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Transient expression of heterologous model gene in plants using *Potato virus X*-based vector

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Abstract

To optimize the efficiency of expression of foreign proteins using *Potato virus X* (PVX) – based vector, the gene for the coat protein (CP) of other virus (*Potato virus A*, PVA) was cloned into the vector, propagated in *E. coli* and subsequently inoculated or agroinfected into the host plants. Host range studies showed that the best host plant is *N. benthamiana*. By means of RT PCR the presence and the stability of the construct were tested. Both ELISA and Western blot analysis were applicable for expressed protein detection. Expression level of PVA CP achieved approximately 5–10 per mille of total soluble proteins. The results demonstrated that agroinfection is the most suitable method for the propagation of our model gene using PVX–based vectors.

Abbreviations: CP – coat protein; DAS-ELISA – double-antibody sandwich ELISA; PBS + T – phosphate buffered saline + Tween 20; SDS-PAGE – polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate; PVA – *Potato virus A*; PVX – *Potato virus X*; RT-PCR – reverse transcription–polymerase chain reaction; TAS ELISA – triple antibody sandwich ELISA

Introduction

The use of plants as expression systems for recombinant proteins usually relies on integration of a transgene into the plant genome. Unfortunately, this strategy often deals with variable levels of expression of the transgene in different plant lines produced with the same construct (Hobbs et al., 1990). The alternative approach is based on transient expression systems which are fast, flexible, unaffected by chromosomal positional effects and can be used in fully differentiated plant tissues (Fischer et al., 1999). Transient systems based on virus allow expression of foreign genes at higher levels in infected tissues than is normally the case in transformed plants (Yusibov et al., 1999). Because of its simplicity and rapidity, this way of protein expression is the most promising one for the industrial production as well as a research tool for preparation and biochemical characterization of a wide range of proteins.

For these purposes several viral vectors were constructed using the full-length cDNA of plant RNA viruses. The systems most frequently used are *Cowpea mosaic virus*, *Potato virus X* (PVX) and *Tobacco rattle virus* (O'Brien et al., 2000; Ratcliff et al., 2001; Liu and Lomonossoff, 2002). In most cases, cDNA of the genomic viral RNA was coupled to different strong promoters (CaMV 35S) and to the 'noster' region, the transcriptional terminator from the nopaline synthase gene of *Agrobacteium tumefaciens* (Angell and Baulcombe, 1997). Then, as in the case of PVX-based vector, a multiple cloning site was introduced in the vicinity of the 5′ region of coat protein gene (Angell and Baulcombe, 1997).

Our project is focused on the expression of the heterologous proteins in plant host cell using viral

PVX-based expression vector pGR106 (Angell and Baulcombe, 1997). The conditions that led to the increase of yields in heterologous protein production were screened. As a model protein for expression the coat protein (CP) from *Potato virus A* (PVA, genus *Potyvirus*) was chosen because of the efficient and reliable way of detection by means of our own polyclonal and monoclonal antibodies. There was no need to optimize the coding sequence of the plant viral model gene, and no reasonable homology that could impair the expression of the model gene by means of gene silencing was found for PVX CP and PVA CP (Al-Kaff et al., 1998).

Appropriate primers for cloning PVA-CP into plasmids suitable for propagation in *E. coli* and for the recloning into PVX-based vector were designed. Optimization of infection namely the choice of host plant, comparison of inoculation with or without *Agrobacterium tumefaciens*, and comparison of different intervals for passaging was done. To screen plant sap from infected plants ELISA, Western blot and RT-PCR tests were used.

Materials and methods

DNA construct

The PVA CP sequence was inserted into the cloning site of pGR106, kindly provided by D. Baulcombe, The Sainsbury Laboratory, Norwich, United Kingdom (Figure 1).

Cloning of CP gene.
For cloning of PVA CP into pGR106 we came out from our construct prepared for raising antibodies against recombinant PVA CP (Čeřovská et al., 2002). On the immuno-captured PVA virus particles propagated in *Nicotiana tabacum* cv. Samsun reverse transcription was performed as described in Čeřovská et al. (2002). The PVA-CP-3D primer (AAAAA**AGATCT**TTTCCCACTCAAACTCAC TGTTG) used for this purpose was designed according to the sequence of isolate PVA LIN, Acc. No. Z21670, (Puurand et al., 1994) and modified to carry the restriction site for enzyme *Bgl*II (underlined). The same downstream primer was used in the subsequent PCR together with the upstream primer PVA-CP-5A: (AAAA**CC*ATGG*** AAGCCGGAACTCTTGATGC) that contains *Nco*I restriction site on its 5′end (underlined) and also provides the ATG translation start codon (in italics). The amplified product of 964 bp was cleaved with above mentioned enzymes and inserted into pMPM4Ω expression vector (Mayer, 1995). From this vector the construct was recloned into pBluescriptSK II + (Stratagene) that contains suitable restriction sites *Not*I and *Cla*I for further recloning of PVA CP into pGR106. Sequence authenticity was confirmed by sequencing the construct in pBluescript.

Plant infection

Several host plants, namely *N. benthamiana*, *N. clevelandii*, *N. debney, Nicotiana tabaccum Samsun* and *Nicotiana tabaccum SR1* were infected with vector pGR106 carrying PVA CP construct, isolated from *E. coli*. The infection was accomplished by abrading the surfaces of two leaves per plant with carborundum and inoculating each leaf with approximately 20 μg of plasmid DNA. The infection was verified by ELISA and immunocapture and reverse transcription-PCR (IC RT-PCR) 7, 14 and 21 days after inoculation. The plasmid with the cloned fragment isolated from *E. coli* was also used for transformation by electroporation of *Agrobacterium tumefaciens* and bacteria containing the plasmid were selected on L-agar plates containing 50 μg ml^{-1} kanamycin. Bacterial suspension for agroinfection of plants was prepared to saturation in L-broth containing 50 μg ml^{-1} kanamycin at 28 °C. The agroinfection

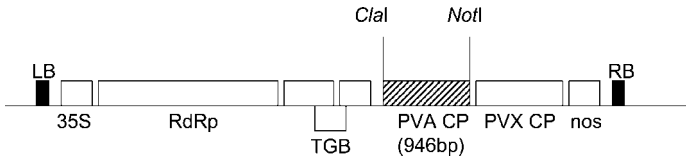

Figure 1. Schematic diagram of the genomic organization of PVX-based vector with our PVA CP construct; RdRp- RNA-dependent-RNA polymerase; TGB- triple gene block; LB and RB – left and right borders.

of plants was accomplished by abrading the surfaces of two leaves per plant with carborundum.

ELISA

The presence of PVX or PVA CP in the sap of infected plants was assayed by enzyme-linked immunosorbent assay (ELISA) and immunobloting.

Individual samples of infected leaves were subjected to various types of ELISA using our antibodies according to Filigarová et al. (1994). All types of ELISA arrangements were employed using buffers described by Clarck and Adams (1977).

IC RT-PCR

Briefly, the tubes were coated with 100 μl anti-PVX IgG (1 μg ml^{-1}) in coating buffer for 3 h at 37 °C. The wells were then washed (3 × 150 μl PBS + T) and 100 μl of the homogenate of pGR106 infected leaves in conjugate buffer (1:10) was added (Čeřovská et al., 1998). The samples were incubated overnight at 4 °C and washed again three times in PBS + T. After the last wash the reverse transcription and amplification by the Access reverse transcription PCR (RT-PCR) Kit (Promega) under manufacturer's conditions was done. The presence of PVX RNA was tested using primers for the PVX coat protein (5-PVX-CP: TAGCACAACACAGCCCATAGG and 3-PVX-CP: GGCAGCATTCATTTCAGCTTC, giving rise to the fragment of 661 bp). The stability of construct was tested using primers PVA-CP-5A and PVA-CP-3D that were used in the cloning procedure.

Polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE)

The 1 ml of the sap extracted from leaves of infected plants was pelleted by 1 min centrifugation, resuspended in 100 μl of the Laemmli buffer, boiled for 2 min and aliquots were loaded on 12% polyacrylamide gel containing SDS (Laemmli, 1970). For visualization of the separated proteins Coomassie Brilant Blue R250 was employed.

Western blot analysis

For these purposes, the proteins separated by SDS-PAGE were electroblotted to a nitrocellulose membrane (0.45 μm, Sleicher & Schuell Protran) in semidry system (OMNI-TRANS apparatus, Omnibio Brno, Czech Republic) according to Hirano and Watanabe (1990). After blotting the membrane was stained nonspecifically by Ponceau S (Sigma Aldrich). The membrane was then incubated 1 h in 4% bovine serum albumin (BSA) in PBS and then washed four times in PBS. For PVA–CP detection the polyclonal antibodies prepared in our laboratory (Šubr et al., 1996) were used. The bands of interest were visualized by reaction with a substrate BCIP/NBT (Sigma) according to Sambrook et al. (1989).

The concentration of expressed PVACP was estimated on the basis of recombinantly prepared PVA CP (Čeřovská et al., 2002).

Results and discussion

To assess whether the level of expression of foreign proteins by means of pGR106 might affect the range of plants the virus can infect, several different host plants were used, namely *N. benthamiana, N. clevelandii, N. debneyi*, *N. tabacum Samsun* and *N. tabacum* SR1. The host range for our chimaera proved to be identical to that of wild type PVX, suggesting that the additional heterologous gene did not cause the changes in the virus capability to infect the host plants.

Two different ways of primary inoculation were performed: mechanical inoculation (application of the plasmids on to plants after gentle rubbing the leaves with carborundum), or alternatively agro-infection (electroporation of the plasmid into *Agrobacterium tumefaciens*, growing transformed bacteria to log phase and mechanical inoculation of the bacteria onto tobacco leaves). Additionally, in both approaches, the passaging of the infectious material to the new host was performed from the leaves harvest 1 week after the initial inoculation.

The ELISA, RT-PCR and Western blot analysis for testing the presence of both PVX vector and PVA construct were done in plant samples in course of time after inoculation. The RT-PCR confirming the presence of PVX was done with primers covering the 5′ and the 3′ region of PVX coat protein; the presence of inserted PVA CP gene was tested with primers that were used in cloning procedure (see Material and methods).

The highest value in ELISA testing for the presence of PVX coat protein was achieved in infected *N. benthamiana*, and they were always higher in agroinfected plants comparing to plants inoculated with plasmid DNA. The material from infected *N. benthamiana* was tested in course of the time. Seven days, 14 and 21 days post infection, the presence of viral particles and intact PVX coding region was confirmed by RT-PCR. However, ELISA showed detectable amounts of PVX CP only in the case of plants inoculated by agroinfection, and the values were even slightly elevated during the course of time (Table 1).

Similarly, the RT-PCR pattern of presence of PVA CP gene in viral RNA gave the best results in the case of agroinfected plants. The PCR fragment of approximately 946 bp was amplified only in the sap from agroinfected leaves and PVA fragment was detectable even 3 weeks after agroinfection (Figure 2). When the passaging of the infectious material was performed 1 week after the primary infection, 7 and 14 days after the passaging, PVX and PVA-CP was detectable by ELISA only when agroinfection was the primary route of inoculation.

As for PVX, the highest value in ELISA for the presence of PVA CP was determined in infected *N. benthamiana*, however the achieved values of absorbance were lower than for PVX measurements (Table 2). The positive values of absorbance in ELISA for PVA CP riched the highest values in 14 days after the vector inoculation.

The more susceptible approach for the detection of presence of PVA CP in infected leaves was performed. Total soluble protein extracts were prepared from apical leaves and subjected to immunoblotting. A product of expected molecular mass (approximately 32 kD) was identified in the leaf extracts from *N. benthamiana* infected by the construct PVA CP in pGR106 by means of agro-

Table 1. Time course detection of PVX in agroinfected tissue in direct DAS-ELISA

Material tested	7 days after inoculation	14 days after inoculation	21 days after inoculation
Buffer	0.20[a]	0.20	0.20
Healthy plants (*Nicotiana benthamiana*) (dilution 1:10)	0.27	0.21	0.21
Infected leaves (*Nicotiana benthamiana*) (dilution 1:10)	1.20	1.8	2.1
Infected leaves (*Nicotiana tabacum SR1*) (dilution 1:10)	1.18	1.02	0.97
Infected leaves (*Nicotiana debney*) (dilution 1:10)	0.59	–	–
Infected leaves (*Nicotiana tabacum,* cv. Samsun) (dilution 1:10)	0	0	0
Infected leaves (*Nicotiana clevelandii*) (dilution 1:10)	0	0	0

[a] A_{405} values following 60 min incubation in DAS-ELISA: Plates were coated with antibodies against PVX (1 μg ml^{-1}) in standard coating buffer and incubated at 37 °C for 4 h. The ELISA plates were washed with PBST four times. Plant samples in conjugate buffer were coated and the plates were incubated overnight at 4 °C. Anti PVX antibody conjugated to alkaline phosphatase diluted 1:1000 were added and incubated 3 h at 37 °C. Finally the plates were washed and substrate (0.1 mg *p*-nitrophenyl phosphate/ml of 0.1 M diethanolamine buffer, pH 9.8) was added.

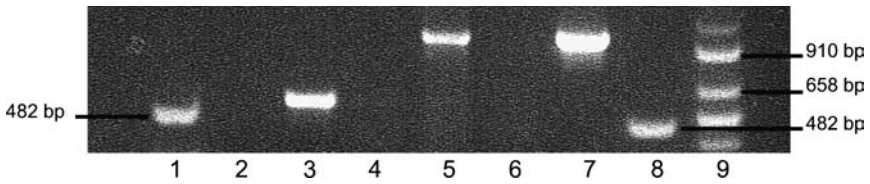

Figure 2. The electrophoresis of IC RT PCR for PVX and PVA CP genes from the *N.benthamiana* harvested 3 weeks after initial infection. PCR for presence of PVX CP: lane 1: marker for PVX CP gene; lane 2: healthy plant (negative control); lane 3: agroinfected plant; lane 4: plant infected by mechanical inoculation of plasmid DNA. PCR for presence of PVA CP gene: lane 5: agroinfected plant; lane 6: plant infected by mechanical inoculation of plasmid DNA; lane7: PCR on pGR vector with cloned PVA CP gene (positive control); lanes 8 and 9: markers. PCR with samples from other host plants and from the healthy plant for the presence of PVA CP were negative (data not shown).

Table 2. Detection of PVACP in pGR106 infected *N. benthamiana* tissue in direct DAS and TAS ELISA after 14 days post-inoculation

	A_{405}	
	Detection with MAb 151 (TAS ELISA)	Detection with polyclonal antibodies (DAS ELISA)
Buffer	0.15	0.15
Healthy plants (dilution 1:10)	0.24	0.15
pGR106 with insereted PVA CP-infected leaves (dilution 1:10) – first transmission	0.636	0.358

A_{405} values following 60 min incubation. MAb 151 was obtained by immunization of mice with PVA-LI isolate (Filigarova et al., 1994). DAS ELISA was done identically as in Table 1. TAS-ELISA: plates were coated with polyclonal anti-PVA IgG diluted in standard coating buffer 3 h at 37 °C. The plates were washed with PBST four times and plant samples in conjugate buffer were coated and the plates were incubated overnight at 4 °C. After washing MAb 151 (1 μg ml^{-1}) in conjugate buffer were added and incubated for 2 h at 37 °C. Swine anti-mouse IgG conjugated to alkaline phosphatase (SWAM-AP, Sigma Aldrich), diluted 1:10,000, was added and incubated for 3 h at 37 °C. Finally, the plates were washed and the substrate (0.1 mg ml^{-1} p-nitrophenyl phosphate in 0.1 mol l^{-1} diethanolamine buffer, pH 9.8) was added.

infection (healthy and plants inoculated with empty pGR106). We detected this product in all samplings in time course of the experiment, it means after 7, 14 and 21 days after inoculation. Determined expression level of PVA CP achieved approximately 5–10 per mille of total soluble proteins. This results were estimated on the basis of the scale prepared from PVA CP expressed in *E. coli* and its evaluation by means of our anti-PVA polyclonal antibodies in Western blot analysis.

The above results demonstrate the other evidence that agroinfection is an efficient way of initiating infections with a RNA virus (Liu and Lomonossoff, 2002), in our case PVX. This demonstration will be exploited to successfully agroinfect *N. bethamiana* with PVX-based chimaeras expressing epitopes from *Human pappiloma virus* proteins either in fusion with foreign gene (PVA CP), or in fusion with PVX CP.

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