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Virus-like particles of potato leafroll virus as potential carrier system for nucleic acids*

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Potato leafroll virus is a member of the polerovirus genus. The isometric virion is formed by a coat protein encapsidating single-stranded, positive-sense, mono-partite genomic RNA with covalently attached viral protein at the 5′ end. The coat protein of the virus exists in two forms: i) a 23 kDa protein, the product of the coat protein gene, and ii) a 78 kDa protein, the product of the coat protein gene and an additional open reading frame expressed by read-through of the coat protein gene stop codon. The aim of this work was the expression of potato leafroll virus coat protein-based proteins that would be able to assemble into virus-like particles in insect cells. These modified particles were tested for their ability to encapsidate nucleic acids. Two types of N-terminally His-tagged coat protein constructs were used for the expression in insect cells: one, encoding a 23 kDa protein with the C-terminal amino-acid sequence corresponding to the wild type coat protein and the second with additional clathrin binding domain at the C-terminus. The expression of these two proteins by a recombinant baculovirus was characterized by Western immunoblotting with antibodies directed against potato leafroll virus. The protection or putative encapsidation of nucleic acids by these two coat protein derivatives was shown by DNase I and RNase A protection assays.

Keywords: Virus-like particles, encapsidation

Virus-like particles formed by the structural elements of viruses have received considerable attention over the past two decades. When expressed in a suitable heterologous system, viral structural proteins involved in capsid or envelope formation often self-assemble into VLPs in the absence of other viral components usually required for virus assembly, such as multiple structural or non-structural proteins and viral genomes. The protein–protein interactions in VLPs are relatively strong and can result in the formation of stable structures. Depending on the nature of the viral protein, such structures can be modified on their surface in order to introduce foreign epitopes, and they may be used to encapsidate non-viral nucleic acids or small proteins.

Potato leafroll virus particles consist of a single-stranded positive sense RNA about 5.8 kb long encapsidated in the major coat protein of 23 kDa (Mayo *et al*., 1989; van der Wilk *et al*., 1989). Sequence analysis of a Polish isolate of PLRV (Palucha *et al*., 1994) confirmed the presence of several open reading frames. The viral CP gene is expressed in two forms: as a 23 kDa protein and a 78 kDa fusion protein after read-through of the stop codon during translation of the CP gene (Bahner *et al*., 1990).

The assembly of VLPs of PLRV in insect cells infected by a recombinant baculovirus containing the CP gene, modified at the N-terminus by a His-tag (MHHHHHHGDDDDKDAMG), was previously reported (Lamb *et al*., 1996). The sedimentation coefficient of such VLPs in sucrose gradients was similar to that of viral particles, suggesting the presence of encapsidated nucleic acids. In our study we expressed two types of VLPs, one with the C-terminus of the CP corresponding to the wild type protein and the second with a clathrin binding domain attached to the end of the CP. Protection of small nucleic acids by the PLRV CP was shown in a DNase I and RNase A protection assay.

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Abbreviations: BCIP, 5-bromo-4-chloro-3-indolyl-phosphate; CP, coat protein; NBT, 4-nitro blue tetrazolium chloride; PBS, phosphate-buffered saline; PLRV, potato leafroll virus; VLPs, virus-like particles.

MATERIALS AND METHODS

Materials. Appropriate fragments of the CP cDNA were amplified by PCR using one 5′ primer (h) and two 3′ primers (CP and CPc). Primer h was 5′ ATGCATCACCATCACCATCACGGGGACGATGACG ATAAAGACGCCATGGGT**ATG**AGTACGGTCGTGGTTAAAGG, primer CP was 5′ AAGCTT**CTA**TTTGG GGTTTTGCAAAG and primer CPc was 5′ AAGCTT**CTA***ATCCAGATCCAGCAG*TTTGGGGTTTTGCAAAG. The initiation codon (primer h) and the complement of the termination codon (primers CP and CPc) are in bold. The complement of the clathrin binding domain is in italics (primer CPc). PCR products were cloned into pGEM-T Easy cloning system and then subcloned to the pFastBac™1 vector in order to obtain plasmids pFhCP and pFhCPc suitable in construction of recombinant baculoviruses.

Purification and expression of recombinant baculoviruses. Using the Bac-to-Bac® system, purified DNA of donor plasmids pFhCP and pFhCPc was transformed into competent DH10Bac™ *Escherichia coli* cells in order to obtain, after *in vivo* transposition, recombinant BhCP and BhCPc Bacmid DNA. Transfection of *Spodoptera frugiperda* (Sf9) cells and expression of particular recombinant virus was managed according to the manufacturer's protocol.

Fractionation and purification of VLPs. Insect cells expressing appropriate VLPs were incubated for 72 and 96 h. Then, the cells were collected by centrifugation at 1000 r.p.m. for 10 min at 15°C from 25 ml cultures and resuspended in 10 ml of 0.1 M sodium citrate, pH 6, containing 0.4 M NaCl. After vortexing for 10 min with glass beads, the extracts were centrifuged at 8000 r.p.m. for 15 min at 10°C (Sigma 3K30, 12158) and the supernatant fraction was additionally centrifuged for 10 min at 15000 r.p.m. at 10°C. Particles were recovered from the final supernatant by centrifugation for 3 h at 45000 r.p.m. at 10°C. Pellets were resuspended in 0.5 ml of 10 mM sodium phosphate, pH 7.

Immunoblotting. Proteins were separated on 12% polyacrylamide/SDS gels and electroblotted onto Hybond-C membranes as described by Ausubel *et al*. (1987). Membranes were blocked for 4 h using 5% non-fat milk and 0.1% Tween 20 in PBS buffer at room temperature and then incubated for 1 h with anti-potato leafroll virus antibody conjugated with alkaline phosphatase (Boehringer Mannheim GmbH) diluted in blocking buffer at 1/5000. Blots were then washed in PBS and the alkaline phosphatase activity was developed using NBT/BCIP solution (Roche).

RNase A and DNase I treatment. Purified protein extracts (200 µl) carrying PLRV VLPs and extracts from insect cells infected with non-recombinant baculovirus were mixed with 200 µl of 2 x reaction mixture containing 40 mM Tris/HCl, pH 8.3, 4 mM $MgCl_2$, 20 units of deoxyribonuclease I (Sigma, D5307) and 200 µg of ribonuclease A (ICN, 193280). After incubation for 1 h at 37°C the remaining nucleic acids were extracted by vortexing with phenol and phenol:chloroform and precipitated by 2 vol. of ethanol after addition of 1/10 vol. of 3 M sodium acetate, pH 4.8.

RESULTS AND DISCUSSION

To express in insect cells the coat protein of potato leafroll virus modified at the N-terminus, two recombinant baculoviruses were constructed. BhCP, which contain cDNA encoding the CP unmodified at the C-terminus and BhCPc carrying cDNA with the gene modified by addition of a clathrin binding domain sequence. The modified constructs were obtained using PCR amplification and subcloning of PCR products (Fig. 1). The cloned DNA from pFhCP and pFhCPc was amplified by PCR using universal primers and the products were sequenced. These plasmids were used for all protein expression experiments.

Proteins present in extracts from Sf9 cells infected with non-recombinant baculoviruses or from Sf9 cells infected with a recombinant one containing either the hCP or hCPc constructs were fractionated by SDS/PAGE. Staining with Coomassie brilliant blue showed a polypeptide of about 27 kDa in cells infected with BhCP and of about 28 kDa in cells infected with BhCPc, that were not present in cells infected by non-recombinant baculovirus (Fig. 2).

In immunoblotting experiments two major polypeptides of 27 kDa for the BhCP recombinant and 28 kDa for the BhCPc recombinant, corresponding to the Coomassie-stained bands, were detected. Additional polypeptides of about 24 kDa and 25 kDa appeared for hCP and hCPc, respectively, probably as a result of protease activity in insect cells (Fig. 3).

A fraction of soluble proteins carrying VLPs from recombinant baculoviruses BhCP and BhCPc and similarly prepared proteins from non-recombinant baculovirus were treated with DNase I and RNase A. After extraction the remaining nucleic acids were separated on 1.5% agarose (Fig. 4). It was shown that small heterologous nucleic acids were protected in the presence of the modified coat proteins of PLRV. The size of the putatively encapsidated nucleic acids is rather small and lies between the size of tRNA and the size of a single-stranded RNA of 200 nucleotides.

Our preliminary results show that the coat protein of potato leafroll virus is a good candidate for further experiments leading to the engineering of native or modified virus-like particles which could protect or encapsidate small nucleic acids. Additional *in vitro* assembly studies on PLRV VLPs will be

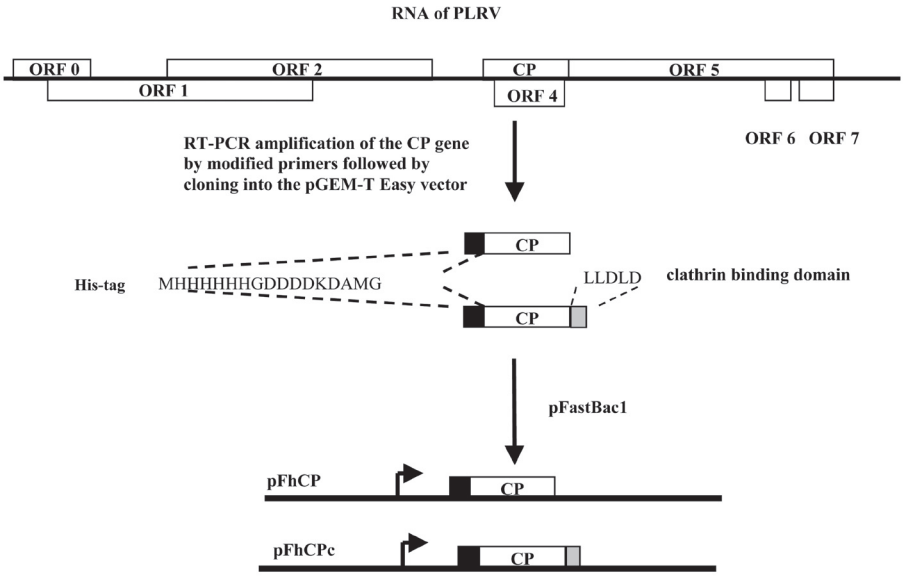

Figure 1. Scheme of cloning of the PLRV CP gene and construction of donor FastBac plasmids.

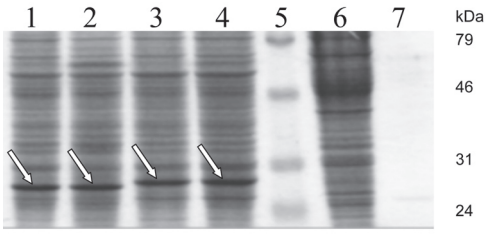

Figure 2. SDS/PAGE of proteins from Sf9 cels.
Lane 1, cells infected with BhCP after 72 h; lane 2, cells infected with BhCP after 96 h; lane 3, cells infected with BhCPc after 72 h; lane 4, cells infected with BhCPc after 96 h; lane 5, prestained molecular mass marker (Fermentas), lane 6, cells infected with non-recombinant baculovirus; lane 7, CP of PLRV. Arrows indicate the infection-specific polypeptides. The gel was stained with Coomassie brilliant blue.

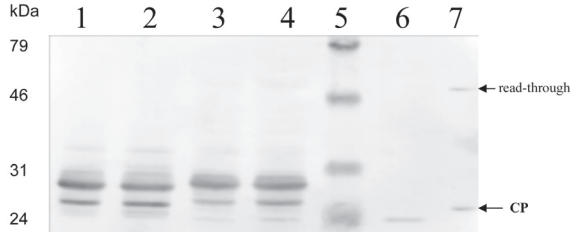

Figure 3. Immunoblotting of proteins from Sf9 cels.
Lane 1, cells infected with BhCP after 72 h; lane 2, cells infected with BhCP after 96 h; lane 3, cells infected with BhCPc after 72 h; lane 4, cells infected with BhCPc after 96 h; lane 5, prestained molecular mass marker (Fermentas), lane 6, cells infected with non-recombinant baculovirus; lane 7, CP of PLRV. Anti-PLRV antibodies linked to alkaline phosphatase were visualized by the NBT/BCIP assay.

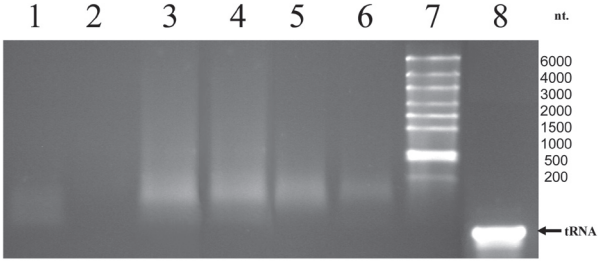

Figure 4. Non-denaturing 1.5% agarose gel with nucleic acids derived from PLRV VLPs treated with DNase I and RNase A for 1 h at 37°C.
Lanes 1 and 2, cells collected after 72 and 96 h after infection with non-recombinat baculovirus; lanes 3 and 4, cells collected 72 and 96 h after infection with BhCP; lanes 5 and 6, cells collected 72 and 96 h after infection with BhCPc; lane 7, RNA Ladder High Range (Fermentas) 0.2, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0 and 6.0 kb; lane 8, yeast tRNA.

focused on encapsidation of nucleic acids like oligonucleotides, PNA or siRNA.

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