New positions for peptide presentation in *Potato virus X* capsid protein

Abstract: *Potato virus X* (PVX) is widely used as a peptide presentation system in plant biotechnology, mostly for transient expression of desired peptides fused to the N-terminus of its capsid protein (CP). Here we describe testing/investigation of new positions for peptide presentation based on seven putative surface loops of PVX CP. We performed bacterial expression of fourteen different PVX CPs modified by the insertion of the epitope derived from the E7 oncoprotein (E7 epitope) of *Human papillomavirus* type 16 fused with His tag. The expression from vector pMPM-A4Ω in *Escherichia coli* MC1061 was performed to evaluate the capacity of the PVX CP platform to tolerate the insertion of E7 epitope fused with His tag in the seven putative surface loops. The immunological characteristics of expressed epitopes were assessed by Western blot using both anti-PVX CP and anti-His antibodies and by immunoelectron microscopy.

Keywords: Bacterial expression, *Potato virus X*, *Human papillomavirus*, E7 oncoprotein

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1 Introduction

Presentation of peptides on virus particles is a promising strategy for the development of peptide-based vaccines. Currently, the development of peptide-based vaccines is limited because the need of suitable strong adjuvants [1,2]. The peptide presentation system based on plant viruses represents potentially a promising new delivery and adjuvant system mainly for the following reasons: (i) the capsid of plant virus represents a scaffold exposing desired epitopes to the immune system in an abundant and organized manner and can serve as an adjuvant by itself, (ii) plant viruses are not infectious to animals, lowering the safety risk associated with their deployment, and (iii) scalability [3,4].

*Human papillomavirus* (HPV) infection is one of the most common sexually transmitted infections worldwide. Infection by high risk HPVs is causally associated with cervical cancer, which represents the second most common cancer among women (after breast cancer) [5]. The most common oncogenic HPV genotypes are 16 and 18, together causing approximately 70 % of all cervical cancers, while HPV 16 alone causing 50 % of all cervical cancers [6]. The E7 oncoprotein plays an important role in both malignant transformation and in the maintenance of the transformed state of HPV infected cells. This represents a promising target for immunotherapy because the E7 oncoprotein is expressed in all cervical tumors and in precancerous lesions [7]. The main known role of E7 oncoprotein in virus cycle is to prevent cell death by binding tumor-suppressor protein Rb (pRb), resulting in the dissociation of pRB from E2F-family transcription factors and the premature cell progression into the S phase of the cell cycle which leads to uncontrollable cell division [8].

*Potato virus X* (PVX) belongs to the order *Tymovirales*, family *Alphaflexiviridae* and genus *Potexvirus*. It is a filamentous, positive single stranded RNA (+ssRNA) virus. Virus particles are about 515 nm long and have about 13 nm in diameter. The PVX genome is capped, has a poly(A) tail and encodes five open reading frames (viral replicase, three movement proteins belonging to the triple-gene block family and capsid protein - approximately 1 300 CP subunits per virion) [9]. The PVX CP can be used for presentation by direct or indirect fusion of a desired protein or peptide to the N-terminus [10-12]. Recently, it has been also shown that the C-terminus of PVX CP can be used for protein presentation [13]. However, the
presentation of proteins/peptides this way is limited due to their isoelectric point, tryptophan content and perhaps their conformation which can influence the formation and stability of modified viral particles [14]. The E7 oncoprotein from HPV 16 is an attractive candidate for anti-cancer therapeutic vaccine development. The effort to express this epitope is related to previous work on the expression of mutated E6 and E7 oncoproteins of HPV 16 using a PVX-based expression vector [15-18].

The latest model representing the PVX CP tertiary structure in a virion predicts loops, which are likely exposed on the virion surface (Fig. 1) [19]. These putative surface loops could be promising candidates for new positions to present proteins or peptides. To examine this hypothesis, we decided to insert an epitope derived from the E7 oncoprotein (E7 epitope; amino acids 44-60; QAEPDRAHYNIVTFCCK) of Human papillomavirus type 16 (HPV 16) fused with 6xHis tag into these loops.

2 Methods

2.1 DNA constructs

The PVX CP sequence was amplified by PCR from the binary vector pGR106 (kindly provided by Dr. D.C. Baulcombe, Department of Plant Science, University of Cambdrige, United Kingdom). The E7 epitope sequence was prepared by PCR from vector pBSC-E7ggg (kindly provided by M. Smahel, IHBT Prague, Czech Republic). The cloning strategy and primers were designed for creating fourteen different constructs of PVX CP in pMPM-A4Ω (Fig. 2A, 2B; Table 1) [20]. The nucleotide sequences of short peptides comprising 6xHis tag fused either to the 5’or 3’ terminus of the E7 epitope (6xHisE7 and E76xHis, respectively) were inserted by splicing the overlap extension (SOE) PCR [21] into seven putative PVX CP surface exposed loops: 66/67 nt (region of predicted loop A: 67-72 nt), 459/460 nt (region of predicted loop B: 457-468 nt), 576/577 nt (region of predicted loop C: 574-582 nt), 669/670 nt (region of predicted loop D: 661-681 nt), 159/160 (region of predicted loop E: 154-162 nt), 213/214 (region of predicted loop F: 211-213 nt) and 534/535 (region of predicted loop G: 526-552 nt) [19].

2.2 Expression of modified PVX CP proteins in E. coli

Each construct in pMPM-A4Ω, an arabinose-inducible expression vector, was expressed in E. coli MC1061. We performed an optimization of expression at two different temperatures (22°C and 37°C) and three different durations of expression (2, 4 and 16 h after the induction). To estimate the production of fusion proteins PVX CP-6xHisE7 and PVX CP-E76xHis in bacteria, the cells were lysed and separated into soluble and insoluble fractions. The fractions were subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) and subsequently Western blot analysis was performed.

Fig. 1: Predicted structure of PVX CP in a virion[15]. Positions of loops A, B, C, D, E, F and G are indicated.
Fig. 2: Schematic illustration of cloning strategy: (A) PVX CP modified with 6xHisE7. (B) PVX CP modified with E76xHis. (C) Restriction cloning into pMPM-A4Ω. pCP - partial capsid protein; PBAD - arabinose promoter; term - transcription terminator. Restriction sites EcoRi and SalI, and putative surface loops A, B, C, D, E, F and G are indicated.

A) PVX CP modified with 6xHisE7 B) PVX CP modified with E76xHis C) Restriction cloning into pMPM-A4Ω
Table 1: Deoxyribonucleotides used for preparation of fusion constructs in pMPM-A4Ω.

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2.3 Preparation of soluble and insoluble fractions

The bacterial soluble and insoluble fractions were prepared according to Cerovska et al., 2003 [23]. Sediments containing insoluble proteins (denatured proteins and protein bodies) were solubilized with 1% SDS. Samples for SDS-PAGE were prepared from purified soluble and insoluble fractions and detection of fusion proteins was performed by Western blot analysis. Protein concentration in each soluble and insoluble fraction was determined by DC Protein Assay kit (Bio-Rad). The purity of pelleted capsid protein was estimated from Coomassie blue stained gel.

2.4 SDS-PAGE and Western blot analysis

Samples for SDS-PAGE were prepared according to a previously published protocol [24]. Proteins separated by SDS-PAGE were electroblotted to a nitrocellulose membrane (Protran 0.2 µm, Schleicher & Schuell) in a semidry system (OMNI-TRANS apparatus, Omnibio Brno, Czech Republic) [25]. After electroblotting, the membrane was blocked in 5% nonfat dry milk for 1 h. Incubation was performed overnight with the primary mouse anti-His antibody (1:3,000 in PBS, GE Healthcare Life Sciences). After washing, the secondary goat anti-mouse IgG (anti whole molecule, 1:30,000 in PBS, Sigma-Aldrich) conjugated to alkaline phosphatase was added. Visualization was performed according to Sambrook et al. [26].
2.5 Immunoelectron microscopy

Immunoelectron microscopy was performed according to Cerovska et al., 2008 [17]. Briefly, modified viral proteins were immobilized either by mouse monoclonal anti-6xHis antibody (GE Healthcare Life Sciences, dilution 1:1000 in PBS, 30 min) or by rabbit polyclonal anti-PVX-CP antibody (PrimeDiagnostics, dilution 1:1000 in PBS, 30 min) followed by washing the grids and detection with respective gold conjugate (goat anti-mouse 10 nm Au, goat anti-rabbit 5 nm Au, both Sigma, 1:20 in PBS). In a set of experiments the viral proteins were decorated with both antibodies simultaneously. After additional washing the samples were stained with 2% ammonium molybdate (pH 7.0). Electron microscopy was carried out either on the JEM 1010 transmission electron microscope (Jeol, Japan, facility of Biology Centre of Academy of Sciences, Ceske Budejovice, kindly performed by Dr. Synkova, IEB AS CR, Prague) or on FEI Morgagni 268D (FEI, Oregon USA) at IEB, Prague.

3 Results

3.1 Insertion of the E7 epitope into the seven putative surface exposed loops of the PVX CP

Both experimental epitopes (E7-6xHis and 6xHis E7) were inserted by SOE PCR in all seven loops creating a total of 14 constructs (Fig. 2). Primers designed for this purpose are listed in Table 1. Each construct was cloned into the bacterial expression vector pMPM-A4Ω using restriction sites EcoRI and SalI (Fig. 2C). The resulting plasmids were named PVX CP-6xHisE7 (A, B, C, D, E, F and G) and PVX CP-E76xHis (A, B, C, D, E, F and G).

Our objective was to evaluate the capacity of the PVX CP platform to tolerate insertion of E7 epitope fused with His tag in the seven different positions. We used the His tag to simplify verification of peptide presence by antibodies.

Insertions were made at seven specific sites in the PVX CP taking into consideration the bioinformatic prediction of the PVX CP structure based on tritium planigraphy [15, 27]. Thereby, fusions were made in the seven different loops connecting α-helices and β-strands of PVX CP which are assumed to be located on the exterior part of the molecule.

The first loop A (amino acids AT) was located between the putative β2-strand and α1-helix, the second one B (amino acids KPEH) between β3-strand and α6-helix, the third one C (amino acids QTA) between α4-strand and β5-strand, the fourth one D (amino acids ITGTTTA) between α7-helix and β6-strand. The fifth loop E (amino acids VAT) was located between α2-helix and α3-helix, the sixth loop F (amino acid P) between α3-helix and α4-helix, the seventh loop G (amino acids KEGLIRPPS) between α6-helix and β4-strand.

All mentioned constructs were transformed into *Escherichia coli* (*E. coli*) strain MC1061 by heat-shock method [22] and verified by sequencing.

Optimization of expression was performed. To illustrate the results, the immunoblots for four loops (A,B,C,D) are depicted in Fig. 3.

The optimal conditions for high yields of protein expression were determined using a volume of 50 ml LB. PVX CPs containing 6xHisE7 in the loops A, B, D, E, F and G and PVX CPs containing E76xHis in the loops B, D, E, F and G were most efficiently expressed at 37 °C (4 h induction with 0.2% arabinose). Alternatively, PVX CP containing 6xHisE7 in the loop C was expressed at 22 °C (2 h induction with 0.2% arabinose) and PVX CPs containing E76xHis in loop A and C were expressed at 22 °C (4 h and 2 h induction with 0.2% arabinose, respectively). Results from the optimization showed that conditions for high yields of proteins depend on the orientation of His tag relative to E7 epitope only in the loop A (4 h and 37 °C for 6xHisE7 and 4 h and 22 °C for E76xHis). As shown in Fig. 3, all insoluble fractions contained the expressed PVX-CP fusion proteins. On the contrary, expression in other loops resulted in proteins prone to degradation, most probably due to disruption of their proper three-dimensional structure.

3.2 Evaluation of immunological properties of expressed constructs

The immunoreactivity of fusion proteins was first verified by Western blot. All fusion constructs were efficiently recognized by both anti-PVX and anti-His antibodies. The same antibodies were used to decorate fusion proteins in transmission electron microscopy. From the examined...
Fig. 3: Expression of PVX CP-6xHisE7/E76xHis in *E. coli* MC1061. Western blot analysis of fusion proteins purified on sucrose cushion (25%): PVX CP-6xHisE7 (N) and PVX CP-E76xHis (C) located in four different surface loops (A, B, C and D) in soluble (SF) and insoluble (IF) fractions. M = marker Spectra™ Multicolor Broad Range Protein Ladder 10 – 260 kDa (Fermentas), PC = positive controls (induced expression of PVX CP-E76xHis in loop D in 10 ml LB for expressions in the loops A, B and D and induced expression of PVX CP-6xHisE7 in loop C for expressions in the loop C, which were obtained from preliminary experiments), NC = negative control (noninduced expression in 10 ml LB). All samples were loaded in a volume of 10 μl, marker 5 μl. Proteins were immunochemically stained with mouse anti-His antibody (GE Healthcare Life Sciences) and anti-mouse IgG (whole molecule) – Alkaline phosphatase (Sigma-Aldrich).

Table 2: Total amount of proteins in soluble (SF) and insoluble (IF) bacterial cytoplasmic fractions (expression in 50 ml LB) purified on sucrose cushion (25%).

<table>
<thead>
<tr>
<th>Expression</th>
<th>Loop</th>
<th>SF</th>
<th>IF</th>
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<td>4 h, 37 °C</td>
<td>PVX CP-6xHisE7 A</td>
<td>0.3 mg</td>
<td>0.5 mg</td>
</tr>
<tr>
<td>4 h, 22 °C</td>
<td>PVX CP-E76xHis A</td>
<td>-</td>
<td>0.9 mg</td>
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<tr>
<td>4 h, 37 °C</td>
<td>PVX CP-6xHisE7 B</td>
<td>-</td>
<td>0.6 mg</td>
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<tr>
<td>4 h, 37 °C</td>
<td>PVX CP-E76xHis B</td>
<td>-</td>
<td>0.1 mg</td>
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<tr>
<td>2 h, 22 °C</td>
<td>PVX CP-6xHisE7 C</td>
<td>0.4 mg</td>
<td>0.9 mg</td>
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<tr>
<td>2 h, 22 °C</td>
<td>PVX CP-E76xHis C</td>
<td>0.3 mg</td>
<td>0.7 mg</td>
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<tr>
<td>4 h, 37 °C</td>
<td>PVX CP-6xHisE7 D</td>
<td>0.4 mg</td>
<td>0.2 mg</td>
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<tr>
<td>4 h, 37 °C</td>
<td>PVX CP-E76xHis D</td>
<td>0.2 mg</td>
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<tr>
<td>4 h, 37 °C</td>
<td>PVX CP-6xHisE7 E</td>
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<td>4 h, 37 °C</td>
<td>PVX CP-6xHisE7 E</td>
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<tr>
<td>4 h, 37 °C</td>
<td>PVX CP-E76xHis F</td>
<td>-</td>
<td>0.8 mg</td>
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<td>4 h, 37 °C</td>
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<td>PVX CP-6xHisE7 G</td>
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<td>PVX CP</td>
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<tr>
<td>4 h, 22 °C</td>
<td>PVX CP</td>
<td>0.1 mg</td>
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constructs, only fusions carrying the 6xHisE7 epitope inserted in loop A (PVX-CP-6xHisE7 A) form aggregates of modified PVX with no regular structures accessible to antibody binding on their surface. Even after thorough observation of the grids, we were unable to find similar units for other constructs. This agrees with the results obtained by SDS-PAGE, where only the fusion proteins in the loop A were proteolytically stable, whereas other expressed constructs were degraded and showed significant instability.

4 Discussion

The main objective of our study was to evaluate the capacity of the PVX CP platform to tolerate insertion of E7 epitope fused with His tag in the seven different positions. We used the fusion proteins labeled with the His tag to simplify verification of peptide presence because of lack of specific anti-E7 antibodies. Insertions of labeled epitopes were made at the seven specific sites in the PVX CP taking into consideration the bioinformatic prediction of the PVX CP structure based on tritium planigraphy [15,27]. Thereby, fusions were made in the seven different loops connecting α-helices and β-strands of PVX CP which are assumed to be located on the exterior part of the molecule (Fig. 1).

The engineering of *Papaya mosaic virus* (PapMV), another member of the family *Flexiviridae* in genus *Potexvirus* has been recently described [28]. PapMV CP was expressed in *Escherichia coli* and showed to self assemble into nucleocapsid like particles (NLPs). Twenty percent of the purified protein was found as NLPs of 50 nm in length and 80% was found as a multimer of 450 kDa (20 subunits) arranged in a disk. [28]. Two new tested insertion sites in CP led to the production of recombinant proteins capable of assembly into PapMV nanoparticles [29].

Unlike PapMV, PVX, even though it belongs to the same family and genus, was not able to form particles without the presence of RNA. Recently, the PVX assembly was found to be initiated at the 5´ terminus of genomic RNA, where the cap structure is an important condition for virus assembly [30]. Since bacteria are not able to create RNA, where the cap structure is an important condition for virus assembly [30]. This was also confirmed by our results. During TEM observation of engineered PVX CP fusion proteins, viral nanoparticles were not detected, only aggregates of modified PVX CP with no regular structures.

Our laboratory has previously confirmed that the PVX CP had the ability to present heterologous proteins fused to the N- or C-terminus of PVX CP [17,31,32]. However, this approach was not always successful and optimization of the topology of inserted proteins or peptides in PVX CP was necessary. Finding new positions inside the PVX CP for protein/peptide presentation showed a new approach to heterologous protein expression. We plan to apply our results concerning the new positions within PVX CP to design improved systems for transient expression in hosts enabling cap creation.

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