extracted from gel and used for recombinational cloning with binary vector pGreen-HDVrz. The pGreen-HDVrz vector is a modified pgR107 without PVX-coding sequence and with 94 nucleotides of *Hepatitis D virus* ribozyme (HDVrz) introduced upstream nopaline synthase (NOS) terminator. This was done by means of PCR with 20 ng of pgR107 template, pgrHDVrz_F and pgrHDVrz_R primers (125 ng each, Table 1) in a presence of deoxyribonucleotides, and *Pfu*Ultra II Fusion HS DNA Polymerase (Agilent Technologies). After the PCR, the remained plasmid template was digested with *Dpn*I (Thermo Fisher Scientific), whereas the resulted PCR product was 5′- phosphorylated, self-ligated and used for *Escherichia coli* (TOP10 strain, Thermo Fisher Scientific) transformation. The plasmid DNA was isolated using PureYield Plasmid Miniprep System (Promega, Fitchburg, WI, USA) and the presence of HDV ribozyme in pGreen-HDVrz was confirmed by sequencing. For cloning purposes the plasmid was linearised in PCR reaction (with primers HDVrzF and pGrdelR, Table 1).

Seamless recombinational cloning was done as described previously (Wieczorek et al., 2015). In details, 50 ng of the linearized and *Dpn*I-digested pGreen-HDVrz plasmid (vector) was mixed with 200 ng of cDNA copies of PSV-P genomic RNAs and satRNA, and 5 μl of GeneArt 2 × Enzyme Mix (Thermo Fisher Scientific) was added. The mixture was incubated at room temperature for 15 min and used for *E. coli* transformation (One Shot DH10B $T1^R$ SA cells,