Transient expression of *Human papillomavirus* type 16 L2 epitope fused to N- and C-terminus of coat protein of *Potato virus X* in plants

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Transient expression of foreign genes based on plant viral vectors is a suitable system for the production of relevant immunogens that can be used for the development of a new generation of vaccines against a variety of infectious diseases. In the present study the epitope derived from HPV-16 L2 minor capsid protein (amino acids 108–120) was expressed from *Potato virus X* (PVX)-based vector pGR106 as N- or C-terminal fusion with the PVX coat protein (PVX CP) in transgenic *Nicotiana benthamiana* plants. The fusion protein $L2_{108-120}$-PVX CP was successfully expressed in plants at a level of 170 mg/kg of fresh leaf tissue. The C-terminal fusion protein PVX CP- $L2_{108-120}$ was expressed using mutated vector sequence to avoid homologous recombination at a level of 8 mg/kg of fresh leaf tissue. Immunogenicity of $L2_{108-120}$-PVX CP virus-like particles was tested after immunization of mice by subcutaneous injection or tattoo administration. In animal sera the antibodies against the PVX CP and the $L2_{108-120}$ epitope were found after both methods of vaccine delivery.

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1. Introduction

Human papillomavirus (HPV) causes cervical cancer and is the second biggest cause of female cancer mortality worldwide with 288,000 deaths yearly. It is firmly established that infections with high-risk (HR)-HPV, particularly with HPV-16, contribute to the development of cervical cancer (zur Hausen 2002, 2006). The availability of preventive vaccines against HR-HPVs (based on HPV-16, HPV-18, HPV-6, and HPV-11 L1 structural proteins assembled in virus-like particles, VLPs) represents a milestone in the prevention of this infection (Koutsky *et al.* 2002; Harper *et al.* 2006), establishing the base for a significant reduction of the rate of cervical cancer in future. Although the minor capsid protein L2, which is not required for capsid formation, is thought to participate in encapsidation of the viral genome and plays a number of essential roles in the viral infectious entry pathway, it became the subject of oncological research (Day *et al.* 1998; Florin *et al.* 2006; Buck *et al.* 2008).

To lower the cost connected with the production of prophylactic vaccines, the capsid protein HPV-L1 has been already produced in plants (Biemelt *et al.* 2003; Varsani *et al.* 2003; Fernández-San Millán *et al.* 2008; Maclean *et al.* 2007). Recently, the HPV-L1 protein expressed in plants was used as a carrier for E7 and E6 epitopes (De la Rosa *et al.* 2009).

Keywords. Human papillomavirus (HPV-16); L2- and E7-derived epitopes; transient expression

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As to therapeutic vaccines, several HPV-specific E7-based vaccine formulations have been tested in animal models (Smahel *et al.* 2001), some of them were produced also in plants (Massa *et al.* 2007) and have been advanced into phase II and III of clinical trials (Billich 2003; Frazer 2004).

Plant and plant cells possess many advantages over other eukaryotic expression hosts, such as high biomass, ease of scale-up, cost-effectiveness and low risk of contamination (Ma *et al.* 2003; Twyman *et al.* 2003). Phytovirus-based vector is a useful tool for efficient expression of a target protein in plants. To date, the most efficient means of achieving high-level transient expression of foreign proteins in plants has involved the use of vectors based on RNA plant viruses (Porta and Lomonossoff 2002; Gleba *et al.* 2004; Lindbo 2007). These systems take advantage of the ability of RNA viruses to replicate to high titers within infected cells. Vectors based on full-length viral replicons, which can move throughout a plant, suffer from problems of biocontainment (Sainsbury and Lomonossoff 2008).

Genetically engineered, self-assembling peptide-carrier vaccine systems have been composed on the basis of plant-virus expression vectors (Rybicki 2010). *Potato virus X* (PVX) has been investigated as a possible presentation system (Nattila *et al.* 2006).

Several viral vectors were constructed using the full-length cDNA of PVX coupled to different strong promoters and the transcriptional terminator from the nopaline synthase gene of *Agrobacterium tumefaciens* (Chapman *et al.* 1992; Marusic *et al.* 2001). The main advantage of the use of plant-virus-based vectors is the speed and large amounts of protein obtained in days after initial molecular cloning event, rather than the months necessary for transgenic expression. On the other hand, the genetic instability of the constructs *in planta* very often results in the loss of the inserted gene by illegitimate recombination (Angell and Baulcombe 1997).

Here we present data regarding the stability of HPV-derived epitope fusions to N- or C-terminus of PVX-CP transiently expressed in plants. The possibility to use them for experimental vaccine development was assessed.

2. Materials and methods

2.1 *Plant propagation*

Cell-to-cell movement of *Potato virus X* can be fully compensated by the *Tobacco mosaic virus* movement protein (TMV-MP) (Fedorkin *et al.* 2001). Therefore, for expression of designed fusion proteins in plants the transgenic *Nicotiana benthamiana*, 3H carrying the TMV-MP (kindly provided by R Beachy, Donald Danforth Plant Science Center, St. Louis, USA) were used. All plants were grown in a growth chamber in controlled conditions (20–25°C over day, 15–20°C over night, 16 h light/8 h dark cycle). Eighteen plants per group were inoculated at four-leaf stage. All experiments were performed in triplicate.

2.2 *Cloning of fusion constructs*

The sequences encoding fusion proteins were inserted into the binary vector pGR106, kindly provided by DC Baulcombe, The Sainsbury Laboratory, Norwich, UK. To evaluate the influence of N- and C-terminally fused antigens on VLPs production, two different PVX CP constructs in pMPM-A4Ω (PVX CP-BH/pMPM-A4Ω and PVX CP-NB/pMPM-A4Ω) were used (Plchova *et al.* 2011). The coding sequence of the epitope from the HPV-16 L2 capsid protein (aa 108–120 – LVEETSFIDAGAP) (Kawana *et al.* 1998) was prepared by annealing of oligonucleotides carrying appropriate restriction sites (table 1) in a buffer containing 10 mM Tris (pH 7.5) and 50 mM NaCl at 95°C for 5 min and 75°C for 4 h. The sequences were then fused with either the 3′- or 5′-terminus of the PVX CP sequence in the bacterial expression vector pMPM-A4Ω using restriction sites *Nco*I, *Bgl*II and *Hin*dIII. The resulting constructs (figure 1a, b) were then subcloned into pGEM-T Easy using *Eco*RI/*Sal*I and subsequently cut out by *Not*I/*Sal*I. The constructs were then inserted into pGR106 cleaved by *Not*I and *Xho*I to produce the final $L2_{108\text{-}120}$-PVX CP/pGR106 and PVX CP-$L2_{108\text{-}120}$/pGR106 expression plasmids carrying the $L2_{108\text{-}120}$ epitope fused to 5′- or 3′-terminus of PVX CP, respectively (figure 1c, d). All constructs were checked for their accuracy by DNA sequencing. The strategy using repetition of the pGR106 sequence to ensure proper viral replication, especially of constructs having the $L2_{108\text{-}120}$ epitope fused to the 3′-terminus of PVX CP, was the same as already described (Plchova *et al.* 2011). To avoid the homologous recombination between the two repetitive identical sequences and subsequent loss of the $L2_{108\text{-}120}$ epitope at the 3′-terminus of PVX CP, the first repetition was mutated as follows: The PVX CP- $L2_{108\text{-}120}$-mut/pGR106 expression plasmid (figure 1e) was prepared by SOE-PCR (Horton 1995). In the first round of PCR, two amplicons were prepared using PVX CP-$L2_{108\text{-}120}$/pGR106 as a template and two flanking primers and two central mutagenic primers. The PCR products were gel-purified, mixed together and used as a template for second round of PCR, in which only flanking primers were used. Taq polymerase (Fermentas) was used in both rounds of PCR, and the final product was subcloned into pJET1.2/blunt (Fermentas) using the T/A overhangs, again cut out with *Not*I/*Spe*I and cloned into pGR106. The correct sequence was confirmed by DNA sequencing. For the construction of PVX CP- $L2_{108\text{-}120}$-mut/pGR106 we used two central mutagenic primers XCP-mutAS and XCP-mutS, which created amplicons

Table 1. Oligonucleotides used for preparation of chimeric constructs and IC PCR detection

Oligonucleotides	Sequences (5′-3′)
L2108120Ns	CATGGCTTTAGTGGAAGAAACCAG CTTTATTGATGCAGGTGCACCGA
L2108120Nas	GATCTCGGTGCACCTGCATCAATAA AGCTGGTTTCTTCCACTAAAGC
L2108120Cs	GATCTTTAGTGGAAGAAACCAGCTT TATTGATGCAGGTGCACCGTAAA
L2108120Cas	AGCTTTTACGGTGCACCTGCATCAAT AAAGCTGGTTTCTTCCACTAAA
pGR-S2	TGTGGTAACAATCATAGCAGTCA
XCP-mutAS	CAATGTAACTACTGCTTCTGCAGTAG TAGTACCAGTGATACGACCTCGAG
XCP-mutS	TACTGCAGAAGCAGTAGTTACATTGC CTCCGCCTAGATCTTTAGTGGAAGA
Nos-ter AS	CATCGCAAGACCGGCAACA
pGR106-5	GCAAACTAGATGCAGAAACC
pGR106-3	CATACAATCAAACCAGAAAATAC

with 25 nt overlap with two flanking primers pGR-S2 and Nos-ter AS, respectively (table 1).

2.3 *Agrobacterium transformation*

The plasmids pGR106 carrying the PVX CP fusion genes were isolated from *E. coli* and electroporated into *Agrobacterium tumefaciens* (GV3101). Bacteria containing the plasmids were selected on L-agar plates with 30 mg/L kanamycin. Bacterial suspension for agroinfection of plants was prepared in L-broth containing 30 mg/L kanamycin at 28°C.

2.4 *Plant inoculation*

The primary infection of host plants was performed by infiltration using 1 mL hypodermic syringe (Hoffmeisterova *et al.* 2008). As a negative control, healthy leaves inoculated with MMA medium (Murashige and Skoog Medium, micro- and macro-elements, 4.3 g/L (Duchefa), 10 mM MES, 10 mM glutamin, 20 g/L sucrose, pH adjusted to 5.6, 200 μM acetosyringone) were used. As a positive control, *Nicotiana benthamiana* 3H inoculated with the pGR106 vector was used in all cases. The infected plants were analysed approximately 12 days post inoculation.

2.5 *IC RT-PCR detection of chimeric gene*

IC RT-PCR was done as described previously (Canizares *et al.* 2006). The presence of sequence coding for $L2_{108-120}$ epitope in desired constructs was tested using epitope-specific primers and primers specific to the pGR106 vector – pGR106-5 and pGR106-3 (table 1) by Access RT-PCR System (Promega).

2.6 *PTA-ELISA*

PTA-ELISA was done as described previously (Mowat 1985). As primary antibodies, the rabbit polyclonal anti-PVX CP (anti-PVX CP) or anti-HPV-16 L2 (anti-L2, kindly provided by M Sapp, Institute for Medical Microbiology, Johannes Gutenberg-University Mainz, Germany) were used. As secondary antibodies, alkaline phosphatase (AP)-conjugated goat anti-rabbit IgG (ICN, USA) were used. All ELISA measurements were performed in duplicates and repeated three times.

2.7 *SDS-PAGE and Western blot analysis*

Both procedures were carried out on systemic leaves as described previously (Cerovska *et al.* 2004). The approximate amount of produced proteins was estimated on the basis of comparison with different dilutions of the purified PVX CP. Its concentration was measured either by measuring absorption at 280 nm or colorimetrically by Bradford's method (Bradford 1976).

2.8 *N-terminal amino acid sequencing*

Proteins separated by SDS-PAGE were transferred to the PVDF membrane, visualized by Coomasie brilliant blue and subjected to N-terminal amino acid sequencing using protein sequencer Procise 491 (Applied Biosystems) following the Manual Instructions.

2.9 *Purification of modified virus*

The purification was conducted by gradient centrifugation as described (Cerovska *et al.* 1991). The presence of virus was tested by PTA-ELISA (dilutions of the virus 1 mg/L and 10 mg/L in 15 mM Na_2CO_3, 35 mM $NaHCO_3$, 3 mM NaN_3, pH 9.6).

2.10 *Electron microscopy*

The binding of antigens to copper grids and their immunolabelling were done according to Cerovska *et al.* (2008). Electron microscopy was carried out using a JEM 1010 transmission electron microscope (Jeol, Japan, facility of Biological Centre of Sciences, Ceske Budejovice, Czech Republic).

2.11 *Immunization of mice*

Six- to eight-week-old female C57BL/6 mice ($H\text{-}2^b$) (Charles River, Germany) maintained under standard conditions

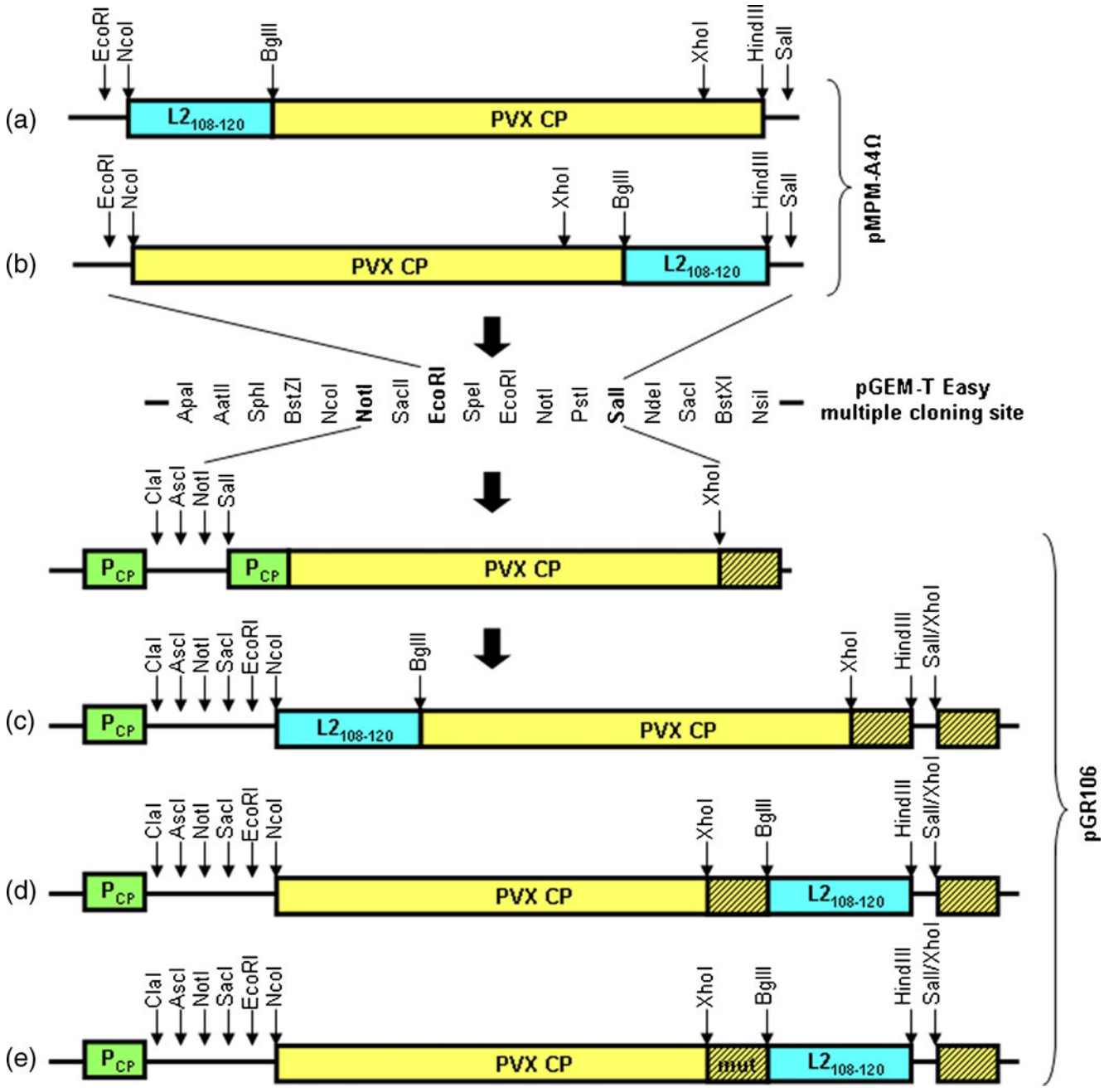

Figure 1. (**a**) Cloning strategy shows how the coding sequence of the epitope from the HPV-16 L2 capsid protein (aa 108–120) was fused with the 5′-terminus of the PVX CP sequence in the bacterial expression vector pMPM-A4Ω using indicated restriction sites. (**b**) Cloning strategy shows how the coding sequence of the epitope from the HPV-16 L2 capsid protein (aa 108–120) was fused with the 3′-terminus of the PVX CP sequence in the bacterial expression vector pMPM-A4Ω using indicated restriction sites. Both ((a) and (b)) resulting constructs were then subcloned into pGEM-T Easy using EcoRI/SalI and cut out by NotI/SalI. The constructs were then inserted into pGR106 cleaved by NotI and XhoI, so that the PVX CP present in pGR106 was replaced by the chimeric constructs. (**c**) The final $L2_{108\text{-}120}$-PVX CP/pGR106 construct having the $L2_{108\text{-}120}$ epitope fused to the 5′-terminus of PVX CP in the plant expression vector pGR106. (**d**) The final PVX CP-$L2_{108\text{-}120}$/pGR106 construct having the $L2_{108\text{-}120}$ epitope fused to the 3′-terminus of PVX CP in the plant expression vector pGR106. (**e**) The final PVX CP-$L2_{108\text{-}120}$-mut/pGR106 construct derived from PVX CP-$L2_{108\text{-}120}$/pGR106 having the the mutated copy of the 3′-terminus of PVX CP in the plant expression vector pGR106. The P_{CP} are PVX CP promoters. The duplicated 3′-terminus of PVX CP is indicated by crosshatched squares and the mutated copy of the 3′-terminus of PVX CP is shown as crosshatched square with the sign mut.

at the Center for Experimental Biomodels (Charles University, Prague) were immunized three times at two-week intervals with 5 μg of $L2_{108-120}$-PVX CP. This construct was administered either by s.c. injection in 200 μL PBS or by using a tattoo machine Rotary 12000 AL (Bortech Tattoogrosshandel, Wuppertal, Germany) in 20 μL PBS into the shaved skin at a dorsum as described previously (Pokorna *et al.* 2009). To enhance immune responses, 25 μg of immunostimulatory CpG motifs (ODN 1826: TCCATGACGTTCCTGACGTT; Generi Biotech, Hradec Kralove, Czech Republic) or monophosphoryl lipid A (MPL-A; InvivoGen, San Diego, CA) or a combination of both adjuvants (12.5+12.5 μg) was co-delivered with $L2_{108-120}$-PVX CP. The experiments proposed in this project have been approved by the Commission for work with laboratory animals; animal experiments were performed by workers with certificates for work with laboratory animals.

2.12 *Detection of induced antibodies*

Sera of immunized mice were collected two weeks after the last immunization. L2-specific antibodies were detected in 1:50 diluted sera by ELISA using the $L2_{107-122}$ synthetic peptide as described previously (Pokorna *et al.* 2009). Antibodies against PVX CP were tested by ELISA using purified PVX CP as described (Cerovska *et al.* 2008).

3. Results

3.1 *Cloning of fusion constructs*

The sequences encoding fusion proteins were inserted into the binary vector pGR106 based on PVX. Two different PVX CP constructs ($L2_{108-120}$-PVX CP and PVX CP-$L2_{108-120}$) with the epitope $L2_{108-120}$ (aa 108–120) from the HPV-16 L2 capsid protein fused either to 5′- or 3′-terminus of PVX CP were prepared first in bacterial expression vector pMPM-A4Ω (figure 1a, b) and then in pGR106 (figure 1c, d) using oligonucleotides listed in table 1 according to the method described in the experimental section. The strategy using repetition of the pGR106 sequence to ensure proper viral replication especially of constructs having the $L2_{108-120}$ epitope fused to the 3′-terminus of PVX CP was as already described (Plchova *et al.* 2011). To avoid the homologous recombination between the two repetitive identical sequences and subsequent loss of the $L2_{108-120}$ epitope at the 3′-terminus of PVX CP the first repetition was mutated without changing the amino acid sequence and the PVX CP-$L2_{108-120}$-mut/pGR106 expression plasmid (figure 1e) was prepared using mutated oligonucleotides (table 1).

3.2 *Expression and purification of chimeric proteins in Nicotiana benthamiana 3H*

Preliminary studies have shown that the optimal approach to infect the experimental host plants was to initiate the infection cycle using *Agrobacterium*-mediated infection. In our experiments we used syringe agroinfiltration. The presence of fusion genes and their products in harvested *Nicotiana benthamiana* 3H leaves was determined by immunocapture (IC) RT-PCR and by plate-trapped antigen (PTA) ELISA and Western blot, using IgG antibodies specific to PVX CP and L2. As a negative control, healthy leaves inoculated with MMA medium were used. As a positive control, *Nicotiana benthamiana* 3H inoculated with the pGR106 vector was used in all cases. The ELISA absorbances of samples prepared from leaves of experimental plants inoculated with MMA were comparable to the absorbances of healthy leaves without MMA treatment.

We confirmed the presence of both desired fusion constructs by IC RT-PCR in *N. benthamiana* 3H plants (data not shown). However, by Western blot analysis we detected only the $L2_{108-120}$-PVX CP product of the appropriate size (34 kDa) (figure 2). The presence of the L2 epitope in the produced recombinant protein was examined by determination of N-terminal amino acid sequence that proved the completely correct sequence of the epitope.

The PVX CP-$L2_{108-120}$ protein was not expressed, and therefore we isolated RNA from appropriate infected plants and performed RT PCR. After sequencing of the obtained product we found out that the 3′-terminal epitope was lost together with one copy of the PVX CP 3′-terminus (figure 1d) most probably because of the recombination event between these two identical sequences. Therefore, we designed the mutated version of the plasmid expressing the C-terminal construct PVX CP-$L2_{108-120}$-mut/pGR106 (figure 1e). Using this PVX CP-$L2_{108-120}$-mut construct, we finally obtained the expression of the fusion protein PVX CP-$L2_{108-120}$.

The amount of expressed chimeric protein was determined by PTA-ELISA or SDS-PAGE/Western blot analysis using anti-PVX CP antibodies. The concentration of both expressed constructs was based on the series of diluted wild-type PVX CP purified from plants. The estimated yield for $L2_{108-120}$-PVX CP was approximately 170 mg/kg and PVX CP-$L2_{108-120}$-mut 8 mg/kg of fresh leaf tissue.

Western blot analysis of different plant parts infected with $L2_{108-120}$-PVX CP/pGR106 showed that $L2_{108-120}$-PVX CP was distributed throughout the infected plants and thus accumulated in plants systemically as did the wild-type virus itself (data not shown). We used infected leaf tissue

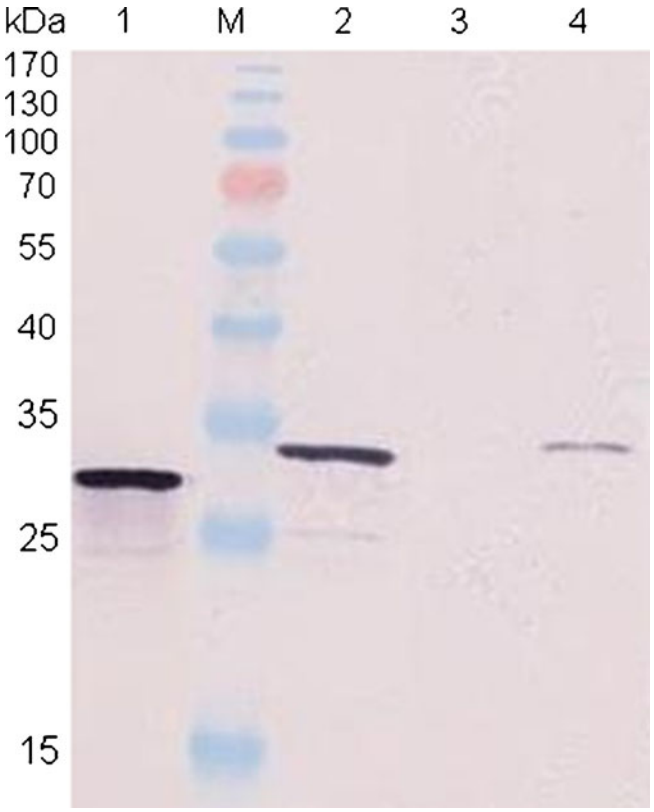

Figure 2. Western blot analysis of fusion constructs expressed in *N. benthamiana* 3H plants. Proteins were electrophoretically separated by SDS-PAGE, transmitted to nitrocellulose membrane and immunochemically stained with anti-PVX antibodies. Lane 1, PVX CP; Lane 2, $L2_{108\text{-}120}$-PVX CP; Lane 3, PVX CP $L2_{108\text{-}120}$; Lane 4, PVX CP $L2_{108\text{-}120}$-mut; M, Molecular weight markers (GeneRuler, Fermentas; 10 - 170 kDa).

for further mechanical transmission of the viral vector containing the $L2_{108\text{-}120}$-PVX CP construct. By Western blot analysis we confirmed the stability and infectivity of this construct even after three subsequent re-infections. The PVX CP-$L2_{108\text{-}120}$-mut construct did not infect the plants systemically, and therefore it was not possible to use it for mechanical inoculation.

To confirm the assembly of monomers of the fusion products to particles, we purified the chimeric $L2_{108\text{-}120}$-PVX CP particles from *N. benthamiana* 3H agroinfected leaves and subjected them to electron microscopy. Immunogold labelling using polyclonal antibodies against both PVX CP and L2 confirmed the presence and accessibility of desired epitopes on the surface of the purified particles (figure 3). The PVX CP-$L2_{108\text{-}120}$ fusion product probably does not form VLPs because it could not be purified as VLPs and no particles were found using immunogold transmission electron microscopy.

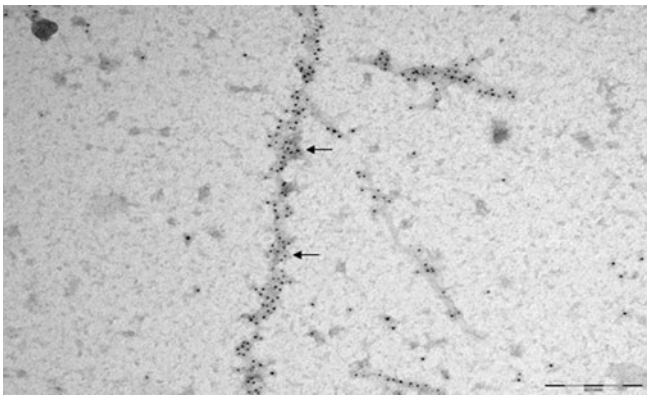

Figure 3. Immunoelectron microscopy of modified virus isolated from *N. benthamiana* 3H plants. $L2_{108\text{-}120}$-PVX CP VLPs were detected by rabbit anti-PVX antibodies decorated by secondary goat anti-rabbit gold-labelled antibodies (5 nm) followed by staining with mouse anti-L2 antibodies and secondary goat anti-mouse gold-labelled antibodies (15 nm). Abscissa indicates 200 nm.

3.3 *Immunogenicity of plant-produced $L2_{108\text{-}120}$-PVX CP*

For immunization of mice only $L2_{108\text{-}120}$-PVX CP purified from plants was used due to its ability to form VLPs. The product was injected subcutaneously (s.c.) or administered into a skin by a tattoo device. Antibodies against the PVX CP and the $L2_{108\text{-}120}$ epitope were determined in animal sera. Both methods of vaccine delivery induced anti-L2 (figure 4) and anti-PVX CP antibodies (data not shown), but their levels were lower after tattooing. We also tried to enhance antibody production by adjuvants – immunostimulatory CpG motifs and monophosphoryl lipid A (MPL-A). While CpG motifs supported anti-L2 formation after both s.c. and tattoo delivery of $L2_{108\text{-}120}$-PVX CP, MPL-A increased L2-specific antibodies only after tattooing and it reduced these antibodies after s.c. immunization.

4. Discussion

This study is the continuation of our work on the transiently expressed HPV-16 epitopes in plants. We demonstrated previously that the L2-ACP-E7 construct consisting of the coat protein of Potato virus A (ACP) fused with E7 and L2 epitopes of HPV-16 and expressed from PVX-based vector pGR106 was capable of forming stable VLPs and both epitopes were incorporated into their surface (Cerovska *et al.* 2008). It has been demonstrated that the N-terminus of PVX CP was exposed on the surface of a virion with the first 33 amino acid residues of CP forming a β-strand (Baratova *et al.* 1992). Consequently, PVX CP represents a good candidate carrier for foreign peptides, as it self-assembles in an ordered fashion and accumulates to high

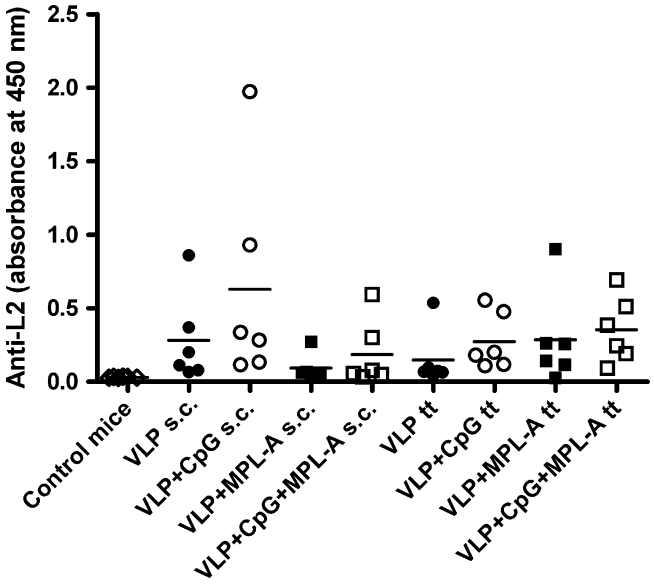

Figure 4. Analysis of $L2_{108-120}$-specific antibodies by ELISA. Mice (n=6) were immunized three times at two-week intervals with 5 μg of $L2_{108-120}$-PVX CP VLPs by s.c. needle injection or using a tattoo device (tt). CpG motifs (25 μg), MPL-A (25 μg) or CpG motifs+MPL-A (12.5+12.5 μg) were used as adjuvants. Two weeks after the final immunization, sera from mice were collected and $L2_{108-120}$-specific antibodies were detected by ELISA.

levels in infected host tissues. In addition, it does not reveal size and packaging constraints usually found in icosahedral viruses (Baratova *et al*. 1992).

Here we provide the evidence that the pGR106 vector can also be used for the expression of the N-terminal fusion construct $L2_{108-120}$-PVX CP in plants. Displayed epitopes of this construct remained immunoreactive, which was demonstrated by their binding to the epitope-specific antibodies in immunomicroscopy. Western blot analysis of the purified $L2_{108-120}$-PVX CP protein obtained from leaves taken from different parts of plants showed that this genetically modified virus was distributed throughout the infected plants and thus accumulated in plants systemically as did PVX itself. Hence, epitopes were transiently expressed as N-terminal fusions with PVX CP engendering the formation of modified virus that likely displays $L2_{108-120}$ epitope in an accessible and functional format. The main advantages of this transient-expression system are the rapidity of the procedure and the yields such as those reported here (17 mg of recombinant protein per 100 g of infected tissue). The main constraints to this approach are, in general, low stability and infectivity of the chimeric structures carrying exogenous sequences fused at the N-terminus of CP, traits that depend mainly on the fused sequence length and amino acid composition (Bendahmane *et al.* 1999; Uhde-Holzem *et al.* 2007). However, in our work we demonstrated that the mentioned chimeric construct retains both viral stability and infectivity even after three subsequent mechanical re-infections. Therefore, the additional fused $L2_{108-120}$ sequence seems not to interfere with virus assembly, cell-to-cell and long distance movement. Moreover, analysis of the infected plant extracts showed that the CP of the modified virus was present as a single band on Western blots, indicating its stability to protease degradation.

The expression of epitopes at the C-terminus of PVX CP was not studied so far. Therefore, we prepared the construct carrying the coding sequence of HPV-16 $L2_{108-120}$ epitope in fusion with 3′-terminus of PVX CP. To ensure proper viral replication we used repetition of the 3′-terminus of PVX CP without any fusion and the usual wild-type sequence of pGR106 with its *cis*-acting elements in the 3′-untranslated region, which followed the chimeric construct (Hu *et al.* 2007; Verchot-Lubitz *et al.* 2007; Plchova *et al.* 2011) (figure 1d). The experiments revealed that the PVX CP-$L2_{108-120}$ construct was not expressed. Therefore, we sequenced the chimeric construct PVX CP-$L2_{108-120}$ in the pGR106 vector again after the purification from *N. benthamiana* 3H plants and found out that the sequence coding for the C-terminal epitope $L2_{108-120}$ was lost together with one copy of the PVX CP 3′-terminus. This result suggests that the 3′-terminal epitope was eliminated from the vector by recombination (most probably between two 60-nt-long identical nucleotide repetitions of PVX CP 3′-terminus). In comparison to our previous successful expression of HPV-16 E7ggg (97 amino acids) fused to the 3′-terminus of PVX CP this elimination event seems to be specific for shorter (in this case 13 amino acids) 3′-terminal fusions, such as epitopes (Plchova *et al.* 2011). To prevent this epitope elimination we prepared the construct PVX CP-$L2_{108-120}$-mut in pGR106, where the first copy of the 3′-terminus of PVX CP was mutated using another codon coding for identical amino acids. In this case, the inoculated leaves of *N. benthamiana* 3H plants contained fusion protein of the expected electrophoretic mobility of approximately 34 kDa. We could not detect protein of this size in upper (non-inoculated) leaves. By electron microscopy we proved the inability of PVX CP-$L2_{108-120}$-mut to form virions. It seems that this C-terminal modification of the PVX CP makes virion assembly *in vivo* impossible.

Because the PVX CP-$L2_{108-120}$-mut product did not form particles the immunogenicity only of $L2_{108-120}$-PVX CP VLPs purified from plants was verified by immunization of mice. Antibodies were produced against both PVX CP and the L2 epitope. In our previous study, we showed the advantage of tattooing for delivery of peptide vaccines in some conditions when compared with s.c. needle injection (Pokorna *et al.* 2009). Therefore, we also tested tattoo delivery of $L2_{108-120}$-PVX CP VLPs in this study. However,

tattoo administration of $L2_{108-120}$-PVX CP VLPs induced lower levels of antibodies. This effect could be caused by mechanical damage of particles during tattooing procedure. Furthermore, while the addition of immunostimulatory CpG motifs increased L2-specific antibodies after both s.c. and tattoo delivery, MPL-A-enhanced generation of these antibodies only after tattooing. Moreover, MPL-A reduced antibodies against the L2 epitope which could be caused by ethanol used for dissolution of this substance that might alter $L2_{108-120}$-PVX CP conformation and thus lower immunogenicity of subcutaneously administered vaccine.

In conclusion, on the basis of the results presented here, we widen the spectrum of knowledge concerning the epitope display experiments based on *Potato virus X* as a carrier for peptide epitopes. Our attempts to express HPV-16 epitope at the C-terminus of PVX CP in pGR106 pointed out the difficulties with stability of nucleotide sequences of such short peptides connected immediately to the 3′-terminus of PVX CP and simultaneously inserted between two identical sequences of the 3′-terminus of PVX CP. Furthermore, the expression of the HPV-16 L2 epitope $L2_{108-120}$ is the first documented case of the expressed epitope fused to the C-terminus of PVX-CP for the purpose of vaccine design.

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References

Angell SM and Baulcombe DC 1997 Consistent gene silencing in transgenic plants expressing a replicating potato virus X RNA. *EMBO J.* **16** 3675–3684

Baratova LA, Grebenshchikov NI, Dobrov EN, Gedrovich AV, Kashirin IA, Shishkov AV, Efimov AV, Järvekülg L, *et al.* 1992 The organization of potato virus X coat proteins in virus particles studied by tritium planigraphy and model building. *Virology* **188** 175–180

Bendahmane M, Koo M, Karrer E and Beachy RN 1999 Display of epitopes on the surface of tobacco mosaic virus: impact of charge and isoelectric point of the epitope on virus-host interactions. *J. Mol. Biol.* **290** 9–20

Biemelt S, Sonnewald U, Galmbacher P, Willmitzer L and Muller M 2003 Production of human papillomavirus type 16 virus-like particles in transgenic plants. *J. Virol.* **77** 9211–9220

Billich A 2003 HPV vaccine MedImmune/GlaxoSmithKline. *Curr. Opin. Investig. Drugs* **4** 210–213

Bradford MM 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72** 248–254

Buck CB, Cheng N, Thompson CD, Lowy DR, Steven AC, Schiller JT and Trus BL 2008 Arrangement of L2 within the papillomavirus capsid. *J. Virol.* **82** 5190–5197

Canizares MC, Liu L, Perrin Y, Tsakiris E and Lomonossoff GP 2006 A bipartite system for the constitutive and inducible expression of high levels of foreign proteins in plants. *Plant Biotechnol. J.* **4** 183–193

Cerovska N, Filigarova M, Branisova H, Zak P and Dedic P 1991 Some factors influencing purification of potato virus A (PVA). *Acta Virol.* **35** 469–471

Cerovska N, Hoffmeisterova H, Pecenkova T, Moravec T, Synkova H, Plchova H and Veleminsky J 2008 Transient expression of HPV16 E7 peptide (aa 44–60) and HPV16 L2 peptide (aa 108–120) on chimeric potyvirus-like particles using Potato virus X-based vector. *Protein Expr. Purif.* **58** 154–161

Cerovska N, Pecenkova T, Moravec T and Veleminsky J 2004 Transient expression of heterologous model gene in plants using potato virus X-based vector. *Plant Cell Tissue Organ Cult.* **79** 147–152

Chapman S, Kavanagh T and Baulcombe D 1992 Potato virus X as a vector for gene expression in plants. *Plant J.* **2** 549–557

Day PM, Roden RB, Lowy DR and Schiller JT 1998 The papillomavirus minor capsid protein, L2, induces localization of the major capsid protein, L1, and the viral transcription/replication protein, E2, to PML oncogenic domains. *J. Virol.* **72** 142–150

De la Rosa GP, Monroy-García A, de Lourdes Mora-García M, Reynaga Peña CG, Hernández-Montes J, Weiss-Steider B and Gómez Lim MA 2009 An HPV 16 L1-based chimeric human papilloma virus-like particles containing a string of epitopes produced in plants is able to elicit humoral and cytotoxic T-cell activity in mice. *Virol. J.* **6** 2

Fedorkin O, Solovyev A, Yelina N, Zamyatnin A Jr, Zinovkin R, Mäkinen K, Schiemann J and Morozov Syu 2001 Cell-to-cell movement of potato virus X involves distinct functions of the coat protein. *J. Gen. Virol.* **82** 449–458

Fernández-San Millán A, Ortigosa SM, Hervás-Stubbs S, Corral-Martínez P, Seguí-Simarro JM, Gaétan J, Coursaget P and Veramendi J 2008 Human papillomavirus L1 protein expressed in tobacco chloroplasts self-assembles into virus-like particles that are highly immunogenic. *Plant Biotechnol. J.* **6** 427–441

Florin L, Becker KA, Lambert C, Nowak T, Sapp C, Strand D, Streeck RE and Sapp M 2006 Identification of a dynein interacting domain in the papillomavirus minor capsid protein L2. *J. Virol.* **80** 6691–6696

Frazer IH 2004 Prevention of cervical cancer through papillomavirus vaccination. *Nat. Rev. Immunol.* **4** 46–54

Gleba Y, Marillonnet S and Klimyuk V 2004 Engineering viral expression vectors for plants: the 'full virus' and the 'deconstructed virus' strategies. *Curr. Opin. Plant. Biol.* **7** 182–188

Harper DM, Franco EL, Wheeler CM, Moscicki AB, Romanowski B, Roteli-Martins CM, Jenkins D, Schuind A, *et al.* 2006 Sustained efficacy up to 4.5 years of a bivalent L1 virus-like particle vaccine against human papillomavirus types 16 and 18: follow-up from a randomised control trial. HPV Vaccine Study group. *Lancet* **367** 1247–1255

Hoffmeisterova H, Cerovska N, Moravec T, Plchova H, Folwarczna J and Veleminsky J 2008 Transient expression of

chimeric potyvirus-like particles carrying two HPV-16 epitopes using different means of inoculation of *Nicotiana benthamiana* and *Brassica rapa*, cv. Rapa plants. *Plant Cell Tissue Organ Cult.* **94** 261–267

Horton RM 1995 PCR-mediated recombination and mutagenesis. SOEing together tailor-made genes. *Mol. Biotechnol.* **3** 93–99

Hu B, Pillai-Nair N and Hemenway C 2007 Long-distance RNA–RNA interactions between terminal elements and the same subset of internal elements on the potato virus X genome mediate minus- and plus-strand RNA synthesis. *RNA* **13** 267–280

Kawana K, Matsumoto K, Yoshikawa H, Taketani Y, Kawana T, Yoshiike K and Kanda T 1998 A surface immunodeterminant of human papillomavirus type 16 minor capsid protein L2. *Virology* **245** 353–359

Koutsky LA, Ault KA, Wheeler CM, Brown DR, Barr E, Alvarez FB, Chiacchierini LM and Jansen KU 2002 A controlled trial of a human papillomavirus type 16 vaccine. Proof of Principle Study Investigators. *New Engl. J.* **347** 1645–1651

Lindbo JA 2007 TRBO: a high-efficiency tobacco mosaic virus RNA-based overexpression vector. *Plant Physiol.* **145** 1232–1240

Ma JKC, Drake PMW and Christou P 2003 The production of recombinant pharmaceutical proteins in plants. *Nat. Rev. Genet.* **4** 794–805

Maclean J, Koekemoer M, Olivier AJ, Stewart D, Hitzeroth II, Rademacher T, Fischer R, Williamson AL and Rybicki EP 2007 Optimization of human papillomavirus type 16 (HPV-16) L1 expression in plants: comparison of the suitability of different HPV-16 L1 gene variants and different cell-compartment localization. *J. Gen. Virol.* **88** 1460–1469

Marusic C, Rizza P, Lattanzi L, Mancini C, Spada M, Belardelli F, Benvenuto E and Capone I 2001 Chimeric plant virus particles as immunogens for inducing murine and human immune responses against human immunodeficiency virus type 1. *J. Virol.* **75** 8434–8439

Massa S, Franconi R, Brandi R, Muller A, Mett V, Yusibov V and Venuti A 2007 Anti-cancer activity of plant-produced HPV16 E7 vaccine. *Vaccine* **25** 3018–3021

Mowat WP 1985 Simplified enzyme immunoassay for plant virus detection and identification; in *Report of the Scottish Crop Research Institute for 1984* (eds) BD Harrison and AF Murant (Kew, p. Commonwealth) p 188

Nattila A, Hammond RW and Nemchinov LG 2006 Epitope presentation system based on cucumber mosaic virus coat protein expressed from a potato virus X-based vector. *Arch. Virol.* **151** 1373–1386

Plchova H, Moravec T, Hoffmeisterova H, Folwarczna J and Cerovska N 2011 Expression of Human papillomavirus 16 E7ggg oncoprotein on N- and C-terminus of Potato virus X coat protein in bacterial and plant cells. *Protein Expr. Purif.* **77** 146–152

Pokorna D, Polakova I, Kindlova M, Duskova M, Ludvikova V, Gabriel P, Kutinova L, Müller M and Smahel M 2009 Vaccination with human papillomavirus type 16-derived peptides using a tattoo device. *Vaccine* **27** 3519–3529

Porta C and Lomonossoff GP 2002 Viruses as vectors for the expression of foreign sequences in plants. *Biotechnol. Genet. Eng. Rev.* **19** 245–291

Rybicki EP 2010 Plant-made vaccines for humans and animals. *Plant Biotechnol. J.* **8** 620–637

Sainsbury F and Lomonossoff GP 2008 Extremly high-level and rapid transient protein production in plants without the use of viral replication. *Plant Physiol.* **148** 1212–1218

Smahel M, Sima P, Ludvikova V and Vonka V 2001 Modified HPV16 E7 genes as DNA vaccine against E7-containing oncogenic cells. *Virology* **281** 231–238

Twyman RM, Stoger E, Schillberg S, Christou P and Fischer R 2003 Molecular farming in plants: host systems and expression technology. *Trends Biotechnol.* **21** 570–578

Uhde-Holzem K, Fischer R and Commandeur U 2007 Genetic stability of recombinant potato virus X virus vectors presenting foreign epitopes. *Arch. Virol.* **152** 805–811

Varsani A, Williamson AL, Rose RC, Jaffer M and Rybicki EP 2003 Expression of Human papillomavirus type 16 major capsid protein in transgenic Nicotiana tabacum cv. Xanthi. *Arch. Virol.* **148** 1771–1786

Verchot-Lubicz J, Ye CM and Bamunusinghe D 2007 Molecular biology of potexviruses: recent advances. *J. Gen. Virol.* **88** 1643–1655

zur Hausen H 2002 Papillomaviruses and cancer: from basic studies to clinical application. *Nat. Rev. Cancer* **2** 342–350

zur Hausen H 2006 Perspectives of contemporary papillomavirus research. *Vaccine* **24S3** S3/iii–S3/iv

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