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Transient expression of human papillomavirus type 16 virus-like particles in tobacco and tomato using a tobacco rattle virus expression vector

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Abstract

The major capsid protein L1 of human papillomavirus type 16 (HPV16) was transiently expressed in tobacco (*Nicotiana benthamiana* and *Nicotiana tabacum*) and tomato (*Lycopersicon esculentum*) leaves using *Agrobacterium tumefaciens*. The expression vector pTV00 was derived from tobacco rattle virus (TRV). The highest L1 expression 15 μg g^{-1}(f.m.) was achieved when the coding sequence of L1 was optimized for expression in humans that caused an increase of the guanine and cytosine (GC) content from 38.2 % in wild type HPV16 to 64.1 % in optimized sequence. L1 monomers readily self-assembled into capsomeres and further into virus like particles (VLPs). Immunological characterization and electron microscopy showed that 89 % of L1 retained VLP structure also in extracts prepared from freeze-dried leaves. Plant expressed L1 in crude extracts was highly immunogenic without any additional adjuvant as vaccinated mice developed strong humoral and cellular immune response, comparable to that elicited by purified VLPs derived from insect cells. Further, the induced antibodies effectively neutralized infection of 293TT cells with pseudovirions. This finding demonstrates that the TRV expression system is comparable to other plant expression systems and due to the broad host range of TRV is particularly attractive when expression in plants with low content of toxic alkaloids is desired. Moreover, a monoclonal anti-L1 antibody E2 raised in the course of immunization with crude extract from freeze-dried leaves expressing L1 is specific preferentially against HPV VLPs and could be used in direct ELISA for monitoring of VLPs assembly and VLP purification protocols.

Additional key words: ELISA, *Lycopersicon esculentum, Nicotiana benthamiana, Nicotiana tabacum*, VLP specific antibody.

Introduction

Infection by human papilloma virus (HPV) attracted attention in connection with cervical cancer in humans (Zur Hausen 1996). HPV type 16 alone accounts for approximately 50 % of all cases of cervical cancer. The virus icosahedral capsid is composed of the L1 major and the L2 minor proteins (Finnen *et al.* 2003). L1 alone has the capacity to self-assemble into virus-like particles (VLPs) without participation of L2 or other proteins (Kirnbauer *et al.* 1992). Because of similar immunogenicity compared to infectious virions, VLPs can be produced and used as a safe preventive vaccine against viral transmission of cervical cancer (Schiller 1999). During recent years two highly efficient VLP-based HPV vaccines (*e.g. Gardasil*, *Merck MSD* and *Cervarix*, *GlaxoSmithKline*) have become available (Schiller *et al.* 2008).

For commercial production of vaccines and recombinant therapeutics, plants are often considered as a

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Abbreviations: BSA - bovine serum albumin; ELISA - enzyme linked immunosorbent assay; ELISPOT - enzyme-linked immunosorbent spot assay; GC - guanine and cytosine; GFP - green fluorescent protein; HPV - human papilloma virus; i.p. - intraperitoneal application; mAb - monoclonal antibody; PAGE - polyacrylamide gel electrophoresis; PBS - phosphate buffered saline; RT-PCR - polymerase chain reaction coupled with reverse transcription of RNA template; s.c. - subcutaneous application; T-DNA - transfer DNA of *Agrobacterium* Ti plasmid; TRV - tobacco rattle virus; VLPs - virus like particles; TSP - total soluble protein; YEB - yeast extract broth.
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cost-effective alternative with several benefits. Firstly, production in plants can be easily scaled up in the case of acute demand for production and secondly, produced proteins are unlikely to be contaminated by human or animal pathogens, toxins and oncogenic sequences. Moreover, plants provide a convenient environment for protein expression and storage including the possibility of direct administration as edible vaccine if expressed in the appropriate plant tissue. Antigens as the Norwalk Virus capsid protein (Mason *et al.* 1996), HPV-11 VLPs (Scholthof *et al.* 1996, Warzecha *et al*. 2003) and the Hepatis-B-Virus surface antigen (Richter *et al.* 2000) have been expressed in potato and the Rabies Virus-G protein in tomato (McGarvey *et al.* 1995) and positively tested in animals for the induction of an immune response after oral delivery.

The bioactive proteins could be produced in transgenic plants or transiently from regular binary vectors containing expression cassettes or vectors derived from plant viruses. Transgenic or transient approaches have their pros and cons; nevertheless transient expression is straightforward, flexible and can rapidly provide large amounts of product for characterization and testing. Moreover, the production from viral vectors is possible at a field scale (Scholthof *et al*. 1996). Protein expression from viral vectors can be further improved by agro-infection when an expression cassette with viral control sequences is delivered into cells on T-DNA with the help of *Agrobacterium* functions (Shen and Hohn 1995).

When expressing L1 in plants, several groups observed none or very low expression from sequences of original HPV isolates and this is why codon usage has been adapted either for expression in plant hosts (potato or tobacco) or in mammalian (human) cells (Nakamura *et al.* 2000, Biemelt *et al.* 2003). Mossadegh *et al*. (2004) showed that expression of HPV11 L1 with codons adapted for human cells results in 100-fold higher content of expressed protein in mammalian epithelial and fibroblast cells when compared to the unmodified L1.

Warzecha *et al.* (2003) and Biemelt *et al.* (2003) reported immune responses in mice fed with potato tubers expressing L1. Further, it was suggested that plant tissue could have an adjuvant stimulatory effect on the immune response. Indeed, Maclean *et al*. (2007) and Fernández-San Millán *et al.* (2008) observed that non-adjuvant plant-derived VLPs elicited higher neutralizing titers than VLPs delivered along with Freund's or alumina adjuvant. This indicates that the adjuvant could be dispensable or even deleterious for application of plant-expressed antigens.

In this study we explored plant tobacco rattle virus (Ratcliff *et al*. 2001) for transient expression of the HPV 16 L1 protein in leaves of *Nicotiana benthamiana*, *Nicotiana tabacum* and *Lycopersicon esculentum*.

Materials and methods

Construction of vectors for transient expression: pTV00, a tobacco rattle virus (TRV) vector based on RNA 2 of TRV strain PPK20 was used for L1 expression in plants. This vector is derived from pCa7, a 35S driven full-length infectious clone of PPK20 RNA 2 (Hernandez *et al*. 1995) transferred to the pGreenI *Agrobacterium* binary vector (Hellens *et al.* 2000a). pTV00 requires for its function RNA-dependent RNA polymerase (RdRp), movement protein (Mp) and 16 kb protein (16k) encoded by TRV RNA1 that is carried on the separate vector pBINTRA6 and this is why pBINTRA6 must be introduced to plants as well as pTV00 to obtain infection (Ratcliff *et al.* 2001).

Original HPV16 L1 coding sequence (wt) and sequences with adapted codon usage for expression in potato (p) and humans (h) from plasmids described by Leder *et al.* (2001) were cloned between *Xma*I and *Hin*dIII sites of the pTV00 and designated as pTVL1wt, pTVL1p and pTVL1h. These constructs were electroporated into *Agrobacterium tumefaciens* GV3101::pMP90(RK) strain (Hellens *et al.* 2000b).

Transient expression of L1 in plants and preparation of leaf extracts: *Agrobacterium tumefaciens* cultures were grown to saturation in YEB medium with antibiotics, centrifuged and resuspended in 10 mM $MgCl_2$, 10 mM MES containing 150 mM acetosyringone for 2 h at room temperature to induce T-DNA transfer functions (Hellens *et al.* 2000b). For TRV infection, separate *Agrobacterium* cultures carrying pTVL1 and pBINTRA6 plasmids (Ratcliff *et al.* 2001) were mixed in a 1:1 ratio. After addition of carborundum powder, two bottom leaves of 4-week-old tobacco (*Nicotiana benthamiana* L., *Nicotiana tabacum* L.) or tomato (*Lycopersicon esculentum* Mill.) plants were inoculated by gentle rubbing of the mixture. Plants were grown in a greenhouse with controlled temperature not exceeding 22 °C. After indicated time periods, leaves were collected and either immediately frozen in liquid nitrogen and stored at -75 °C until use or freeze-dried. Crude extracts were prepared from frozen leaf tissue either by grinding in liquid nitrogen to a fine powder or by homogenization with steel balls in *Mixer Mill MM301* (*Retsch,* Haan, Germany). For 100 mg of fresh leaves 100 mm^3 of phosphate buffered saline (PBS) containing a cocktail of protease inhibitors (*Sigma,* St. Louis, USA) was used. Suspension was cleared from tissue debris by centrifugation at 12 000 *g* for 5 min at 4 °C and supernatants were kept on ice until use. From freeze-dried leaves proteins were directly extracted by vortexing at concentration 10 mg of dry mass per 100 mm^3 of PBS for 1 min at room temperature and cleared from debris by centrifugation.

Quantitative detection of L1 mRNA by RT-PCR: Total plant RNA was isolated using RNeasy plant mini kit (*Qiagene,* Hilden, Germany) following the supplied protocol. Enhanced *Avian* RT-PCR kit (*Sigma*) was used to synthesize cDNA from 1 mg of total RNA with the help of anchored oligo$(dT)_{23}$ in 20 mm^3 reaction as described in supplied protocol. Heat denaturated cDNA (1 mm^3) reaction mixture was used for quantitative RT-PCR assay using *Brilliant SyberGreen QPCR* master mix (*Stratagene,* La Jolla, USA) in *MX 3005P* cycler (*Stratagene*) with the following primers: L1p forward: 5'-CAGGACTTCAATACAGGGTTT-3', L1p reverse: 5'-GTTCAAGTGGTGGACAATCTC-3', L1h forward: 5'-GTGAGCAAGGTGGTGAGCACC-3', L1h reverse: 5'-GATGCACTCCCTGTTGTCCAC-3', L1wt forward: 5'-TGCAGTTGGACATCCCTATT-3', L1wt reverse: 5'-GTCCACTAACAGGTGGTAATCT-3'. Aliquots of the PCR reactions were checked on agarose gels for size and purity. Values were normalized for variation against 7S-RNA used as an internal standard.

Antibodies: Anti-HPV16L1 monoclonal (1.3.5.15, 25C and E10) antibodies and rabbit anti-L1 polyclonal antibody #4543 were produced at DKFZ (Heidelberg, Germany) and routinely used. In early experiments a monoclonal anti-HPV16 L1 antibody CamVir-1 (*Chemicon,* Tamecula, USA) was also used.

Mouse monoclonal antibody against L1-VLPs was raised from splenocytes of mice immunized with the crude extract from freeze-dried leaves of *N. benthamiana* expressing L1 protein using a standard protocol. For this purpose, a group of four B10.AxBalb/C mice was immunized twice, at 2-week intervals, firstly intraperitoneal (i.p.) and followed by subcutaneous (s.c.) application of plant extract prepared from leaves expressing L1 (2 mice) and two control mice with plant extract alone. Plant-expressed L1 induced a high titer of specific antibody already after the first immunization (1:100 and 1:50), and increase in antibody titer (1:500 and 1:100) was found after the second booster. Both mice challenged with L1 were used for preparation of monoclonal antibodies. For this purpose, the third booster was applied to mice three months after the second booster. The mouse with a higher titer of anti-L1 antibody died and so only one immunized mouse was used for preparation of hybridoma cells. The hybridoma supernatants were analyzed by ELISA against the purified VLPs and further tested by native and denaturing Western blot. The hybridoma E2, whose supernatant showed the highest signal on the native and no signal on the denaturing Western blot, was grown in mice and in cell culture.

Detection of L1 protein by ELISA: Plant extracts were prepared as described above. 96-well microtiter plates were coated overnight at 4 °C with extracts or PBS only. After washing, plates were blocked with 3 % nonfat milk in PBS and 0.05 % *Tween 20* and incubated with primary anti-L1 CamVir-1 diluted 1:500 for 1 h at 37 °C. Goat anti-mouse antibody conjugated with horseradish peroxidase (*Sigma*) diluted 1:4000 was used as secondary antibody for detection. After a final wash, 100 mm^3 of freshly prepared staining solution containing 100 mM sodium acetate, 50 mM NaH_2PO_4, pH 4.2, 1 mg cm^{-3} ABTS (2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid; *Sigma*) and 0.012 % H_2O_2 was added and after 15 min the signal was read in a *VersaMax* (*Molecular Device,* Sunnyvale, USA) plate reader.

Detection of L1 structure by antigen-capture ELISA: 96-well microtiter plates were coated overnight at 4 °C either with anti HPV16L1 mAb 1.3.5.15 diluted 1:500 or incubated with PBS only for background determination. After coating, plates were washed and blocked with 3 % nonfat milk in PBS and 0.05 % *Tween 20* for 1 h at 37 °C. For analysis, plant extracts diluted 1:1 in 1.5 % milk in PBS were added into wells of coated plates and incubated for 1 h at 37 °C. VLP-specific polyclonal rabbit antiserum #4543 diluted 1:4000 in 1.5 % nonfat milk and 0.05 % *Tween20* in PBS was used as a secondary antibody to detect L1. After incubation with the secondary antibody for 1 hour at 37 °C, plates were washed and a goat anti-rabbit antibody conjugated with peroxidase (*BioRad,* Hercules, USA) diluted 1:4000 in 1.5 % nonfat milk in PBS and 0.05 % *Tween 20* was added. Staining using ABTS substrate was performed as described above. As positive control purified VLPs diluted 1:500 were used.

Western blot analysis of L1 expression: Protein concentration was determined in extracts by *BioRad* protein assay using bovine serum albumin as standard. Equal amounts of total protein were processed for SDS-PAGE in Laemmli sample buffer (*BioRad*) and loaded on 15 % SDS polyacrylamide gels and electrophoresed in *Mini Protean II* apparatus (*BioRad*) at 20 mA per gel, for 60 min. Separated proteins were semi-dry electro-blotted in 25 mM Tris, 190 mM glycine, 20 % methanol buffer onto *Immobilon-P* membranes (*Millipore,* Billerica, USA) at a constant current 0.8 mA cm^{-2} for 1.5 h. Native proteins were analyzed similarly, but omitting denaturizing and reducing agents and conditions in native sample buffer (*BioRad*). L1 protein on blots was detected with primary mAb 1.3.5.15 diluted 1:200 in 1 % nonfat milk in PBS with 0.1 % *Tween 20* and secondary goat anti-mouse antibody conjugated with alkaline phosphatase (*Sigma*) diluted 1:15000 in 1 % nonfat milk, 0.1 % *Tween 20* in PBS. Membranes were stained with an alkaline phosphatase conjugate substrate kit (*BioRad*).

Localization of L1 in plant cells: Small pieces of leaves were fixed in a mixture of 3 % paraformaldehyde and 0.5 % glutaraldehyde in PBS and sequentially equilibrated with 0.11, 0.22, 0.44, 0.88 and 1.76 M sucrose in PBS at 4 °C and after final equilibration step frozen at -20 °C. Cryosections (6 μm) were adhered to polyethylene coated slides and sucrose from sections was removed in several steps with decreasing sucrose concentration.

After final washes in PBS, slides were immediately processed for L1 detection. Microscopic specimens were permeabilized in 1 % *TritonX-100* in TBS (Tris 50 mM, NaCl 150 mM, pH 7.6) for 20 min and blocked with 10 % goat serum. L1 was detected by overnight incubation with primary anti-L1 polyclonal antibody #4543 diluted 1:4000 at 4 °C, followed by 2 h incubation with secondary goat anti-rabbit antibody conjugated with alkaline phosphatase (*Sigma*) at room temperature. Alkaline phosphatase in samples was detected with *HNPP* fluorescent detection set (*Roche,* Indianapolis, USA). Nuclei were counterstained with *DAPI* (0.1 mg cm^{-3}) in 1,4-diazabicyclo[2.2.2]octane (DABCO) based anti-fade mounting media. Images were captured on CCD camera on a top of *Nikon Eclipse 800* epifluorescence microscope, evaluated with *Lucia* software (*LIM, Czech Republic*) and processed with *PaintShop Pro 7*.

Electron microscopy: For negative staining of L1 particles, carbon-coated copper grids were discharged and 10 mm^3 aliquots of plant extract (10 mg of freeze dried leaves extracted in 100 mm^3 of PBS) were allowed to adsorb for 60 s. After wash in water, excess liquid was removed and the specimens were stained for 30 s with 2 % aqueous solution of uranyl acetate, dried and examined with *Zeiss EM10A* electron microscope at 80 kV.

Immunization of mice: Each of 15 female C57BL/6 mice was vaccinated s.c. with 5 μg of L1 protein in 250 mm^3 of extract from 50 mg freeze-dried leaves expressing L1 on days 0, 15, 30. As a negative control 5 mice received only extract from 50 mg freeze-dried leaves infected with an empty vector pTV00 and as a positive control 5 mice received the same extract as negative control, but supplemented with 5 μg of purified VLPs. The booster dose of 100 mm^3 of vaccine was applied 30 days after the last immunization. No plant extract-related toxicity was noticed in any of vaccinated mice. Serum samples were collected from immunized mice one week after booster and analyzed for presence of L1-specific antibodies by ELISA.

Detection of anti-L1 responses in mice: Microtiter plates were coated with 50 ng of purified VLPs in PBS per well during overnight incubation at 4 °C. Wells treated with PBS only served as a negative control. After washing three times with PBS and 0.05 % *Tween 20* wells were blocked with 3 % nonfat milk in PBS, 0.05 % *Tween 20* for 1 h at 37 °C. Sera from vaccinated mice were diluted 1:25 with 1.5 % nonfat milk, 0.05 % *Tween 20* in PBS and 100 mm^3 added to precoated wells and incubated for 1 h at 37 °C. After washing, anti-L1 antibodies were detected with a goat anti-mouse antibody conjugated to horseradish peroxidase (*Sigma*) diluted 1:3000 with 1.5 % nonfat milk and 0.05 % Tween 20 in PBS using ABTS peroxidase substrate. Absorbtion was measured 15 min after addition of substrate at 450 nm. *MAb 1.3.5.15* was used as positive control.

ELISPOT assay: Induction of cytotoxic T-lymphocytes (CTLs) was monitored by ELISPOT assay as previously described (Ohlschläger *et al*. 2003, Thönes and Müller 2007). Splenocytes recovered from immunized C57BL/6 mice one week after the second booster were stimulated with a $L1_{(165\text{-}173)}$ peptide AGVDNRECI. The number of IFN-γ producing L1 specific T-cell precursors was determined using an anti-IFN-γ antibody. Spots were determined on ELISPOT reader *Zeiss-Vison C* (*Zeiss,* Jena, Germany).

Neutralization assay: Neutralizing capacity of antibody raised by crude extracts from plants expressing L1 in mice was assayed by the neutralization of pseudovirions (Pastrana *et al.* 2004) as described in detail by Thönes and Müller (2007). Target 293TT cells for the neutralization assay, were seeded at concentration of 22 500 cells per well on 96-well plates in *DMEM* media (*Sigma*), supplemented with 10 % fetal calf sera (*Gibco BRL,* Eggenstein, Germany), 1 % penicillin/streptomycin (*Life Technologies,* Eggenstein, Germany) and 125 μg cm^{-3} hygromycin (*Roche*). The following day, the pseudovirions (Psv) were diluted in DMEM (1:5 000) and mixed with the sera of immunized mice at different dilutions and after 15 min of incubation at room temperature the medium of the 293TT cells was replaced with 200 mm^3 of the neutralized pseudovirion solution. As a negative control, cells were incubated with pseudovirions alone. Rabbit anti-L1 polyclonal antibody #4543 diluted 1:5000 and monoclonal 1.3.1.15 antibodies diluted 1:500 were used as positive controls. Quantification of secreted alkaline phosphatase (SEAP) in cell culture supernatant was performed 5 d later with the chemiluminescent SEAP reporter gene assay kit (*Roche*) following the manufacturer's instructions. To calculate the percent neutralization, the differences were normalized between the Psv/alone control and the pseudovirions incubated with the sera (Psv/alone - Psv/serum) to the difference between the signals of the controls (Psv/alone - Psv/#4543).

Results

Transient expression of L1 protein in plants: The L1 coding sequence was cloned into the TRV-based viral expression vector. Beside the wild type L1 sequence *L1wt*, we used also *in vitro* synthesized genes, in which the codon usage was adapted for expression in either potato *L1p* or human cells *L1h* (Leder *et al.* 2001). Their analysis did not revealed presence of cryptic splice-sites. Transcript levels measured by RT-PCR were detectable

2 - 14 d after inoculation (Fig. 1*A*). Although the mRNA levels did not differ significantly, the level of the expressed L1 protein from pTVL1h was one order of a magnitude higher than those from pTVL1p and pTVL1wt vectors (Fig. 1*A*). L1 was detectable 3 d after inoculation and the infection spread systematically to apical leaves in one week reaching maximum of the expression 10 - 14 d after inoculation (Fig. 1*A*). We have also tested co-expression of L1 and GFP. The highest L1 expression coincided with the peak GFP fluorescence observed *in situ* (data not shown), when leaves were co-infected with PVXGFP (potato virus X based vector expressing GFP reporter protein, Voinnet *et al.* 2000). In early experiments we found that infection spreads evenly from inoculated leaves to the top of the plants and so the co-infection was further omitted. It should be noted that co-infection of pTVL1h with pTV00-based GFP expression vector did not produce any fluorescence signal indicating that expression vectors derived from pTV00 are not suitable for simultaneous expression of two different proteins by means of super infection.

With direct ELISA we first detected monomeric L1 protein three days after inoculation, however, assembly into capsomers and VLPs was delayed for several days as detected by antigen capture ELISA using rabbit polyclonal antibody specific for higher order structures (Fig. 1*B*). This finding was later confirmed with direct ELISA when the VLP-specific mAb E2 became available.

Transiently expressed L1 in cells accumulates in the cytoplasm as depicted on leaf sections as detected by immunohistochemistry (Fig. 2*B*). The size of the expressed protein was determined by Western blot from extracts from apical leaves harvested 12 d after inoculation. A single band with a M_r of 56 kDa was detected after infection with the pTVL1h vector, whereas no protein was detected when the empty vector pTV00 was used (Fig. 3).

The yield of the L1 protein in leaves after *Agrobacterium* infection: The time-course of expression was similar in *N. tabacum*, *N. benthamiana* and *L. esculentum*, but the expression levels of L1 from pTVL1h varied considerably according to plant. The

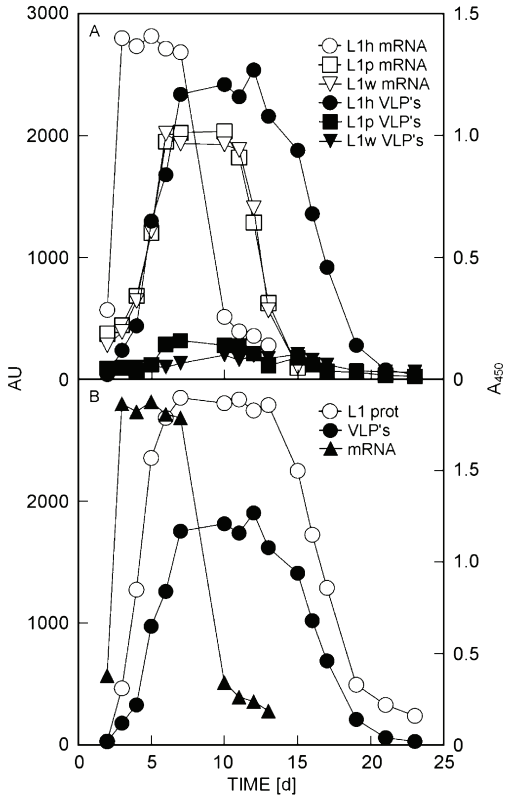

Fig. 1. Time course of the transient expression of L1 in *N. benthamiana*. *A* - The effect of codon usage was monitored at the level of mRNA transcripts (*open symbols*) with RT-PCR (arbitrary units, left y-axis) and of L1 protein (*black symbols*) by direct ELISA (A_{450}, right y-axis) over the period of three weeks after inoculation. The expression of L1h is by an order of magnitude higher than that of L1p and L1wt. *B* - Maturation of expressed L1h into VLPs. mRNA transcripts (*open circles*) are present instantly after leaf inoculation arbitrary units, left y-axis). Synthesis of L1 protein is first detected 3 d after inoculation and its assembly into VLPs is approximately delayed for another 2 d.

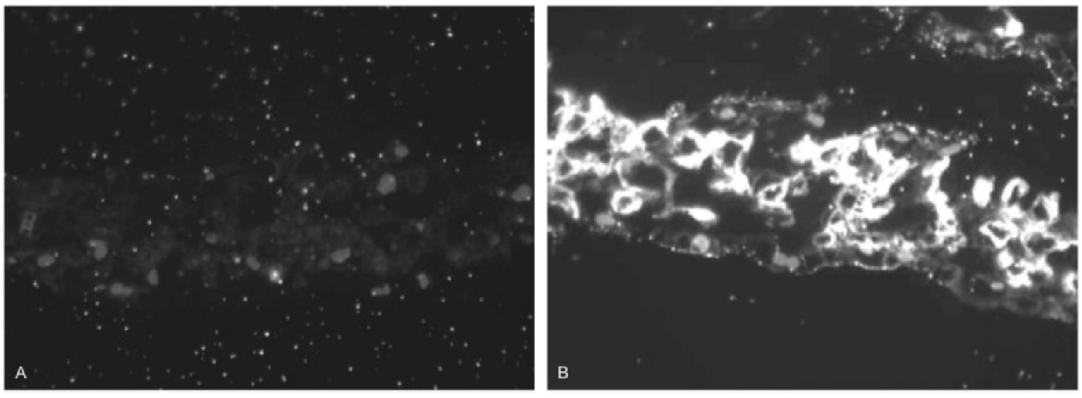

Fig. 2. Localization of L1 in sections of *N. benthamiana* leaves. *A* - No signal is detected in leaves inoculated with an empty pTV00. *B* - L1 accumulates in the cytoplasm of cells after inoculation of leaves with pTVL1h. The signal was detected by fluorescence microscopy at 400× magnification. Nuclei were counterstained with DAPI.

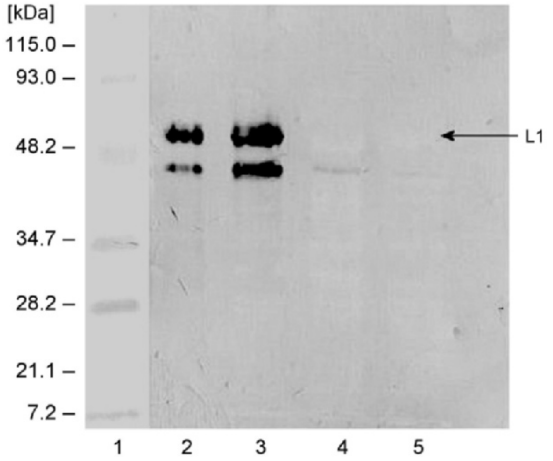

Fig. 3. Analysis of L1 in leaf extracts by Western blot. *Lanes* from left to right: 1 - Mr markers, 2 - purified HPV16 VLPs from insect cells (positive control), 3 - extracts from leaves of *N. benthamiana* inoculated with pTVL1h, 4 and 5 - extracts from leaves inoculated with pTV00 (negative control).

highest expression was found in tomato leaves with a yield of 15 ± 4 μg(L1) g^{-1}(f.m.) followed by *N. benthamiana* with a yield 10 ± 4 μg g^{-1}. However, *N. benthamiana* with higher leaves mass per plant than tomato is better suited for laboratory production of L1. *N. tabacum* with a yield only 1 μg g^{-1} seems to be least suitable for production of L1.

Stability of L1 assemblages upon freeze-drying and storage: The harvested leaves were frozen in liquid nitrogen, freeze-dried, ground to powder and stored at room temperature. The loss of higher order structure of L1 protein upon freeze-drying was determined as described above with a rabbit anti-L1 polyclonal antibody #4543 specific for assembled L1 proteins. When compared to fresh leaves, no significant difference of signal was detected (Fig. 4). Freeze-drying stabilizes L1 assembled structure in comparison with direct storage of leaves expressing L1 at -80 °C and undergoing freeze/thawing cycle, which leads to partial denaturing of the assembled L1 (not shown). We observed that the freeze-dried tissue retains the integrity of L1 at RT over the period of 1 year. Extracts prepared from freeze-dried powder was further analyzed by electron microscopy and used for mice immunization.

Electron microscopy of plant extracts prepared from freeze-dried leaves: Extracts from freeze-dried leaves expressing L1 were prepared for electron microscopy as described in Materials and methods. This analysis revealed the presence of capsid-like structures with size and morphology consistent with those of native HPV16 virions (55-nm-diameter spherical particles). However, the majority of L1 protein is assembled into capsomers (Fig. 5). In addition, flexible-rods of TRV capsids are also present in the extracts.

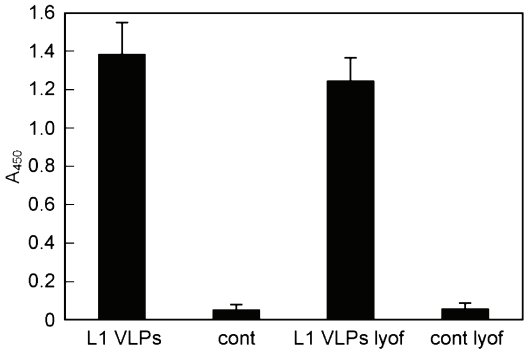

Fig. 4. Stability of L1 VLPs structure expressed in *N. benthamiana* leaves upon freeze-drying and extraction cycle. More than 90 % of L1 retains conformation in extracts from lyofilised leaves (*third column*) in comparison to extract prepared from fresh leaves (first column). Controls are extracts from leaves prepared the same way, but from plants inoculated with an empty pTV00 vector.

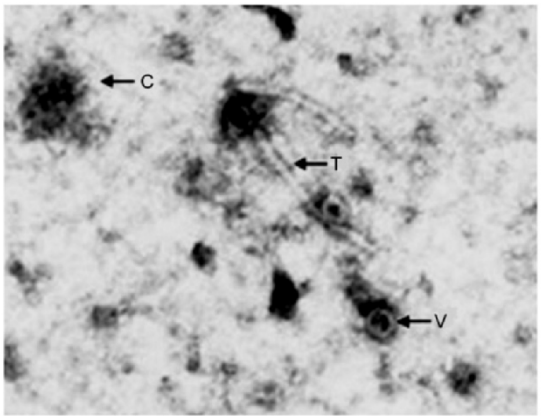

Fig. 5. Electron microscopy of extracts prepared from freeze-dried leaves expressing L1. V - HPV16 L1 VLPs (55-nm particles), C - HPV16 L1 capsomeres, T - flexible rods of TRV.

Immunogenicity of plant-derived HPV16 L1: To study the induction of the humoral immune responses, extracts from freeze-dried leaves of *N. benthamiana* alone or leaves expressing L1 were prepared. A group of 15 C57BL/6 mice was vaccinated s.c. with extracts prepared from L1 expressing leaves (5 μg of L1 per mouse), 5 mice with 5 μg of purified VLPs in plant extract prepared from uninfected leaves (positive control) and 5 mice with plant extract prepared from leaves infected with pTV00 (negative control). ELISA using purified VLPs from insect cell as an antigen was used to measure induction of L1-specific antibodies. Results showed that all mice vaccinated with plant-derived L1 responded and that there is no significant difference in immune induction between plant-derived L1 and purified VLPs (Fig. 6). Antibody titers ranged from 1:100 to 1:1000. No toxicity associated with application of extracts from *N. benthamiana* leaves was observed.

Neutralization activity of induced antibodies: The sera collected from immunized mice were tested *in vitro*

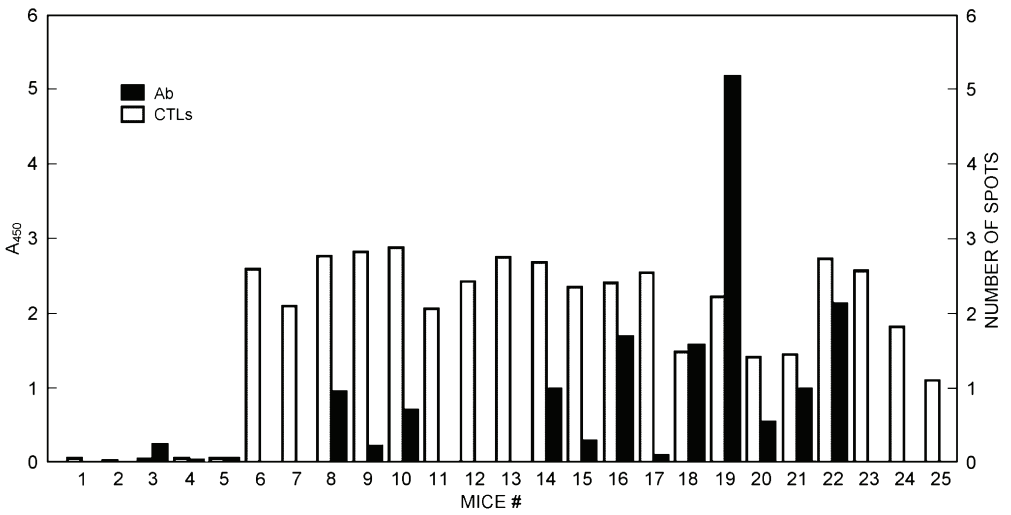

Fig. 6. Antibody (Ab) and cytotoxic T-lymphocytes (CTLs) induction by L1. Response elicited by control plant extracts (mice 1 - 5), control plant extract from *N. benthamiana* containing purified VLPs from insect cells (mice 6 - 10) and plant extracts from leaves expressing L1 (mice 11 - 25) in C57BL/6 mice. Collected sera from individual mice were tested by ELISA for specific antibody induction (*gray columns*, left y-axis in A_{450} units). CTLs induction was measured by ELISPOT analysis of splenocytes recovered from scarified animals. The number of CTLs spots was recorded by an ELISPOT reader and expressed as a mean per 10^6 splenocytes (*back columns*, right y-axis in 100 CTLs spots).

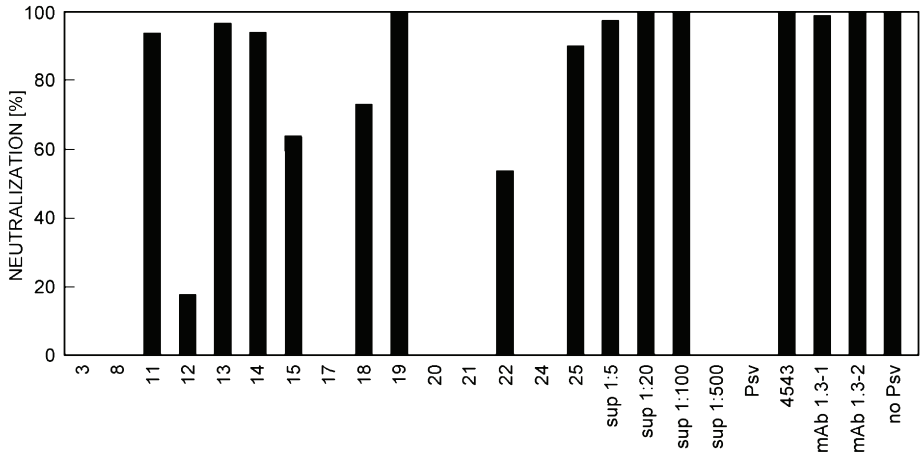

Fig. 7. Pseudovirion neutralization assay. Sera of mice and of E2 hybridoma supernatant were tested for neutralization of infection of 293T cells by pseudovirions (Psv). Percent of neutralization obtained by incubation with mice sera diluted 1:50 and that of E2 hybridoma supernatant diluted 1:5, 1:20, 1:100, 1:500. Neutralization activity of mice sera was compared to a high titer rabbit polyclonal anti-L1 antiserum 4543 (100 %) and to the mAb's 1.3.1 and 1.3.2 as additional positive controls.

for their ability to neutralize infection by pseudovirions. In the group of mice immunized with plant-derived L1 there were two sera that neutralized more than 50 % and six that neutralized more than 70 % of the input pseudovirions at a dilution 1:50 (Fig. 7). However, the included positive-control serum, collected from the mouse #8 immunized with the insect cell-derived VLPs did not show any neutralization activity when diluted 1:50. The sera collected from mice immunized with a control plant extract were also analyzed and as expected the absence of L1-specific antibodies in their sera (mouse #3) was confirmed by the inability of these sera to neutralize the VLPs.

L1 expressed in plants stimulates L1-specific cytotoxic T-cell immune response: The splenocytes isolated from immunized mice were assayed for IFN-γ-producing cells in response to the $L1_{165\text{-}173}$ peptide in ELISPOT. Seven of nine mice were tested positive after vaccination with plant-derived L1 and 2 of 3 mice after vaccination with purified VLPs (Fig. 6). Four mice immunized with plant-derived L1 had higher spot number than mice treated with purified VLPs.

Monoclonal antibody E2 specific for HPV16 L1-VLPs: Monoclonal antibody produced by the hybridoma clone E2 was analyzed for the specificity toward various

assembled structures of L1 and the ability to neutralize pseudovirions. In the neutralization assay supernatant of E2 inhibited pseudovirions up to dilution 1:100 (Fig. 7). Results indicated that mAb E2 preferentially reacts with T7-VLPs (72 pentamers, Thönes and Müller 2007) and to much smaller extent also with a mixture containing T1-particls (12 pentamers) and capsomers (Fig. 8). Analysis and comparison with other anti-L1 monoclonal antibodies used in this study showed that mAb E2 is specific to VLPs (Fig. 8) and could be used in direct ELISA for detection of L1-VLPs instead of antigen-captured ELISA.

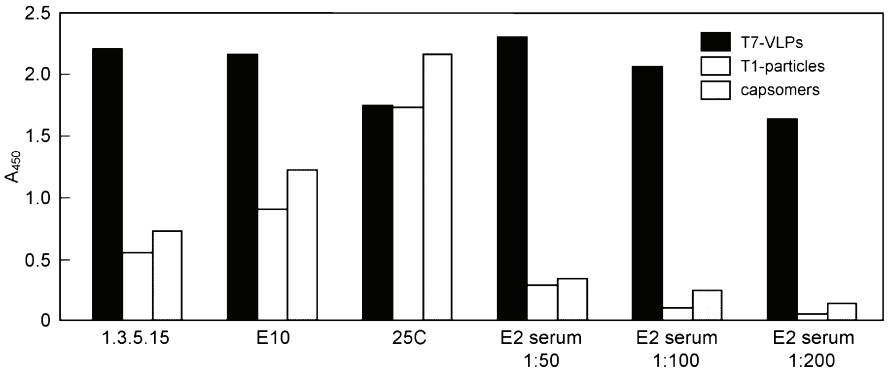

Fig. 8 The specificity of mAb E2 toward various L1 assembly structures. T7-VLPs of 72 pentamers, T1-particles of 12 pentamers and capsomers produced in insect cells were absorbed on the microtiter plates and analyzed by ELISA. Interactions of hybridoma E2 supernatant is compared to antibodies obtained after immunization with insect cell-derived VLPs (1.3.5.15, E10 and 25C).

Discussion

Transient expression of L1 in plants: Plant expression vector pTV00 based on TRV was used to express the major structural protein L1 of HPV-16. This vector overcomes many problems associated with vectors derived from PVX (potato virus X) and TMV (tobacco mosaic virus). TRV induces very mild symptoms, infects large areas of adjacent cells and cloned proteins are also expressed in the growing points of plants. Potential application of pTV00 is not restricted only to *Nicotiana* as TRV has a reported host range of over 60 species from 12 families including tomato, potato and others (Brunt *et al.* 1996). This makes the expression system of TRV particularly attractive when expression in plants with low content of toxic alkaloids is desired.

Yield of TRV expression is in the range 10 - 15 μg(L1) g^{-1}(f.m.) (1 - 1.5 % of TSP) that is similar to transient expression from tobacco mosaic virus (TMV) 5 μg g^{-1} in tobacco leaves (Varsani *at al.* 2006) or to expression 10 - 15 μg g^{-1} in tubers of transgenic potato (Biemelt *et al.* 2003, Warzecha *et al.* 2003).

Our results are in accordance with Biemelt *et al.* (2003), Warzecha *et al.* (2003), Mclean *et al.* (2007) and others and indicate that the expression level of L1 in plants strongly depends on codon usage. The coding sequence of L1 from original (wt) HPV16 isolate evidently reflects control function of the virus life cycle on transcription/translation level, what can underline instability of *L1wt* transcripts reported by Biemelt *et al.* (2003) and also why the codon usage has to be changed for efficient expression in human cells (Leder *et al.* 2001). Adapting original HPV16 sequence to plant (potato, tobacco) codon usage had little or no effect on transient expression of L1 and efficient expression has not been achieved until coding sequence was changed to human consensus codon usage. This led to the increase of GC content from 38.2 % in *L1w* or 34.8 % in *L1p* to 64.1 % in *L1h*. Our results show that transcripts from *L1h* appear earlier and are more efficiently translated into protein. Nevertheless the levels of *L1wt* and *L1p* mRNA transcript were also high in contrast to nearly undetectable expression of the L1 protein. Thus codon optimization besides improving transcription efficiency and mRNA stability evidently alleviates translational block(s). Whether efficient translation from *L1h* is due to removal of mRNA secondary structure hurdles or unknown translation signal(s) is not clear.

Higher levels of L1 transient expression and the role of signals for sub-cellular localization were reported Mclean *et al.* (2007) when he used regular expression cassettes in a plant binary vector and their transfection into cells after infiltration of plants with *Agrobacterium* strains. Particularly targeting of the L1 protein into chloroplast had a dramatic increasing effect on accumulation of L1 up to 0.65 mg g^{-1}(f.m.) in comparison to targeting to cytosol or ER.

Direct insertion of the HPV16 *L1* sequence between *trnI* and *trnA* region in chloroplast genome led to expression up to 3 mg g^{-1} of L1 in fresh leaves with levels reaching 24 % of TSP (Fernández-San Milán *et al.* 2008). In this case, the authors ascribed the high yield of L1 to the increased number of gene copies per cell. Surprisingly, such high level of L1 expression was achieved from an *L1* gene of HPV16 variant (African type 1) with codons of original isolate. These findings

point to the unique chloroplast environment, which on one side increases gene dosage and evidently helps to overcome transcriptional and translational constraints (Fernández-San Milán *et al.* 2008) and on the other supports the accumulation of heterologous proteins (Fernández-San Milán *et al.* 2008, Mclean *et al.* 2007). Nevertheless, which of these factors contributes most to the efficient expression of L1 in chloroplasts remain to be established.

Adjuvant effect of plants extracts: In several studies plant-expressed L1 has been proven to be immunogenic when administrated s.c. or i.p. (Varsani *et al.* 2006, Mclean *et al*. 2007, Férnández-San Millán *et al.* 2008). We found that VLPs assembled in plant leaves are stable upon freezing and drying and crude extracts prepared from such leaves are highly immunogenic. We observed that combined application of the extracts together with Freund's adjuvant elicit hyper-allergic response resulting even in mice death. Férnández-San Millán *et al.* (2008) also observed adverse effect when plant L1 extract was combined with additional adjuvant (Freund's or aluminum hydroxide gel). Evidently some, so far, unidentified plant proteins help elicit strong immune response to antigens when injected. Nevertheless, previous studies showed that immunogenic activity of L1 administrated by ingestion of potato tubers (Warzecha *et al.* 2003, Biemelt *et al.* 2003) is rather weak. Possible explanations are that potatoes do not contain protein(s) with adjuvant effect or that the adjuvant effect of the plant tissue does support induction of immune response across the intestinal mucosa.

Developing L1-VLPs as a diagnostic protein: Since two commercial prophylactic vaccines against cervical cancer, *Gardasil* and *Cervarix* (Schiller *et al.* 2008), are available and widely used, it is not realistic to expect that plant-derived vaccine will replace them, particularly in sight when the production cost contributes to market price at most by 30 %. Nevertheless, because of the robust expression of *L1* and its folding into VLPs reported by several groups and the availability of a highly specific antibody against L1-VLPs like our E2, L1-VLPs could be explored as diagnostic protein of expression. Routine direct ELISA assay of L1 and its assembly into structural VLPs with our E2 mAb monitors not only expression of the protein, but also its assembly in particular tissue when fused with the respective leader sequence. We are currently exploring this diagnostic system in fruits of transgenic tomato.

Conclusions: In this study we used the TRV-based vector pTV00 due to its broad host susceptibility range for transient expression of HPV16 major capsid protein L1 in two *Nicotiana* species and tomato. We showed that for successful expression of the L1, coding sequence has to be adapted by increasing the GC content from 40 to 60 %. The levels of the expressed L1 remain low in comparison to the yield after infiltration of binary vectors or chloroplast expression in transgenic plants, but are similar to other plant virus-derived vectors. This indicates that plant viral expression vectors are not well suited for biotechnological production of desired bioactive proteins. Expressed L1 readily assembles into VLPs, which are stable in foliar tissue upon freeze-drying. Extracts prepared from dried tissue and stored at room temperature for an extended period of time do not loose activity in respect to induce humoral and cytotoxic immunity. Antibodies induced by vaccination with crude extracts effectively neutralize pseudovirions infection without any additional adjuvant. We used this feature to generate monoclonal antibody E2 specific for assembled VLPs. Assembly of L1 into VLPs monitored by E2 in direct ELISA opens the possibility to use *L1h* as an alternative diagnostic protein to study effects of sub-cellular targeting.

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