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Development of new potato virus X-based vectors for gene over-expression and gene silencing assay

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ABSTRACT

Multiple plant viruses, including potato virus X (PVX), have been modified as vectors for expressing heterologous genes or silencing endogenous genes in plants. PVX-based vectors facilitate the functional analysis of genes in plant. However, they can only express one protein in a time. In this paper we report the construction of new vectors based on a 35S promoter-driven PVX infectious clone, pCaPVX100. Vector pCaPVX440 contains two additional subgenomic promoters and can be utilized to express two foreign genes at the same time. Plasmid pCaPVX760 is a CP minus vector and can be used to express foreign proteins through the gene substitution strategy. In addition, plasmid pCaPVX100 was engineered into a gene silencing vector (pCaPVX440-LIC) by introducing a ligation independent cloning (LIC) site into the vector. These results indicate that the newly developed PVX vectors are competent for multiple research purposes.

1. Introduction

In recent years multiple plant RNA and DNA viruses have been modified to serve as vectors for over-expressing and/or silencing genes in plants. Some virus-based vectors were used to produce heterologous proteins or peptides with commercial importance, including antibodies or vaccine antigens, in plant cells (Gellért et al., 2012; Gleba et al., 2007; Nuzzaci et al., 2007; Roy et al., 2010). In general, plant virus-based over-expression systems are more cost-effective and easier to use than the stable transformation technology. The virus-based vectors can also be used to study gene function via virus-induced gene silencing (VIGS) (Burch-Smith et al., 2004; Faivre-Rampant et al., 2004; Kumar et al., 2012; Purkayastha et al., 2010; Zhang et al., 2009, 2012). Silencing genes in plant often results in specific phenotypes that allow quick identifications of gene functions in plants. To date more than a dozen plant virus-based vectors have been used successfully to silence genes in plants, including apple latent spherical virus (Igarashi et al., 2009), barley stripe mosaic virus (Yuan et al., 2011), bean pod mottle virus (BPMV) (Zhang et al., 2009, 2010), brome mosaic virus (Ding et al., 2006), cabbage leaf curl virus (Turnage et al., 2002), cotton leaf crumple virus (Tuttle et al., 2008), grapevine leafroll-associated virus-2 (Kurth et al., 2012), pea early browning virus (Constantin et al., 2004), potato virus X (PVX) (Faivre-Rampant et al., 2004; Lacomme and Chapman, 2008), tobacco mosaic virus (TMV) (Kumagai et al., 1995), tobacco rattle virus (Burch-Smith et al., 2006; Kumar et al., 2012; Ratcliff et al., 2001; Valentine et al., 2004), and tomato golden mosaic virus (Peele et al., 2001).

PVX has a single-stranded genomic RNA of about 6430 nucleotides (nt) in length (Yu et al., 2010). The 5′ end of the PVX RNA contains an m^7GpppG cap structure and its 3′ end has a polyadenylate tail. The PVX genome has five open reading frames (ORFs). The ORF1 encodes a 164 kDa RNA-dependent RNA polymerase (RdRp) followed by three partially overlapped ORFs known as the triple gene block (TGB). The TGB encodes the 25 kDa TGBp1, the 12 kDa TGBp2, and the 8 kDa TGBp3, respectively. The last ORF in the PVX genome, ORF5, is also transcribed through a subgenomic promoter (SGP) and encodes the 25 kDa structural coat protein (CP). The TGBps and CP have been shown to function in PVX cell-to cell movement in plants (Fedorkin et al., 2001; Lough et al., 2000).

PVX genome was cloned behind a T7 RNA polymerase promoter or a cauliflower mosaic virus (CaMV) 35S promoter to serve as expression and/or gene silencing vectors (Lacomme and Chapman,

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2008). The PVX-based vectors have been successfully used to transiently express foreign genes; however, they can only express one protein in a time (Cerovska et al., 2012; Chapman et al., 1992; Lacomme and Chapman, 2008; Plchova et al., 2011). PVX-based vectors have also been used to silence endogenous genes through VIGS, so as to study the function of silenced gene (Faivre-Rampant et al., 2004; Lacomme and Chapman, 2008). However, digestion and ligation procedures limit its application in large scale cloning. In this paper, we constructed new PVX-based expression vectors that can express two foreign genes simultaneously in the same cells, or express foreign proteins through the gene substitution strategy. In addition, a VIGS vector was constructed, which carries a ligation independent cloning (LIC) site and can be used in high throughout silencing studies.

2. Materials and methods

2.1. Growth of test plants

Seeds of *Nicotiana benthamiana*, tobacco (*N. tabacum*) cv. NC89, tomato (*Solanum lycopersicum*) cv. Micro-Tom and potato (*S. tuberosum*) cv. Zaodabai were sown in soil. The seedlings were transplanted into pots at the 3- to 4-leaf stage, and allowed to grow in a growth chamber set at 25 °C and 16 h/8 h (light/dark) conditions.

2.2. Construction of PVX infectious clone

The PVX isolate 1985 (PVX1985; GenBank accession number EU571480) was from a previously published source (Yu et al., 2010) and maintained in *N. tabacum* plants. The PVX1985 sequence was amplified through reverse transcription (RT)-PCR from a total RNA sample extracted from infected *N. tabacum* leaves using a moloney murine leukemia virus (M-MuLV) reverse transcriptase (Transgen, China) and a Phusion DNA Polymerase (Thermo, Finland). To construct the full-length infectious clone of PVX1985, a 35S promoter and the 5′ end 548 bp sequence of PVX genome were fused together through overlapping PCR with primer pairs 1985-pro-F ('F' for forward) and 1985-pro-R ('R' for reverse), 1985-1-F and 1985-1-R (Table S1), and then ligated into vector pMD18-T simple to yield pMD18-T-1. The second fragment representing partial PVX sequence, position 543 to 3354, was amplified with the primer pair 1985-2-F and 1985-2-R. After digestion with *Eco*RI and *Xma*I enzymes, the fragment was ligated into pMD18-T-1 to yield pMD18-T-2. The 3′ half PVX genome, position 3349 to the 3′-end poly (A) tail, was amplified with the primer pair 1985-3-F and 1985-3-R, digested with *Bam*HI and *Xma*I, and then ligated into pMD18-T-2 to yield pMD18-T-PVX100. The full-length 35S promoter and the PVX1985 sequence were then inserted between the *Sal*I and *Xma*I site in pCambia0390. The resulting vector is referred to as pCaPVX100 (Fig. 1A). The PVX100 nucleotide sequence is numbered as the PVX sequence deposited in GenBank.

2.3. Construction of PVX-based over-expression and silencing vectors

To construct a pCaPVX100-based over-expression vector, we planned to introduce a multiple clone site to the region between TGB and CP ORF. However, the candidate restriction site of *Apa*I was also present in the plasmid pCambia0390. Therefore, we first obtained an insert containing three fragments. The first fragment contained the sequence from *Apa*I site (4945) to PVX CP SGP (Chapman et al., 1992; Skryabin et al., 1988) plus an *Asi*SI restriction site (GCG ATC GC). The second fragment contained the TMV CP SGP (Grdzelishvili et al., 2000) fused with the second cloning site (e.g. *Sac*I, *Bst*BI and *Mlu*I; GAG CTCGGT CCG GAG GTT CGA ACG ACG CGT). The third fragment contained the 3′-terminal sequence of PVX1985 genome from the PVX CP SGP to the 3′-end poly (A) tail. These three fragments were amplified separately with the primer pairs 1985-4-F/1985-7-R, 1985-6-F/1985-6-R, and 1985-7-F/1985-3-R, and fused by overlapping PCR with primer pair 1985-4-F and 1985-3-R. The insert was digested with *Apa*I and *Xma*I and cloned into pMD18-T-PVX100 to produce pMD18-T-PVX140. The 35S promoter sequence and the engineered PVX genome in pMD18-T-PVX140 were then cloned into pCambia0390 using the restriction sites *Sal*I and *Xma*I. The thymine in the first PVX CP SGP (position 5651) and TMV CP SGP (position 5859) were changed to guanine through mutagenesis PCR to abolish the start codon. The resultant plasmid was designated as pCaPVX440 (Fig. 2A).

The second over-expression vector pCaPVX760 was produced by replacing most of the PVX CP sequence (ORF5), position 6009 to 6,710, in the pCaPVX440 with three restriction sites (*Nhe*I, *Xho*I and *Nru*I; GCT AGCAAA CTC GAGTTT TCG CGA). The three *Nhe*I sites in pCaPVX440 were removed by changing GC**T** AGC to GC**A** AGC (Fig. 3A).

The fragments encoding the N- or C-terminal parts of an enhanced yellow fluorescent protein (eYFP) were amplified from vectors 35S-SPYNE and 35S-SPYCE (Walter et al., 2004), respectively, using the primer pairs YFP-Sac-F/Lap-YFP-R and Lap-YFP-F/YFP-Mlu-R (Table S1), and then used to generate the full length *eyfp* gene via overlapping PCR. The green fluorescent protein gene (*gfp*) was amplified from pTVBMV-GFP (Gao et al., 2012) through PCR using the primer pairs GFP-Asis-F/GFP-Asis-R and GFP-Nhe-F/GFP-Nru-R. The resultant *gfp* and *eyfp* fragments were inserted individually into vectors pCaPVX440 and pCaPVX760 to generate pCaPVX440-GFP, pCaPVX440-GFP-YFP and pCaPVX760-GFP.

Vector pCaPVX440-LIC (Fig. 4A) was produced by inserting a LIC sequence (AGG GTC TTG TCG TTC GAA CCC GAG AGG AGT A) (Aslanidis and de Jong, 1990; Dong et al., 2007; Yuan et al., 2011) containing a *Bst*BI restriction site (underlined sequence) between the *Sac*I and *Mlu*I sites in pCaPVX440. The *pds* gene was amplified with primer pairs Lic-PDS-F/Lic-PDS-R and Lic-PDSas-F/Lic-PDSas-R, in which the forward primers contained 5′-GGGTCTTGTCGTTCGA-3′ and the reverse primers contained 5′-CTCCTCTCGGGTTCGA-3′ at their 5′-termini. By using primer pairs Lic-PDS-F/Lic-PDS-R and Lic-PDSas-F/Lic-PDSas-R, the same region of the *pds* gene was amplified, but ligated into the silencing vector pCaPVX440-LIC in different orientations. The 717 bp fragment of *gfp* gene was amplified with primers Lic-GFP-F and Lic-GFP-R. The PCR products were purified, and then treated at 22 °C for 30 min with T4 DNA polymerase (New England Biolabs) in 1X reaction buffer containing 5 mM dTTP to generate sticky ends, and heated at 75 °C for 10 min to inactivate the polymerase. The *Bst*BI-linearized pCaPVX440-LIC vector was treated with T4 DNA polymerase in the presence of 5 mM dATP to generate sticky ends complementary to those of the PCR products mentioned above. The treated PCR products (~200 ng) and pCaPVX440-LIC vector (~20 ng) were mixed, incubated at 66 °C for 2 min and 22 °C for 10–30 min to anneal their complementary termini. Then 10 μL aliquots were used for transformation into *Escherichia coli* DH5α. Positive clones identified by plasmid PCR and restriction enzyme digestion were confirmed by sequencing.

2.4. Detection of the pds gene

The *pds* gene accumulation level in *N. benthamiana* was detected at 14 dpi by semi-quantitative RT-PCR using primers PDS-1-F and PDS-1-R, which were complementary to regions outside the one cloned into pCaPVX440-LIC in 2.3. The semi-quantitative RT-PCR reaction was conducted in a final volume of 20 μL, containing 250 ng of RNA and 0.5 μM of each primer. After 27 cycles, the PCR