*Journal of General Virology* (2007), **88**, 1460–1469 DOI 10.1099/vir.0.82718-0

# 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. Maclean,[1,2] M. Koekemoer,[2] A. J. Olivier,[3] D. Stewart,[1] I. I. Hitzeroth,[1] T. Rademacher,[4] R. Fischer,[4] A.-L. Williamson[1] and E. P. Rybicki[1,2]

Correspondence
E. P. Rybicki
ed@science.uct.ac.za

[1]Institute of Infectious Disease and Molecular Medicine, Faculty of Health Science, University of Cape Town, Cape Town, South Africa

[2]Department of Molecular and Cell Biology, University of Cape Town, Cape Town, South Africa

[3]Biovac Institute, Pinelands, Cape Town, South Africa

[4]Fraunhofer Institute, Aachen, Germany

Virus-like particle-based vaccines for high-risk human papillomaviruses (HPVs) appear to have great promise; however, cell culture-derived vaccines will probably be very expensive. The optimization of expression of different codon-optimized versions of the HPV-16 L1 capsid protein gene in plants has been explored by means of transient expression from a novel suite of *Agrobacterium tumefaciens* binary expression vectors, which allow targeting of recombinant protein to the cytoplasm, endoplasmic reticulum (ER) or chloroplasts. A gene resynthesized to reflect human codon usage expresses better than the native gene, which expresses better than a plant-optimized gene. Moreover, chloroplast localization allows significantly higher levels of accumulation of L1 protein than does cytoplasmic localization, whilst ER retention was least successful. High levels of L1 (>17 % total soluble protein) could be produced via transient expression: the protein assembled into higher-order structures visible by electron microscopy, and a concentrated extract was highly immunogenic in mice after subcutaneous injection and elicited high-titre neutralizing antibodies. Transgenic tobacco plants expressing a human codon-optimized gene linked to a chloroplast-targeting signal expressed L1 at levels up to 11 % of the total soluble protein. These are the highest levels of HPV L1 expression reported for plants: these results, and the excellent immunogenicity of the product, significantly improve the prospects of making a conventional HPV vaccine by this means.

Received 15 November 2006
Accepted 30 January 2007

## INTRODUCTION

The identification of a causal link between infection with specific human papillomaviruses (HPVs) and the development of cervical cancer has stimulated considerable interest in the development of HPV vaccines, especially for use in developing countries, where the disease burden is high and resources for widespread screening and follow-up procedures are limited (reviewed by Maclean *et al.*, 2005).

The major structural protein of the HPV capsid, L1, is the antigen of choice for the development of prophylactic vaccines. L1 can self-assemble into virus-like particles (VLPs), which are highly immunogenic and have given excellent results in human phase 2 clinical vaccine trials (Harper *et al.*, 2004; Mao *et al.*, 2006). Recombinant baculoviruses and yeast are currently the systems of choice for VLP production, but these platforms are not ideal for use in developing countries, as production costs are too high.

The use of plants for the large-scale production of heterologous proteins is gradually gaining widespread acceptance and could provide a platform for the cost-effective production of proteins on an agricultural scale. In particular, it has been proposed that plant production of human and animal vaccines may lower the cost of production of the raw material very significantly, especially for oral vaccination (reviewed by Fischer *et al.*, 2004; Mason *et al.*, 2002). Several groups have investigated the production of papillomavirus vaccines in plants: HPV-16 L1 protein has been produced in transgenic tobacco and potato and transiently in tobacco (Biemelt *et al.*, 2003; Liu *et al.*, 2005; Varsani *et al.*, 2003, 2006), HPV-11 L1 in transgenic potato (Warzecha *et al.*, 2003) and cottontail rabbit papillomavirus (CRPV) L1 both transiently and transgenically in *Nicotiana* spp. (Kohl *et al.*, 2006). Although VLPs were produced in all cases except for CRPV, yields of HPV-16 and HPV-11 L1 in potato were

 

too low to allow successful oral immunization and, in all cases, yields were <1 % of total soluble protein (TSP), considered to be the threshold for commercial production (Fischer *et al.*, 2004). However, Kohl *et al.* (2006) were able to protect rabbits successfully against CRPV challenge by injection of concentrated extracts of both transiently and transgenically produced CRPV L1 – the first proof of concept for a plant-produced papillomavirus L1 protein vaccine.

Expression levels of foreign proteins in plants can be increased markedly by use of different vectors and host plants, protein targeting and gene modification. However, making transgenic plants to investigate expression of a large assortment of proteins and/or vectors is not always practical, due to time constraints. In contrast, transient-expression systems are capable of rapidly evaluating the expression of numerous constructs in a variety of plant species.

*Agrobacterium tumefaciens*-mediated gene transfer has been utilized for many years to generate stably transformed plants. This involves the transfer of the T-complex (a complex of the bacterial T-DNA and virulence gene products) from *Agrobacterium* to plant cells. Any DNA located between the 25 bp direct repeats (left and right borders), which delimit the single-stranded T-DNA, is transferred into the plant-cell nucleus (Zupan *et al.*, 2000), where it integrates into the plant chromosome via illegitimate recombination (Somers & Makarevitch, 2004). Many of the T-DNA copies present in the nucleus do not integrate, however, but are transcribed, resulting in transient expression. Transient expression is not affected by position effects and can produce dramatically higher protein levels than stable transformation (Kapila *et al.*, 1997).

Two different *Agrobacterium* tissue-infiltration (agroinfiltration) methods are commonly used: direct injection into the abaxial air spaces of a leaf (Voinnet *et al.*, 2003) and vacuum infiltration (Kapila *et al.*, 1997). *Agrobacterium*-mediated transient expression peaks 60–72 h post-infiltration and then declines sharply as a result of post-transcriptional gene silencing (PTGS) (Voinnet, 2001): this is an adaptive antiviral-defence response that limits virus replication and spread in plants. Certain plant viruses encode silencing suppressors that can inhibit PTGS. Some of these proteins, such as NSs from tomato spotted wilt virus (TSWV) (Takeda *et al.*, 2002) and p19 of tomato bushy stunt virus (Voinnet *et al.*, 2003), are utilized to prolong and amplify *Agrobacterium*-mediated transient expression by the co-infiltration of *Agrobacterium* containing a silencing-suppressor gene.

In this paper, we describe the use of agroinfiltration to investigate the effects that codon optimization and plant cell-compartment targeting have on HPV-16 L1 expression levels, and we demonstrate the assembly of the L1 protein into higher-order and appropriately antigenic structures. We demonstrate the immunogenicity of these transiently expressed, plant-derived VLPs in mice and the elicitation of neutralizing antibodies. Finally, we demonstrate the production of transgenic tobacco expressing high levels of L1 (up to 11 % of TSP) when utilizing optimal expression vectors and strategies.

## METHODS

**Plasmid construction.** Three novel binary *Agrobacterium* vectors were used: pTRAc, pTRAkc-rbcs1-cTP and pTRAkc-ERH (Fig. 1). The pTRAc vector consists of a cauliflower mosaic virus (CaMV) 35S promoter (P35SS) with duplicated transcriptional enhancer, chalcone synthase 5′ untranslated region (CHS) and CaMV 35S polyadenylation signal (pA35S) for foreign gene expression; two copies of the tobacco Rb7 scaffold attachment region (SAR) flanking the expression cassette; the left and right borders for T-DNA integration; origins of replication for *Escherichia coli* and *Agrobacterium*; and the *bla* gene for antibiotic selection. pTRAkc-rbcs1-cTP is a derivative of pTRAc with the chloroplast-transit peptide sequence of the potato *rbcS1* gene. pTRAkc-ERH contains a plant codon-optimized signal-peptide sequence from the murine mAb24 heavy-chain gene and the his6 and endoplasmic reticulum (ER) retention (SEKDEL) sequences. The pTRAkc-rbcs1-cTP and pTRAkc-ERH vectors also include the *npt II* gene for kanamycin resistance in plants.

Four HPV-16 L1 gene variants were cloned into the above-mentioned binary vectors: (i) a South African isolate, SAL1 (GenBank accession no. AY177679); (ii) human codon-optimized L1 (HL1; GenBank accession no. DQ067889); (iii) plant codon-optimized L1 (SYNL1; GenBank accession no. DQ067890); and (iv) a nuclear localization signal (NLS)-deficient, C-terminal 22 aa truncation of HL1, HL1ΔC22.

Restriction-enzyme sites were added to the termini of the L1 genes by PCR amplification. SAL1 was amplified with sense primer 5′-GGACGCGTTAGGTACATGTCTCTTTGGCTGCCT and antisense primer 5′-TCTAGACTCGAGTTACAGCTTACGTTTTTTGCGTTT, digested with *Afl*III/*Xho*I and cloned into pTRAc, forming pTRA-SAL1; or digested with *Mlu*I/*Xho*I and cloned into pTRAkc-rbcs1-cTP, forming pTRACTP-SAL1. SAL1 was amplified with the above sense primer and antisense primer 5′-AGCGGCCGCCAGCTTAC-GTTTTTTGCG, digested with *Afl*III/*Not*I and cloned into the *Nco*I and *Not*I sites of pTRAkc-ERH, forming pTRAERH-SAL1.

SYNL1 was amplified with sense primer 5′-GGACGCGTGAGAT-TCATGAGCCTTTGGCTCCCT and antisense primer 5′-ATCTAGA-CTCGAGTTAGAGCTTCCTCTTCTTCCTCTT, digested with *Bsp*HI/*Xho*I and cloned into the *Afl*III and *Xho*I sites of pTRAc, forming pTRA-SYNL1; or digested with *Mlu*I/*Xho*I and cloned into pTRAkc-rbcs1-cTP, forming pTRACTP-SYNL1. SYNL1 was amplified with the above sense primer and antisense primer 5′-AGCGGCCGCGAG-CTTCCTCTTCTTCCTCTT, digested with *Bsp*HI/*Not*I and cloned into the *Nco*I and *Not*I sites of pTRAkc-ERH, forming pTRAERH-SYNL1.

HL1 was amplified with sense primer 5′-GGACGCGTGAGGTTC-ATGAGCCTGTGGCTGCCC and antisense primer 5′-ATCTAGACT-CGAGTCACAGCTTGCGCTTCTTCCG, digested with *Bsp*HI/*Xho*I and cloned into the *Afl*III and *Xho*I sites of pTRAc, forming pTRA-HL1; or digested with *Mlu*I/*Xho*I and cloned into pTRAkc-rbcs1-cTP, forming pTRACTP-HL1. HL1 was amplified with the above sense primer and antisense primer 5′-AGCGGCCGCCAGCTTGCGC-TTCTTCCGC, digested with *Bsp*HI/*Not*I and cloned into the *Nco*I and *Not*I sites of pTRAkc-ERH, forming pTRAERH-HL1.

HL1ΔC22 was formed by PCR amplification of HL1 with the above sense primer and antisense primer 5′-TCTAGACTCGAGTCAGCCC-AGGGTGAACTTAGG to facilitate termination before the NLS (Zhou

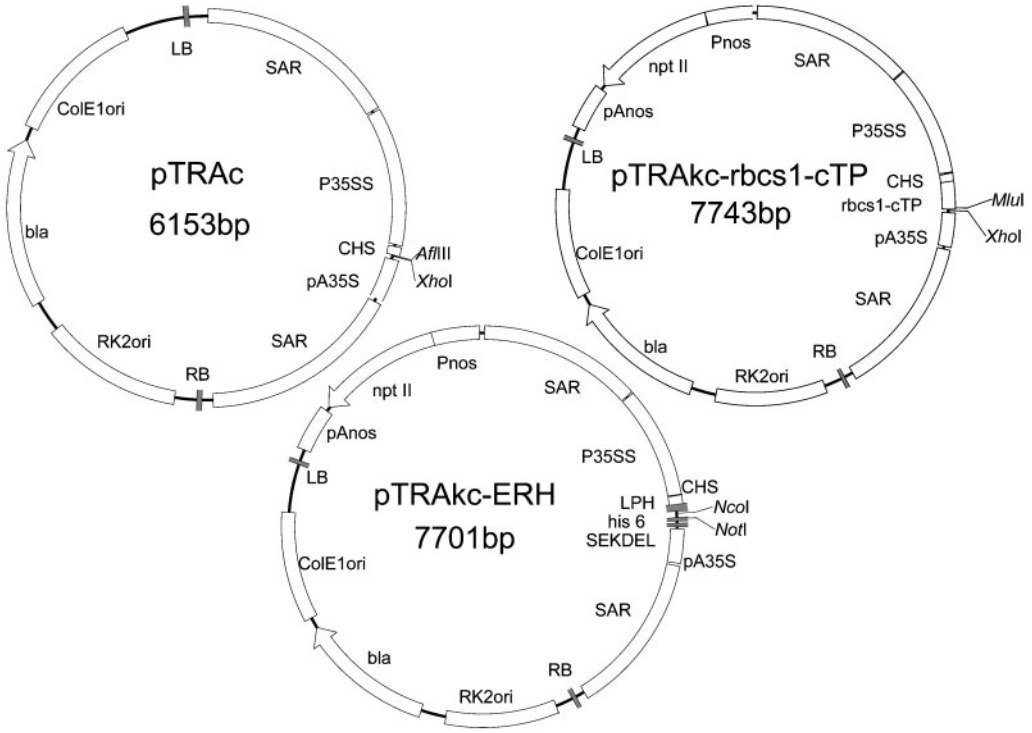


**Fig. 1.** *Agrobacterium* vectors pTRAc, pTRAkc-rbcs1-cTP and pTRAkc-ERH. P35SS, CaMV 35S promoter with duplicated transcriptional enhancer; CHS, chalcone synthase 5′ untranslated region; pA35S, CaMV 35S polyadenylation signal; SAR, scaffold attachment region of the tobacco Rb7 gene; LB and RB, the left and right borders for T-DNA integration; ColE1ori, origin of replication for *E. coli*; RK2ori, origin of replication for *Agrobacterium*; bla, ampicillin/carbenicillin-resistance *bla* gene; LPH, signal-peptide sequence from the murine mAb24 heavy chain; his6, 6 × His tag sequence; SEKDEL, ER-retention signal sequence; rbcs1-cTP, chloroplast-transit peptide sequence of a Rubisco small-subunit gene (rbcS1) from *Solanum tuberosum*; npt II, kanamycin-resistance *npt II* gene; Pnos and pAnos, promoter and polyadenylation signal of the nopaline synthase gene.

*et al.*, 1991). The PCR product was digested with *Mlu*I/*Xho*I and cloned into pTRAkc-rbcs1-cTP, forming pTRACTP-HL1ΔC22.

The 30B-GFPC3 mutant *gfp* gene (GenBank accession no. U62637) was amplified from pBSG1057 (Large Scale Biology Corporation) with sense primer 5′-GGACGCGTTAGGTCCATGGCTAGCAA-AGGAGAAG and antisense primer 5′-ATCTAGATTATTTGTA-GAGCTCATCCATG, digested with *Nco*I/*Xba*I and cloned into pTRAc, forming pTRA-GFP.

***Agrobacterium* transformation.** *A. tumefaciens* GV3101 : : pMP90RK was made electrocompetent (Shen & Forde, 1989) and 100 µl was mixed with 50–200 ng of the HPV-16 L1 binary vector clones in a 0.1 cm electrogap cuvette (BioRad). The cells were transformed by using a GenePulser (BioRad) set at 1.8 kV, 25 µF and 200 Ω. Electroporated cells were incubated in 1 ml Luria–Bertani (LB) broth for 2 h prior to plating on LB medium containing 50 µg carbenicillin $ml^{-1}$, 50 µg rifampicin $ml^{-1}$ and 30 µg kanamycin $ml^{-1}$.

**Agroinfiltration.** *Agrobacterium* cultures containing HPV-16 L1 vector clones were supplemented with 50 µg carbenicillin $ml^{-1}$ and 50 µg rifampicin $ml^{-1}$. *Agrobacterium* LBA4404 (pBIN-NSs) cultures containing the *NSs* silencing-suppressor gene of TSWV were supplemented with 50 µg rifampicin $ml^{-1}$ and 30 µg kanamycin $ml^{-1}$. Cultures were grown with shaking at 27 °C to exponential phase ($OD_{600}$ approx. 0.8) in LB broth containing the appropriate antibiotics. Cells were collected by centrifugation at 4000 ***g***, resuspended in induction medium (LB broth at pH 5.6 containing 10 mM MES, 20 µM acetosyringone and 2 mM $MgSO_4$) with the appropriate antibiotics, and grown as above. The cells were collected by centrifugation at 4000 ***g*** and resuspended in infiltration medium (10 mM $MgCl_2$, 10 mM MES, 2 % sucrose and 150 µg acetosyringone $ml^{-1}$, pH 5.6). The *Agrobacterium* suspensions were diluted in infiltration medium to an $OD_{600}$ of 1.0 and were kept at 22 °C for 2–3 h.

The two infiltration methods used were direct injection and vacuum infiltration. For injection, the *Agrobacterium*-L1 and *Agrobacterium* (pBIN-NSs) suspensions were diluted and combined in infiltration medium, both to a final $OD_{600}$ of 0.25. When *Agrobacterium* (pTRA-GFP) was co-infiltrated with the above suspension, it was used at a final $OD_{600}$ of 0.0125. Leaves from 2–4-week-old *Nicotiana benthamiana* plants were infiltrated by injecting the bacterial suspension into the abaxial air spaces from the underside of the leaf. Six leaves were agroinfiltrated with each bacterial mixture (three plants, two leaves per plant). The plants were grown for 5–6 days under conditions of 16 h light, 8 h dark, 22 °C. For vacuum infiltration, *Agrobacterium* (pTRA-HL1) and *Agrobacterium* (pBIN-NSs) were grown overnight in induction medium. The cells from each culture were combined and resuspended in 1–8 l infiltration medium to a final $OD_{600}$ of 0.25 per culture. Whole *Nicotiana tabacum* L. 'Petite Havana' SR1 plants with roots removed were submerged into the bacterial suspension and subjected to a vacuum of −90 kPa for 5–10 min, with occasional agitation to release trapped air bubbles. The vacuum was released rapidly (approx. 10 kPa $s^{-1}$). The plant

stalks were placed in water-saturated floral foam. The plants were grown for 3 days under conditions of 16 h light, 8 h dark, 22 °C.

**Protein extraction and L1 detection.** *N. benthamiana* leaf discs (cut by using the cap of a microfuge tube) were harvested from agroinfiltrated leaves and ground in 250 µl high-salt phosphate buffer (0.5 M NaCl) per disc. The extract was centrifuged at 13 000 r.p.m. for 5 min, supernatant was collected and the centrifugation was repeated.

For Western blot analysis, plant extracts were incubated at 85 °C for 2 min in loading buffer (Sambrook *et al.*, 1989), separated by SDS-PAGE (10 % gel) and then transferred onto a nitrocellulose membrane by semi-dry electroblotting. L1 protein was detected with H16.J4 mAb (1 : 3000) and then with a goat anti-mouse–alkaline phosphatase conjugate (1 : 10 000; Sigma). Detection was performed with NBT/BCIP tablets (Roche).

L1 protein was quantified from plant extracts by capture ELISA, which was modified from a PVA-blocking ELISA method (Studentsov *et al.*, 2002). A 96-well microtitre plate was coated with mAbs H16.J4 (recognizing a linear HPV-16 L1 epitope) or H16.V5 (recognizing a conformational epitope) for 1 h at 37 °C, washed and blocked. Plant extract was added for 1 h at 37 °C, followed by a washing step and addition of rabbit anti-HPV-16 VLP polyclonal serum (1 : 1000) for 1 h at 37 °C. Detection was with swine anti-rabbit–horseradish peroxidase (HRP) conjugate (1 : 5000; DAKO, Denmark) and 1,2-phenylenediamine dihydrochloride (OPD; DAKO) substrate. Insect cell-derived VLPs of known concentration were used as standards.

TSP was measured with a Lowry assay (BioRad) as per the manufacturer's instructions.

Green fluorescent protein (GFP) was detected by capture ELISA. Plant extracts were diluted in 1 % milk solution [skimmed milk powder in PBS with 0.05 % Tween 20 (PBS-T)] and incubated on a Reacti-Bind Anti-GFP-coated plate (Pierce Biotechnology) for 1 h at 37 °C. The plate was washed four times with PBS-T, followed by the addition of goat anti-GFP–HRP conjugate (Abcam; 1 : 2000 in 1 % milk solution) for 30 min at 37 °C. After four washes with PBS-T, TMB substrate (KPL) was utilized for detection.

**Electron microscopy.** Six days post co-infiltration of *N. benthamiana* with *Agrobacterium*-L1 and *Agrobacterium* (pBIN-NSs), infiltrated leaf material was ground and centrifuged at 13 000 r.p.m. for 5 min. The supernatant was immunotrapped with H16.V5 antibody (1 : 1000) on carbon-coated copper grids. The grids were stained with 2 % uranyl acetate and viewed by using a JEOL 200CX transmission electron microscope.

**Immunization of mice and serology.** *N. tabacum* material (350 g) that had been vacuum-infiltrated with *Agrobacterium* (pTRA-HL1) and *Agrobacterium* (pBIN-NSs) was homogenized with a Waring blender in 2 ml extraction buffer [0.25 M sodium phosphate, 0.1 M sodium metabisulphite, 10 mM EDTA, 4 % polyvinylpolypyrrolidone (PVPP), pH 7.4] (g plant material)$^{-1}$. The extract was filtered through two layers of cheesecloth, centrifuged at 10 000 ***g*** for 20 min and the supernatant was ultracentrifuged at 30 000 r.p.m. for 3 h. Resultant pellets were resuspended in PBS and freeze-dried to reduce volume. The powder was resuspended in 0.1 vol. water.

BALB/c mice were immunized once subcutaneously with 100 µl plant extract (11 µg L1), either with incomplete Freund's adjuvant (1 : 1, v/v; four mice) or without (five mice). Control groups were immunized once with 1 µg insect cell-derived HPV-16 VLP (without adjuvant), twice (4-week interval) with 10 µg of the same antigen, or with PBS. Serum was collected from the retro-orbital plexus 4 weeks post-immunization, pooled by group and frozen at −20 °C. All protocols were done under permit from the University of Cape Town Animal Ethics Committee.

ELISAs were used to detect antibodies to L1 in the mice. Microtitre plates were coated with insect cell-produced HPV-16 L1 VLPs (2 µg ml$^{-1}$) overnight at 4 °C, blocked for 2 h at 37 °C with 5 % milk in PBS + 0.5 % Tween 20, then washed twice with PBS + 0.5 % Tween 20. Pooled sera were diluted 4-fold in 1 % milk in PBS (1 : 40–1 : 40 960) and incubated in duplicate on the plate at 4 °C overnight. After washing four times with PBS + Tween 20, rabbit anti-mouse–HRP conjugate (1 : 10 000; DAKO) was added for 2 h at 37 °C. Detection was performed with OPD substrate (DAKO). The sera were tested on four occasions.

The HPV-16 pseudovirus neutralizing-antibody assay was performed according to the method of Pastrana *et al.* (2004): plasmids required for the assay were obtained from Dr John Schiller (Laboratory of Cellular Oncology, National Cancer Institute, Bethesda, MD, USA). Pooled sera were diluted 4-fold (1 : 25–1 : 102 400) and were tested in duplicate on two occasions.

**Sucrose rate zonal sedimentation analysis.** Protein extracts were prepared from agroinfiltrated *N. benthamiana* leaves as described above. Protein extracts (250 µl) were loaded onto sucrose gradients [5–50 % (w/v) in high-salt phosphate buffer] and centrifuged at 37 000 r.p.m. for 2.75 h (Beckman SW40Ti rotor). Fractions were collected and analysed by H16.V5 mAb capture ELISA as described above.

**Plant transformation and regeneration.** *N. tabacum* L. 'Petite Havana' SR1 leaf discs were transformed as described by Horsch *et al.* (1985). Sterilized leaf discs ($\pm$1 cm$^2$ in size) were dipped into a culture of *Agrobacterium* (pTRACTP-HL1) or *Agrobacterium* (pTRAERH-HL1) grown overnight in induction medium, and placed on co-cultivation medium for 2 days, followed by regeneration medium with kanamycin selection until small shoots appeared from the callus (4–6 weeks). Healthy shoots of 1.5–2.0 cm long were transferred to rooting medium until strong root growth was evident, when they were transplanted into soil and grown to maturity.

**Screening of transgenic plants.** Following DNA extraction (Extract-N-Amp Plant PCR kit; Sigma), putative transgenic plants ($R_0$ generation) were screened for the presence of the HPV-16 L1 gene by PCR using the primers 5′-GGCGTGGATAACAGAGAATGC and 5′-CGCAGGTAGAAGAACAGGCT. Transgenic plants (approx. 30 cm high) were screened by ELISA for HPV-16 L1 protein. Leaf discs were excised with a microfuge tube lid, ground in 250 µl high-salt phosphate buffer (0.5 M NaCl) per disc and centrifuged at 13 000 r.p.m. for 5 min to remove debris. L1 protein was assayed by capture ELISA as described above.

## RESULTS

### Transient expression of HPV-16 L1 in plants

Agroinfiltration was used to compare the expression of numerous HPV-16 L1 constructs in plants (Table 1). L1 constructs were injected into *N. benthamiana* leaves with pBIN-NSs and pTRA-GFP, and L1 protein levels were measured 5 days post-infiltration (Fig. 2). Both codon optimization and plant cell-compartment targeting had considerable influences on L1 accumulation levels. L1 quantification was done by using H16.J4 mAb capture, as this mAb recognizes a linear surface epitope and should therefore bind to monomeric/denatured L1, as well as to assembled species. Of the four HPV-16 L1 genes tested, the

**Table 1.** Summary of *Agrobacterium* expression constructs utilized during this study

| Vector | Insert | Clone name | Cell compartment targeted |
|---|---|---|---|
| pTRAc | SAL1 (South African HPV-16 L1 isolate) | pTRA-SAL1 | Cytoplasm |
| pTRAc | SYNL1 (plant codon-optimized HPV-16 L1) | pTRA-SYNL1 | Cytoplasm |
| pTRAc | HL1 (human codon-optimized HPV-16 L1) | pTRA-HL1 | Cytoplasm |
| pTRAc | *gfp* | pTRA-GFP | Cytoplasm |
| pTRAkc-rbcs1-cTP | SAL1 | pTRACTP-SAL1 | Chloroplasts |
| pTRAkc-rbcs1-cTP | SYNL1 | pTRACTP-SYNL1 | Chloroplasts |
| pTRAkc-rbcs1-cTP | HL1 | pTRACTP-HL1 | Chloroplasts |
| pTRAkc-rbcs1-cTP | HL1ΔC22 (nuclear-localization signal truncation) | pTRACTP-HL1ΔC22 | Chloroplasts |
| pTRAkc-ERH | SAL1 | pTRAERH-SAL1 | ER |
| pTRAkc-ERH | SYNL1 | pTRAERH-SYNL1 | ER |
| pTRAkc-ERH | HL1 | pTRAERH-HL1 | ER |

full-length human codon-optimized variant (HL1) caused the highest accumulation of L1 protein. SAL1 (native, South African isolate) caused the second-highest L1 accumulation, followed by truncated HL1 (HL1ΔC22). The plant codon-optimized gene (SYNL1) did not generate detectable L1 protein [<1 mg L1 (kg plant material)$^{-1}$, <0.1 % TSP]. L1 that was targeted to the chloroplasts accumulated to the highest levels, whilst ER-targeted L1 accumulated to very low levels. SAL1 accumulated to 10 mg (kg plant material)$^{-1}$ (0.3 % TSP) when produced in the cytoplasm, and to levels over 13-fold higher when targeted to the chloroplasts [137 mg (kg plant material)$^{-1}$, 4.5 % TSP]. SAL1 was not detected when targeted to the ER. Cytoplasm-localized HL1 protein levels [379 mg (kg plant material)$^{-1}$, 14.9 % TSP] were 37-fold greater than the SAL1 equivalent. The highest L1 accumulation was detected by chloroplast-targeted HL1 [533 mg (kg plant material)$^{-1}$, 17.1 % TSP). The removal of the NLS from chloroplast-targeted HL1 reduced its accumulation significantly, i.e. by 5-fold to 108 mg (kg plant material)$^{-1}$ (3.6 % TSP), when compared with its full-length equivalent.

*Agrobacterium* (pTRA-GFP) co-infiltration was done at a low bacterial concentration and did not influence L1 accumulation significantly (data not shown). Although GFP expression did vary between samples, this variation was small and considerably lower than the variation in L1 expression.

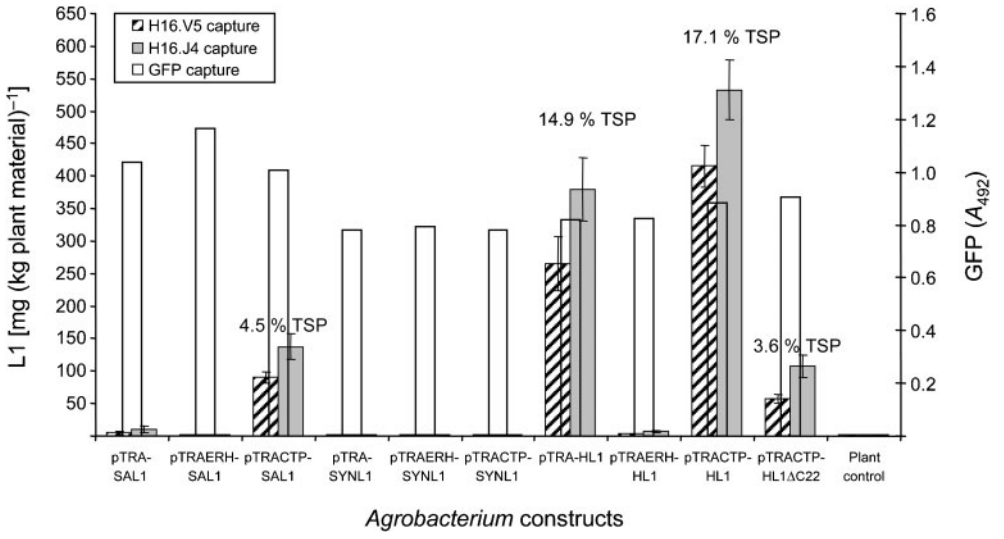


**Fig. 2.** HPV-16 L1 transient-expression levels in *N. benthamiana*. *Agrobacterium*-L1 suspensions were combined with *Agrobacterium* (pBIN-NSs) and *Agrobacterium* (pTRA-GFP) and injected into *N. benthamiana* plants (three plants, two leaves per plant per *Agrobacterium* sample). Five days post-infiltration, the L1 present in the leaf material was quantified by capture ELISA with H16.V5 or H16.J4 mAb. The L1 percentage of the total soluble protein (TSP) is indicated for certain samples, as determined by the H16.J4 ELISA capture assay. *Agrobacterium* (pTRA-GFP) was utilized to measure infiltration-efficiency deviations between samples.

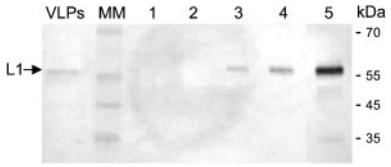


**Fig. 3.** Western blot of *N. benthamiana* leaf samples after infiltration with *Agrobacterium* carrying the HPV-16 L1 gene. Leaves were infiltrated by injection with an *Agrobacterium*-L1 construct alone or by co-infiltration with *Agrobacterium* (pBIN-NSs). The Western blot was performed on crude leaf extracts 6 days post-infiltration, using H16.J4 anti-HPV-16 L1 mAb. The first two lanes contain insect cell-derived HPV-16 VLPs and a molecular mass (MM) marker, respectively. The *Agrobacterium* samples were as follows: 1, pTRA-GFP; 2, pTRA-HL1; 3, pBIN-NSs and pTRA-HL1; 4, pTRACTP-HL1; 5, pBIN-NSs and pTRACTP-HL1.

Western blot analysis of infiltrated *N. benthamiana* leaf samples (Fig. 3) demonstrated the successful expression of the 55 kDa L1 monomer. HL1 expression from the pTRA-HL1 vector (cytoplasmic localization) was not detectable by Western blot (but was detected by capture ELISA; data not shown) unless the vector was co-infiltrated with pBIN-NSs. The pTRACTP-HL1 vector (chloroplast targeting of L1), however, generated L1 levels that were high enough to be detected by Western blot without pBIN-NSs co-infiltration.

The formation of VLPs in plants by transient L1 expression was confirmed by electron microscopy of crude leaf extracts (Fig. 4). The size range of the plant-derived VLPs was 30–65 nm in diameter: approximately 60 % of chloroplast-targeted VLPs were 55–60 nm, whilst 90 % of the cytoplasm-localized VLPs were 40–52 nm in diameter. Sucrose sedimentation analysis (Fig. 5) showed that a high proportion of L1 protein from the plant extracts had a sedimentation coefficient similar to that of insect cell-produced L1, but that there was also a large amount of presumed capsomeres (fractions 18–24) and other aggregates (fractions 1–8) in the plant extracts compared with the insect cell-produced protein. Whilst the sedimentation coefficient of chloroplast-targeted VLPs coincided with that of the insect-cell derived VLPs, the plant cytoplasm-localized VLPs had a slightly lower sedimentation coefficient.

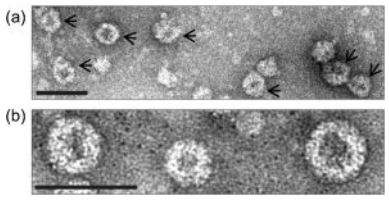


**Fig. 4.** Electron micrographs of H16.V5-immunotrapped crude *N. benthamiana* plant extract 6 days post-infiltration with (a) *Agrobacterium* (pTRA-HL1; cytoplasm-localized) or (b) *Agrobacterium* (pTRACTP-HL1; chloroplast-targeted) VLPs. Bars, 100 nm.

Vacuum infiltration of whole *N. tabacum* plants occurred readily over 70–90 % of the leaf area, with L1 protein yields of 40 mg (kg plant material)$^{-1}$ 3 days post-infiltration (results not shown).

## Immunogenicity of plant-derived HPV-16 L1

Mice were immunized once with a crudely prepared extract of pTRA-HL1-infiltrated *N. tabacum*: this product was chosen because vacuum-infiltrated tobacco gave the highest biomass and because cytoplasm-localized L1

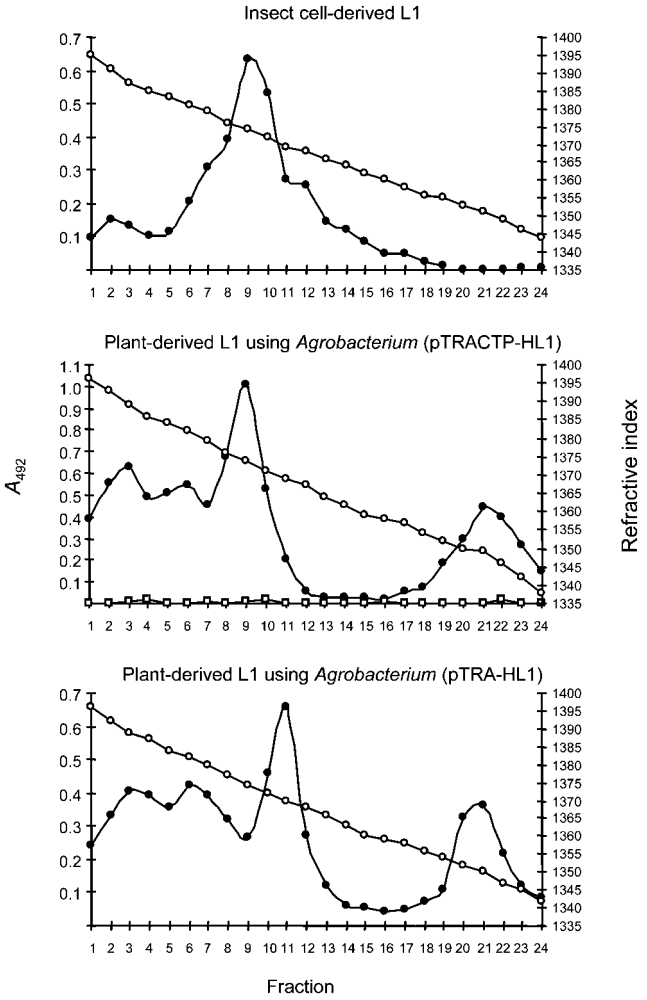


**Fig. 5.** Sucrose-sedimentation analysis of HPV-16 L1 protein derived from transiently expressed L1 in *N. benthamiana* plants or from insect cells. The fractions were analysed for the presence of L1 protein by capture ELISA with H16.V5 (●). □, ELISA on negative-control plant fractions; ○, refractive index. Fraction 1 corresponds to the bottom of the centrifuge tube.

**Table 2.** L1 reactive- and neutralizing-antibody titres induced in mice by immunization with plant-derived HPV-16 VLPs

Sera were taken 4 weeks post-immunization, pooled, diluted 4-fold and used in an ELISA against HPV-16 VLPs. The $A_{492}$ values were measured and the results were recorded as the reciprocal of the highest dilution where the $A_{492}$ was $>2\times$ that of the prebleed at a dilution of 1 : 160. Sera were also tested twice in a SEAP HPV-16 pseudovirus neutralization assay. Neutralization titres were defined as the reciprocal of the highest serum dilution that caused at least a 50 % reduction in SEAP activity. Titres below 25 were considered negative.

| Antigen | End-point titre | Neutralization titre |
|---|---|---|
| PBS | 0 | <25 |
| Plant-derived VLPs (11 μg × 1) | 40 960 | 6400 |
| Plant-derived VLPs + adjuvant (11 μg × 1) | 40 960 | 1600 |
| Insect cell-derived VLPs (1 μg × 1) | 640 | <25 |
| Insect cell-derived VLPs (10 μg × 2) | 40 960 | 1600 |

accumulated best in tobacco over the 3 days allowed. Four weeks post-immunization, the pooled sera were analysed four times against insect cell-derived VLPs and compared with sera from mice immunized with insect cell-derived VLPs: Table 2 shows results from a representative ELISA. The groups that were immunized with plant-derived L1 elicited high HPV-16 VLP-specific reciprocal antibody titres (1 : 40 960), which were equivalent whether administered with or without adjuvant. These titres were equivalent to those induced by two immunizations using 10 μg insect cell-derived VLPs.

An *in vitro* HPV-16 pseudovirus neutralization assay was performed twice by using the mouse sera (Table 2). This assay is based on the ability of neutralizing serum to block the entry of SEAP HPV-16 pseudovirions into cells, thus stopping the transfer of the SEAP reporter gene into the cells and expression and secretion of alkaline phosphatase. Serum from mice that had been immunized with plant-derived L1 had high titres of neutralizing antibodies. These neutralizing-antibody titres were equal or greater to those elicited by two doses of 10 μg insect cell-produced VLPs.

## Expression of L1 in transgenic plants

The presence of the L1 gene was confirmed by PCR (results not shown). High HPV-16 L1 expression was established in three *N. tabacum* lines (Fig. 6a). Line 4 – transformed with pTRAERH-HL1 (ER-retaining) – produced L1 up to 5 % of TSP. The phenotype of this line was highly abnormal, with stunted growth and malformed leaves. The L1 levels detected in lines 7 and 9 – transformed with chloroplast-targeting pTRACTP-HL1 – were up to 11 and 8 % of TSP, respectively. The phenotypes of lines 7 and 9 appeared normal when compared with non-transgenics. L1 expression was assayed in various leaves of the mature line 9 plant, just prior to flower formation (Fig. 6b): there was a generally increasing trend of L1 accumulation with increasing leaf age. The highest L1 level (887 mg $kg^{-1}$)

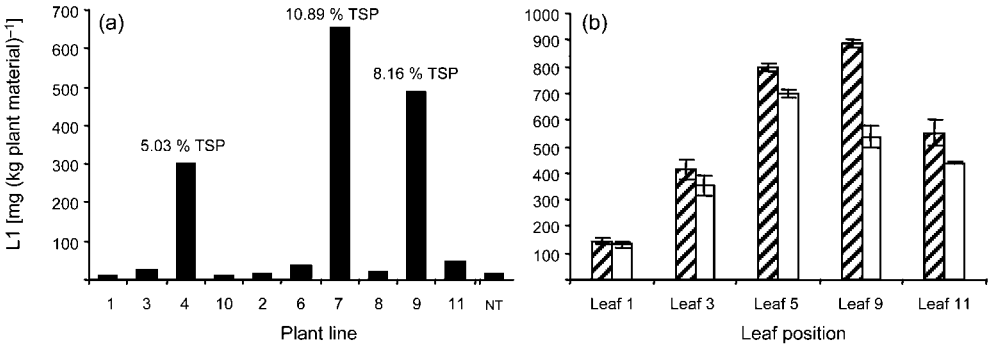


**Fig. 6.** HPV-16 L1 protein expression in *N. tabacum* transgenic plants. (a) Screening of 11 putative transgenic plants by H16.V5 capture ELISA. Lines 1, 3, 4 and 10 were transformed with *Agrobacterium* (pTRAERH-HL1); lines 2, 6, 7, 8, 9, and 11 were transformed with *Agrobacterium* (pTRACTP-HL1). NT, Non-transgenic control plant. The L1 percentage of the TSP is indicated for certain samples. (b) L1 expression in transgenic plant line 9, using H16.V5 (hatched) and H16.J4 (empty) capture ELISA. Leaf 1, topmost/youngest leaf; leaf 11, lowest green leaf.

was detected in the largest leaf on the plant. The observation that the H16.V5 mAb was able to capture L1 suggested the formation of higher-order structures. Western blot analysis of lines 7 and 9 demonstrated the successful expression of the 55 kDa L1 monomer (result not shown).

## DISCUSSION

Whilst human clinical trials of HPV VLP prophylactic vaccines have produced very encouraging results, the vaccines currently in production will probably be too expensive for widespread use in developing countries (Sanders & Taira, 2003; Taira *et al.*, 2004). The use of plants has therefore been explored recently as an alternative and cost-effective VLP production method by a number of groups, including ours. However, the L1 protein levels in all of these cases may be too low – $<1$ % TSP – for the economically viable production of VLPs. For example, Biemelt *et al.* (2003) obtained 0.5 % of TSP of HPV-16 L1 in transgenic tobacco and 0.2 % TSP in potato tubers, representing approximately 12 μg assembled L1 (g tissue)$^{-1}$; Warzecha *et al.* (2003) obtained approximately 20 ng HPV-11 L1 g$^{-1}$ in transgenic potato tubers; we obtained 4 ng HPV-16 L1 (g leaf tissue)$^{-1}$ in transgenic tobacco and approximately 40 ng g$^{-1}$ via a tobacco mosaic virus vector in *N. benthamiana* (Varsani *et al.*, 2003, 2006), and 1 and 0.4 μg CRPV L1 g$^{-1}$ in the same systems (Kohl *et al.*, 2006); Liu *et al.* (2005) obtained approximately 0.05 % TSP in transgenic tobacco.

In order to optimize the expression of HPV-16 L1 in plants, therefore, we investigated transient expression in *Nicotiana* spp. by agroinfiltration of numerous binary vectors containing codon-optimized L1 genes and plant cell compartment-targeting signal sequences. We showed that codon optimization of the L1 gene and targeting of the L1 protein to specific plant-cell compartments can influence the accumulation of L1 protein considerably. We also demonstrated that VLPs assembled in the plant cells and that these VLPs were highly immunogenic by injection after a crude purification protocol.

Agroinfiltration proved very useful for rapid comparison of the expression of numerous HPV-16 L1 constructs in plants. Optimization of the L1 gene for expression in mammalian cells resulted in significantly higher L1 accumulation than the native or plant codon-optimized genes: these results reiterated those of Biemelt *et al.* (2003), who measured the expression levels of HPV-16 L1 in transgenic tobacco and potato and were only able to obtain detectable amounts of L1 with another version of a human codon-optimized gene. In our study, it was surprising that the native L1 sequence (SAL1) caused greater protein accumulation than the plant codon-optimized gene (SYNL1). Biemelt *et al.* (2003) investigated both native and plant codon-optimized HPV-16 L1, but were unable to detect protein expression from either gene in plants. However, although we cannot compare our genes directly with theirs as the sequences are different (human codon-optimized genes are 96.1 % similar; plant-optimized genes are 75.7 % similar), it is possible that our transient-expression system gave much higher expression levels than theirs.

Inhibitory sequences have been identified at the 5′ end of the HPV-16 L1 gene, which act at the levels of transcription and RNA processing (Collier *et al.*, 2002). The exact sequences and inhibitory mechanism are largely unknown; however, Collier *et al.* (2002) found that mutating the first 514 nt of the native L1 sequence from 41 to 67 mol% G+C without altering the amino acid sequence increased L1 expression significantly. They also determined that the inhibition did not act on a translational level and that the presence of rare codons did not reduce translation. In our study, it is unknown why HL1 was expressed more readily than the other L1 sequences, but it may be as a result of the disruption of the above-mentioned inhibitory sequences. At 64 mol%, the G+C content (first 514 nt) of the HL1 gene is significantly higher than that of SAL1 (40.7 mol%, first 514 nt) and is similar to that of the mutated L1 gene used by Collier *et al.* (2002). However, the G+C content of the 5′ end of L1 alone cannot account for the increase in L1 expression, because in our study, SAL1 was expressed at higher levels than SYNL1, which has a G+C content of 52.2 mol% (first 514 nt), higher than that of the native gene. Although the HL1 sequence does not contain many codons that are used rarely in *N. benthamiana* or *N. tabacum*, it certainly contains rarer codons than the plant-optimized sequence, which strengthens the observation of Collier *et al.* (2002) that L1 inhibition acts on a transcriptional and not a translational level.

It is interesting that the highest L1 accumulation in our work was obtained by using the chloroplast-targeting constructs. We believe that L1 is entering the chloroplasts because, during the membrane-translocation process, the rbcs-cTP signal is cleaved, resulting in the 55 kDa L1 protein band seen on the Western blot. This band runs at exactly the same position as cytoplasm-targeted protein, under conditions where a 10 % increase in $M_r$ could easily be seen. The presence of L1 in the chloroplasts has also been confirmed by immunogold labelling of thin plant sections (J. Maclean, unpublished results). Increased accumulation in the chloroplast may be a result of a reduced number of proteases in the chloroplasts or of lower cellular toxicity. It is also possible that the mRNA is more stable or that protein stability in the cytoplasm is increased by the rbcs-cTP signal.

The observation that the H16.V5 mAb was able to capture L1 suggested the formation of at least pentamers and possibly VLPs; this was confirmed by electron microscopy. Crude plant extracts contained VLPs with a large range of sizes: chloroplast-targeted VLPs were generally of a similar size to insect cell-derived VLPs (55–60 nm), whereas cytoplasm-localized VLPs were slightly smaller (40–52 nm). This observation was strengthened by sucrose-gradient sedimentation analysis, which showed that chloroplast-targeted

VLPs sedimented similarly to insect-cell derived VLPs, whereas cytoplasm-localized VLPs sedimented more slowly. Sedimentation analysis also showed that a high proportion of L1 in the plant extracts was in the form of capsomeres and small aggregates.

The plant-derived L1 was at least as immunogenic in mice as insect cell-derived VLPs, eliciting high antibody titres. Moreover, the antibodies produced were strongly neutralizing. Although we used crude plant extracts for the immunizations and the percentages of monomeric/denatured versus pentameric/VLP forms of L1 in the plants was not known, the ultracentrifugation step would have enriched for VLPs and higher-order aggregates, thus probably contributing to the high neutralizing-antibody titre. The addition of Freund's adjuvant to the plant extract did not increase the humoral response elicited to HL1 noticeably and, in fact, unadjuvanted plant extract apparently elicited a higher titre of neutralizing antibodies than adjuvanted extract, indicating that the adjuvant might even be deleterious. Whilst Biemelt *et al.* (2003) obtained similar results with HPV-16 L1 produced in tobacco, it was only by use of far more purified, caesium chloride gradient-fractionated preparations.

Although our objective is to generate commercially extractable transgenic plants, transient agroinfiltration expression can also be scaled up to industrial levels. Medicago Inc. has developed processes to agroinfiltrate up to 7500 alfalfa leaves week$^{-1}$, and Schillberg and colleagues (Institute for Molecular Biotechnology, RWTH Aachen, Germany) have agroinfiltrated batches of up to 100 kg tobacco leaves (Fischer *et al.*, 2004). We have demonstrated the potential for large-scale, high-level expression of HPV L1 in *N. benthamiana* and *N. tabacum* by vacuum infiltration. We found that by vacuum-infiltrating whole plants, instead of loose leaves, it was relatively easy to scale infiltration up to 2 kg *N. tabacum* plant material. This method could prove very useful for expression of proteins that express transiently, but fail to express in transgenic plants, as well as for quick biological testing of novel biopharmaceuticals, including vaccines.

We also generated transgenic *N. tabacum* plants successfully by using some of the higher L1-yielding constructs: although we expected significantly lower L1 yields than with transient expression, our data suggest high L1 yields of up to 11 % TSP in plants containing the human codon-optimized L1 chloroplast-targeting construct (pTRACTP-HL1), with no apparent phenotypic change. This level of L1 is very significantly higher than that recorded by other groups, as discussed above. Although the ER-retention construct transiently produced low levels of L1, significant levels were produced in one transgenic line (5 % TSP), perhaps due to prolonged accumulation. Closer analysis of one of the transgenic lines showed that L1 accumulation was highest [almost 900 mg (kg plant material)$^{-1}$] in the largest leaves, which bodes well for future scale-up initiatives. We are in the process of generating transgenic plants with the other high L1-yielding constructs described in this study and we are currently establishing L1 transgene- and protein-expression stability of our transgenic plants.

## ACKNOWLEDGEMENTS

We thank Dorina Saleh for the GFP and L1 quantification ELISAs, Eric van der Walt for insect cell-produced HPV-16 VLPs, Marcel Prins (Laboratory of Virology, Wageningen, The Netherlands) for the *A. tumefaciens* LBA4404 (pBIN-NSs) strain and Neil Christensen (Department of Pathology, Milton S. Hershey Medical Center, Hershey, Pennsylvania, PA, USA) for the H16.V5 and H16.J4 mAbs. We are grateful to John Schiller (Laboratory of Cellular Oncology, National Cancer Institute, Bethesda, MD, USA) for the plasmids used for the HPV-16 pseudovirus neutralizing-antibody assay.

## REFERENCES

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

**Collier, B., Oberg, D., Zhao, X. & Schwartz, S. (2002).** Specific inactivation of inhibitory sequences in the 5′ end of the human papillomavirus type 16 L1 open reading frame results in production of high levels of L1 protein in human epithelial cells. *J Virol* **76**, 2739–2752.

**Fischer, R., Stoger, E., Schillberg, S., Christou, P. & Twyman, R. M. (2004).** Plant-based production of biopharmaceuticals. *Curr Opin Plant Biol* **7**, 152–158.

**Harper, D. M., Franco, E. L., Wheeler, C., Ferris, D. G., Jenkins, D., Schuind, A., Zahaf, T., Innis, B., Naud, P. & other authors (2004).** Efficacy of a bivalent L1 virus-like particle vaccine in prevention of infection with human papillomavirus types 16 and 18 in young women: a randomised controlled trial. *Lancet* **364**, 1757–1765.

**Horsch, R. B., Rogers, S. G. & Fraley, R. T. (1985).** Transgenic plants. *Cold Spring Harb Symp Quant Biol* **50**, 433–437.

**Kapila, J., De Rycke, R., Van Montagu, M. & Angenon, G. (1997).** An *Agrobacterium*-mediated transient gene expression system for intact leaves. *Plant Sci* **122**, 101–108.

**Kohl, T., Hitzeroth, I. I., Stewart, D., Varsani, A., Govan, V. A., Christensen, N. D., Williamson, A. L. & Rybicki, E. P. (2006).** Plant-produced cottontail rabbit papillomavirus L1 protein protects against tumor challenge: a proof-of-concept study. *Clin Vaccine Immunol* **13**, 845–853.

**Liu, H. L., Li, W. S., Lei, T., Zheng, J., Zhang, Z., Yan, X. F., Wang, Z. Z., Wang, Y. L. & Si, L. S. (2005).** Expression of human papillomavirus type 16 L1 protein in transgenic tobacco plants. *Acta Biochim Biophys Sin (Shanghai)* **37**, 153–158.

**Maclean, J., Rybicki, E. P. & Williamson, A. L. (2005).** Vaccination strategies for the prevention of cervical cancer. *Expert Rev Anticancer Ther* **5**, 97–107.

**Mao, C., Koutsky, L. A., Ault, K. A., Wheeler, C. M., Brown, D. R., Wiley, D. J., Alvarez, F. B., Bautista, O. M., Jansen, K. U. & other authors (2006).** Efficacy of human papillomavirus-16 vaccine to prevent cervical intraepithelial neoplasia: a randomized controlled trial. *Obstet Gynecol* **107**, 18–27.

**Mason, H. S., Warzecha, H., Mor, T. & Arntzen, C. J. (2002).** Edible plant vaccines: applications for prophylactic and therapeutic molecular medicine. *Trends Mol Med* **8**, 324–329.

**Pastrana, D. V., Buck, C. B., Pang, Y. Y., Thompson, C. D., Castle, P. E., FitzGerald, P. C., Kruger, K. S., Lowy, D. R. & Schiller, J. T.**

**(2004).** Reactivity of human sera in a sensitive, high-throughput pseudovirus-based papillomavirus neutralization assay for HPV16 and HPV18. *Virology* **321**, 205–216.

**Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989).** *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

**Sanders, G. D. & Taira, A. V. (2003).** Cost-effectiveness of a potential vaccine for human papillomavirus. *Emerg Infect Dis* **9**, 37–48.

**Shen, W. J. & Forde, B. G. (1989).** Efficient transformation of agrobacterium spp by high-voltage electroporation. *Nucleic Acids Res* **17**, 8385.

**Somers, D. A. & Makarevitch, I. (2004).** Transgene intergration in plants: poking or patching holes in promiscuous genomes? *Curr Opin Biotechnol* **15**, 126–131.

**Studentsov, Y. Y., Schiffman, M., Strickler, H. D., Ho, G. Y., Pang, Y. Y., Schiller, J., Herrero, R. & Burk, R. D. (2002).** Enhanced enzyme-linked immunosorbent assay for detection of antibodies to virus-like particles of human papillomavirus. *J Clin Microbiol* **40**, 1755–1760.

**Taira, A. V., Neukermans, C. P. & Sanders, G. D. (2004).** Evaluating human papillomavirus vaccination programs. *Emerg Infect Dis* **10**, 1915–1923.

**Takeda, A., Sugiyama, K., Nagano, H., Mori, M., Kaido, M., Mise, K., Tsuda, S. & Okuno, T. (2002).** Identification of a novel RNA silencing suppressor, NSs protein of Tomato spotted wilt virus. *FEBS Lett* **532**, 75–79.

**Varsani, A., Williamson, A. L., Rose, R. C., Jaffer, M. & Rybicki, E. P. (2003).** Expression of Human papillomavirus type 16 major capsid protein in transgenic *Nicotiana tabacum* cv. Xanthi. *Arch Virol* **148**, 1771–1786.

**Varsani, A., Williamson, A. L., Stewart, D. & Rybicki, E. P. (2006).** Transient expression of Human papillomavirus type 16 L1 protein in *Nicotiana benthamiana* using an infectious tobamovirus vector. *Virus Res* **120**, 91–96.

**Voinnet, O. (2001).** RNA silencing as a plant immune system against viruses. *Trends Genet* **17**, 449–459.

**Voinnet, O., Rivas, S., Mestre, P. & Baulcombe, D. (2003).** An enhanced transient expression system in plants based on suppression of gene silencing by the p19 protein of tomato bushy stunt virus. *Plant J* **33**, 949–956.

**Warzecha, H., Mason, H. S., Lane, C., Tryggvesson, A., Rybicki, E., Williamson, A. L., Clements, J. D. & Rose, R. C. (2003).** Oral immunogenicity of human papillomavirus-like particles expressed in potato. *J Virol* **77**, 8702–8711.

**Zhou, J., Doorbar, J., Sun, X. Y., Crawford, L. V., McLean, C. S. & Frazer, I. H. (1991).** Identification of the nuclear localization signal of human papillomavirus type 16 L1 protein. *Virology* **185**, 625–632.

**Zupan, J., Muth, T. R., Draper, O. & Zambryski, P. (2000).** The transfer of DNA from *Agrobacterium tumefaciens* into plants: a feast of fundamental insights. *Plant J* **23**, 11–28.