Arch Virol (2003) 148: 1771–1786
DOI 10.1007/s00705-003-0119-4

Archives of Virology
Printed in Austria

Expression of *Human papillomavirus* type 16 major capsid protein in transgenic *Nicotiana tabacum* cv. Xanthi

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Received December 18, 2002; accepted March 26, 2003
Published online June 2, 2003

Summary. The production of vaccine antigens in plants is a safe and potentially very cost-effective alternative to traditional expression systems. We investigated the possibility of transgenic plant expression of the *Human papillomavirus* (HPV) type 16 L1 major capsid protein, with and without nuclear localisation signals, in *Nicotiana tabacum* cv. Xanthi plants. The genes were stably integrated into the *N. tabacum* genome, and both the expressed proteins were capable of assembling into capsomers and virus-like particles. The proteins in concentrated leaf extracts ($L1_{Tr}$) were tested for antigenicity using a panel of characterised monoclonal antibodies (Mabs). Neutralising and conformation-specific Mabs (H16:V5 and H16:E70) were shown to bind to both types of the plant-produced particles. We estimated the $L1_{Tr}$ product yield to be 2–4 μg per kg of fresh leaf material. Rabbits immunised with small doses of plant-produced particles elicited a weak anti-HPV-16 L1 immune response. Our results support the feasibility of using transgenic plants for the production of HPV vaccines.

Introduction

Traditionally most prophylactic vaccines for human viruses have consisted of live attenuated or inactivated virus. Due to the difficulties and risks involved in generating large quantities of these traditional vaccines there has recently been increasing emphasis on developing viral protein subunit vaccines. With the advent

of recombinant technologies, subunit vaccines based on protein expression in bacteria, yeast and insect cells have therefore become popular. However, subunit vaccines produced in this way tend to be expensive, so there has been considerable interest in developing new methods for producing cheap subunit vaccines.

Over the last decade plants have become a popular choice for the production of recombinant proteins. Production of foreign proteins in plants has many economic and qualitative benefits [11]. The cultivation, harvesting, storage, and processing of transgenic crop could use existing infrastructure and would require relatively little capital investment [11, 28, 36]. Kusnadi et al. [21] have estimated that the cost of producing recombinant proteins in plants could be 10- to 50-fold lower than in *E. coli*. Two main strategies have been employed in achieving recombinant protein expression in plants: these are transgenic expression, and transient expression using plant viruses.

Cervical cancer is the second most common cancer among women worldwide, and during the last few decades a growing body of evidence has demonstrated the aetiological association of *Human papillomavirus* (HPV) with a variety of anogenital cancers. HPV DNA has been detected in more than 90% of all tumours of the uterine cervix [34]. HPV types 16 and 18 can be detected in nearly 70% of squamous cell carcinomas of the cervix, with HPV-16 being predominant. HPV types 6 and 11 are commonly detected in condyloma acuminata, but are never found in cervical carcinomas [16]. An effective cheap prophylactic vaccine against oncogenic types of HPV – and especially against HPV-16 – could potentially have a large impact on the world cancer burden.

The virions of papillomaviruses are highly immunogenic, inducing high titres of neutralising antibodies when systemically inoculated [19]. Papillomavirus like-particles (VLPs), made from the major capsid protein L1 alone, or by the co-expression of L1 and the minor L2 capsid protein, have been proven to induce protective immunity in animal models [3, 8, 17, 32], and L1-only VLPs are currently in clinical trials [20]. However, these are normally produced in insect cell or yeast culture systems, and will almost certainly be expensive vaccines.

We investigated the transgenic expression of HPV-16 L1 (full length) and L1ΔC483 (without C-terminal nuclear localisation signals) genes in *Agrobacterium*-transformed *N. tabacum* cv. Xanthi plants. Our data show that a plant-based expression system for the production of the HPV-16 L1 VLPs has potential for low-cost vaccine production.

Materials and methods

Cloning of HPV-16 L1 and L1ΔC483 genes into the binary vector pART27 and Agrobacterium *transformation*

The L1 open reading frame (ORF) of HPV-16 (South African isolate, GenBank accession no. AY177679) was PCR amplified (forward 5′-CCC GGG ATG TCT CTT TGG CTG CCT AG-3′ and reverse 5′-GCC TCG AGT TAC AGC TTA CGT TTT TTG C-3′), cloned into pSK (*Sma*I/*Xho*I) and sequenced. The HPV-16 L1 ORF without the bipartite nuclear localisation signal (NLS; KRK_1atpttsststtaKRKKRKL_2) was PCR amplified (forward 5′ TTA ATT AAA

TGT CTC TTT GGC TGC CTA G-3′ and reverse 5′-CTC GAG TTA TCC TAA TGT AAA TTT TGG TTT GGC-3′) and cloned into pGEM®-T East vector (Promega).

The full length HPV-16 L1 gene and shortened version without the NLS (HPV-16 L1Δ C483) were directionally cloned into the multiple cloning site of the plasmid pART7 (*Cla*I/*Xba*I and *Eco*RI respectively). This placed the L1 genes downstream of the CaMV 35S promoter and upstream of the octopine synthase gene terminator (*ocs*3′). The CaMV35S promoter, L1 ORF and the *ocs*3′ cassette was excised and cloned into the binary vector pART27 (*Not*I). The binary vector pART27 carries the colE1 origin of replication for high copy maintenance in *E. coli*, the neomycin phosphotransferase (npt) gene on the T-DNA for kanamycin resistance as a selection marker in both *E. coli* and *Agrobacterium*.

C58C1 *Agrobacterium tumefaciens* cells were transformed with HPV-16 L1- and L1Δ C483-pART27 plasmid DNA using the freeze-thaw method [14]. The transformed cells were selected on Luria agar plates containing kanamycin (40 μg/ml) and rifampicin (100 μg/ml) at 30 °C and screened by PCR.

Production of HPV-16 L1 VLPs via baculovirus expression in insect cells

The L1 gene described above was subcloned into pFastBac1® vector (Life Technologies) and the resulting DNA was used to transfect DH10bac *E. coli* cells for the preparation of bacmid clones. The resulting bacmid DNA was subsequently transfected into *Spodoptera frugiperda* (*Sf*21) cells using Cellfectin (Life Techologies). The Bac-to-Bac® protocol was then followed to amplify the recombinant virus. Five insect cell tissue culture 75 cm^2 flasks were seeded with 6×10^6 *Sf*21 cells and infected with 40 μl of the respective recombinant baculovirus amplified stock in a total of 10 ml of complete media. The flasks were incubated at 27 °C for 48–72 h. The infected cells were harvested by washing, spun down at 3 000 rpm, resuspended in high salt phosphate buffered saline (PBS with 0.5 M NaCl) and sonicated 4 times at 5 sec intervals. The sonicated material was overlaid onto a 40% sucrose cushion and pelleted at 100 000 × g for 3 h. The pellet was resuspended in CsCl buffer (PBS with 0.4 g/ml CsCl) with sonication (4 times with 5 sec intervals). The suspension was centrifuged at 100 000 × g at 10 °C for 24 h in a Beckman SW50.1 rotor. The distinct band was extracted and dialysed overnight against PBS at 4 °C.

N. tabacum *cv. Xanthi leaf disk transformations*

N. tabacum cv. Xanthi leaf disks were transformed with the HPV-16 L1 *A. tumefaciens* constructs and grown on co-cultivation media for 3 days, followed by regeneration media with kanamycin selection for callus formation [15]. The callus was allowed to differentiate on shooting and rooting plant tissue culture media. Following root induction, the plantlets were transferred to soil and grown to maturity. Flowers of the R_0 generation were self-pollinated and seeds were screened on plant tissue culture containing 300 μg/ml kanamycin. The resistant germinated seeds, once the 3rd leaves had grown, were transplanted to soil and grown to maturity.

Screening of transgenic plants for HPV-16 L1 and L1ΔC483 genes

The transformed *N. tabacum* plants were screened by PCR for the HPV-16 L1 and HPV-16 L1ΔC483 genes using the following primer pairs: forward 5′-ATG TCT CTT TGC TGC CTA GTG AG-3′, reverse 5′-TTA CAG CTT ACG TTT TTT GCG TTT AGC AGT TG-3′ (for full length L1) and reverse 5′-TTA TCC TAA TGT AAA TTT TGG TTT GGC CTT CAA TC-3′ (for L1ΔC483). Plant genomic DNA was extracted from the *N. tabacum* leaves using the method outlined by Dellaporta et al. [9].

Analysis of total mRNA extracted from transgenic plants

Total RNA was extracted from fresh or stored (−70 °C) leaves using the TRIzol™ reagent (Life Technologies). The L1 mRNA was detected by RT-PCR amplification using the Access RT-PCR system, Promega, (L1 amplification product 500 bp, forward 5′-GGT CCA TTA GGT GTG GG-3′, reverse 5′-AGC TGT CGC CAT ATG GTT CTG-3′).

Concentration of transgenic plant material

Transgenic *N. tabacum* plants that tested positive for HPV-16 L1 mRNA were homogenised in 1:2 (weight to volume) cold high salt (0.5 M NaCl) phosphate buffered saline (PBS). The homogenate was strained through cheesecloth and centrifuged at low speed (∼4300 × g; 10 min). The supernatant was ultracentrifuged at ∼178 000 × g for 6 h. The pellet was resuspended in 1/5 initial volume of PBS. The resulting suspension was clarified by centrifuging at ∼27 000 × g for 20 minutes. The supernatant was subjected to a second round of ultracentrifugation at ∼178 000 × g for 3 h. The pellet was resuspended in PBS. For the antibody characterisation of the plant-produced L1 ($L1_{Tr}$) and $L1\Delta C483_{Tr}$ capsomers, the protein was further concentrated using the Centricon® YM-30 centrifugal filters (Amicon Separations).

The baculovirus-produced VLPs ($L1_{Bac}$) at a concentration of 0.63 μg/μl were diluted to 0.13 ng/μl for characterisation of the antigenicity of HPV-16 $L1_{Tr}$ and $L1\Delta C483_{Tr}$. Therefore, based on the absorbance and dilution of the $L1_{Tr}$ and $L1\Delta C483_{Tr}$, an approximate concentration of the capsomers could be worked out (see below).

Antibody characterisation of the transgenic plant-produced L1 protein

Transgenic plant-produced HPV-16 $L1_{Tr}$ and $L1\Delta C483_{Tr}$ protein was tested with the panel of monoclonal antibodies (Mabs) H16:V5, H16:E70, H16:U4, H16:9A, H16:D9, H16:I23 and H16:J4 by capture ELISA. Mabs H16:V5 and H16:E70 both recognise amino acid residues 50, 266 and 282 [35] and are conformation-specific Mabs; however, residue 266 has been shown to be critical only for Mab H16:E70 binding to HPV-16 L1 VLPs [31]. Mabs H16:U4 (epitope unknown) and H16:9A (epitope unknown but in region aa 1–172) recognise conformational epitopes, while H16:D9 (binds aa 428–465), H16:I23 (binds aa 111–130), H16:J4 (binds aa 261–280), recognise linear epitopes [6, 7]. Guinea pig anti-HPV-16 serum was diluted 1:500 and coated overnight onto ELISA plates. The plates were blocked with 1% non-fat milk in PBS. The plant extract was diluted 1:10 and allowed to bind for 2 h. The captured antigen was probed with the Mabs (1:200) for 2 h. Anti-mouse-alkaline phosphatase conjugated secondary antibody (1:2000) was allowed to bind to the Mab for 1 h at 37 °C. The secondary antibody was detected using p-nitrophenyl phosphate and the absorbance was measured using a Titrex ELISA plate reader at 405 nm.

Electron microscopy of transgenic plant extracts

The plant extract was viewed either directly or after immunotrapping the particles with Mabs H16:J4 (binds linear epitope aa 261–280) and H16:V5 (binds conformational epitope) diluted 1:50 on carbon coated copper grids. In the case of immunogold labelling, the extract was immunotrapped with guinea pig anti HPV-16 L1 (1:100) onto carbon coated copper grids. The trapped $L1_{Tr}$ and $L1\Delta C483_{Tr}$ particles were probed with Mab H16:V5 (1:50 dilution) and secondary gold-labelled (10 nm) anti-mouse (1:100). The grids were stained with 2% uranyl acetate and viewed using a JEOL 200CX transmission electron microscope. Concentrated plant protein extracts were viewed by immunotrapping the particles with H16:J4 and H16:V5 Mabs.

Immunisation of rabbits with transgenic plant extracts and serum analysis

Concentrated (65 fold) transgenic HPV-16 $L1_{Tr}$ *N. tabacum* sap extract was injected into three white New Zealand rabbits. For each rabbit the plant extract (500 µl in PBS with ~0.1 µg of L1 antigen) was diluted to and injected 1:1 with Freund's incomplete adjuvant. The HPV-16 $L1_{Tr}$ antigen was administered to two subcutaneous sites and an intramuscular site at days 1, 21, 96 and 118. The rabbit serum at a dilution of 1:50 was analysed for VLP-specific antibodies by ELISA against baculovirus-produced HPV-16 $L1_{Bac}$ VLPs (100 µl per well coated at 1 µg/ml concentration), and by western blotting.

The HPV-16 $L1_{Bac}$ protein was denatured for 10 min at 100 °C in SDS loading dye (without reducing agents). The denatured $L1_{Bac}$ protein was resolved on a 10% SDS PAGE gel. The resolved gel was transferred onto nitrocellulose membrane by semi-dry electrophoresis (BioRad) for 25 min at 25 V. The membrane was blocked using 1% non-fat milk for 2 h and incubated with rabbit serum at a dilution of 1:50 overnight at 4 °C. The membrane was washed with PBS/0.05% Tween-20 and probed with alkaline phosphataselabelled secondary goat anti-rabbit antibody diluted 1:2000, for 1 h at room temperature. Reaction was detected colorimetrically using 5-bromo-4-chloro-3-indoyl-phosphate (BCIP) and 4-nitro blue tetrazolium chloride (NBT) substrates.

Results

Production and genetic analysis of transformed N. tabacum *plants*

Twenty plant lines (ten per construct) resulting from the transformation of leaf discs with HPV-16 L1 and L1ΔC483 *Agrobacterium tumefaciens* were screened by PCR for the HPV-16 L1 gene. Eight plants were found to be positive for each of the HPV-16 $L1_{Tr}$ and $L1\Delta C483_{Tr}$ genes (Fig. 1A and B). Total RNA extracted from these transgenic plants was analysed for expression of the integrated HPV-16 L1 gene by RT-PCR. A 500 bp integrated L1 gene DNA fragment was observed as an amplification product from the RNA analysis (Fig. 1C and D), suggesting active transcription of the HPV-16 L1 gene.

In order to assess the stability of the plant integrated HPV-16$L1_{Tr}$ and $L1\Delta C483_{Tr}$ in the T_1 generation, four of the R_0 transgenic plants (lines 2, 4, 6 and 10 for $L1_{Tr}$ and lines 3, 4, 6 and 10 for the $L1\Delta C483_{Tr}$) were self-pollinated. The criteria used in selecting these lines were that the most vigorous of the L1 mRNA-positive were chosen. The seeds generated were collected and screened on kanamycin tissue culture media to select for transgenics before being transplanted to soil. Four plants from each of the four T1 transgenic lines of $L1_{Tr}$ and $L1\Delta C483_{Tr}$ were randomly screened for the integration of the L1 and L1ΔC483 genes. All the screened T1 generation plants were found to be positive for the genes, suggesting stable integration into the *N. tabacum* genome.

Analysis of HPV-16 $L1_{Tr}$ and $L1\Delta C483_{Tr}$ protein produced in transgenic N. tabacum

The HPV-16 $L1_{Tr}$ and $L1\Delta C483_{Tr}$ proteins could not be detected in the initial analysis of extracts of the R_0 transgenic plant lines by western blot or ELISA, despite confirmation of mRNA expression of both (Fig. 1C and D) in the

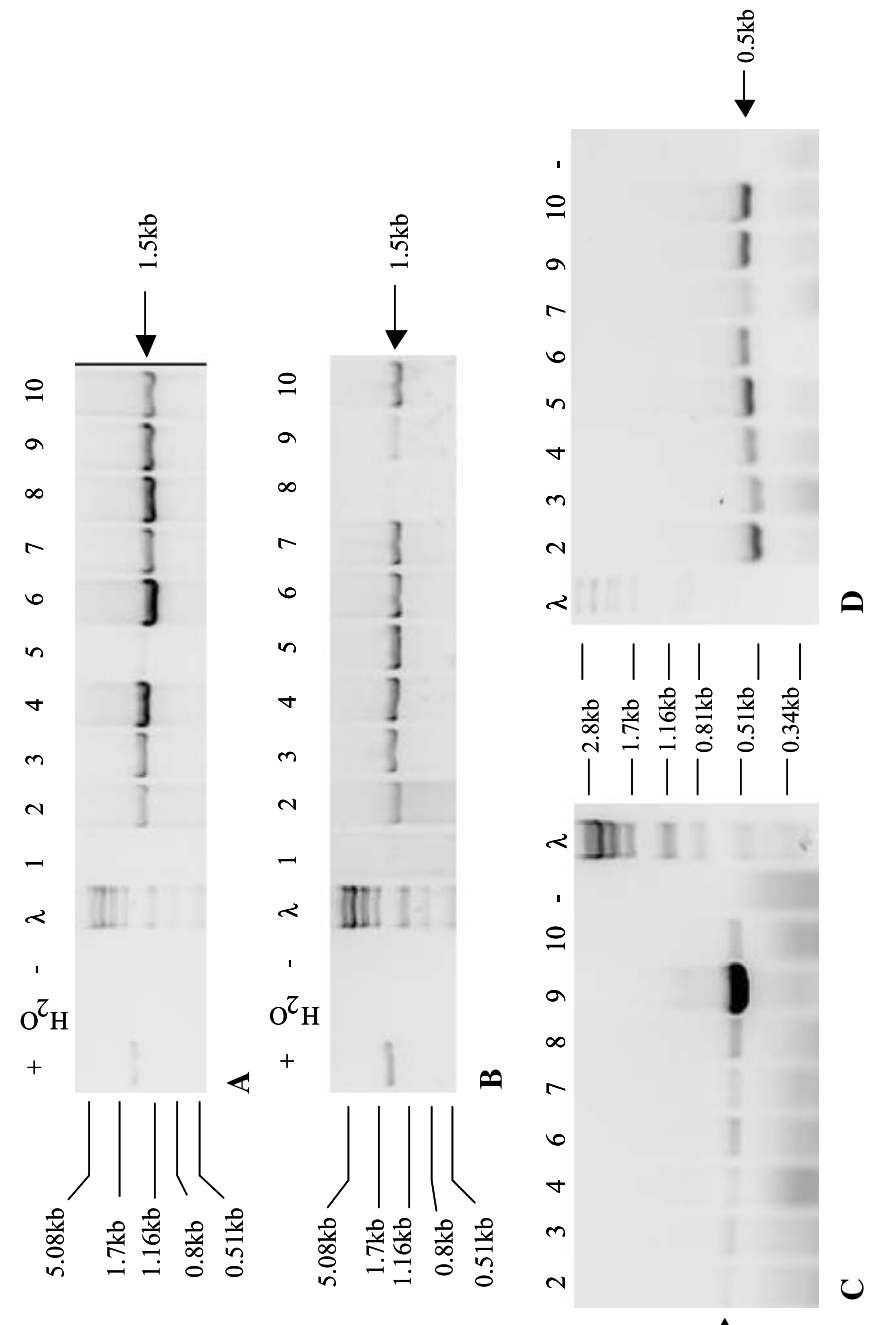


Fig. 1. Genetic analysis of the transgenic *N. tabacum*. PCR analysis of the transgenic plants (R_0) for the HPV-16 L1 (**A**) and L1ΔC483 (**B**) genes. *1–10* represents the different lines of transgenic plants. All transgenics are positive for L1 DNA except #5 and #8. **C** RT-PCR analysis of the total mRNA extracted from the PCR-positive transgenic plants for HPV-16 L1 and (**D**) L1ΔC483. All the PCR-positive transgenic plants expressed the L1 gene as shown by the 500 bp amplification product

PCR-positive transgenic plants; therefore, we assumed that the expression and accumulation of the $L1_{Tr}$ and $L1\Delta C483_{Tr}$ proteins was below the detection limits. We therefore separately pooled leaf material (approximately 500 g) from the four lines of $L1_{Tr}$ T_1 and $L1\Delta C483_{Tr}$ transgenic plants, homogenised the pools in PBS + 0.5 M NaCl, and concentrated the extracts 100-fold by a combination of ultracentrifugation and the use of a centrifugal YM-30 Centricon filter. The $L1_{Tr}$ and $L1\Delta C483_{Tr}$ proteins were separately detected in the respective concentrates by western analysis (data not shown), using H16:J4 monoclonal antibody that binds L1 amino acids (aa) 261–280 [7].

Once we had confirmed the $L1_{Tr}$ and $L1\Delta C483_{Tr}$ protein production in the transgenic plants, it was important to determine the antigenicity of the protein product with a panel of characterised monoclonal antibodies. The binding of the conformation-specific antibodies H16:V5, H16:U4 and H16:E70 in the assays indicated the formation of higher order L1 structures such as capsomers or VLPs (Fig. 2).

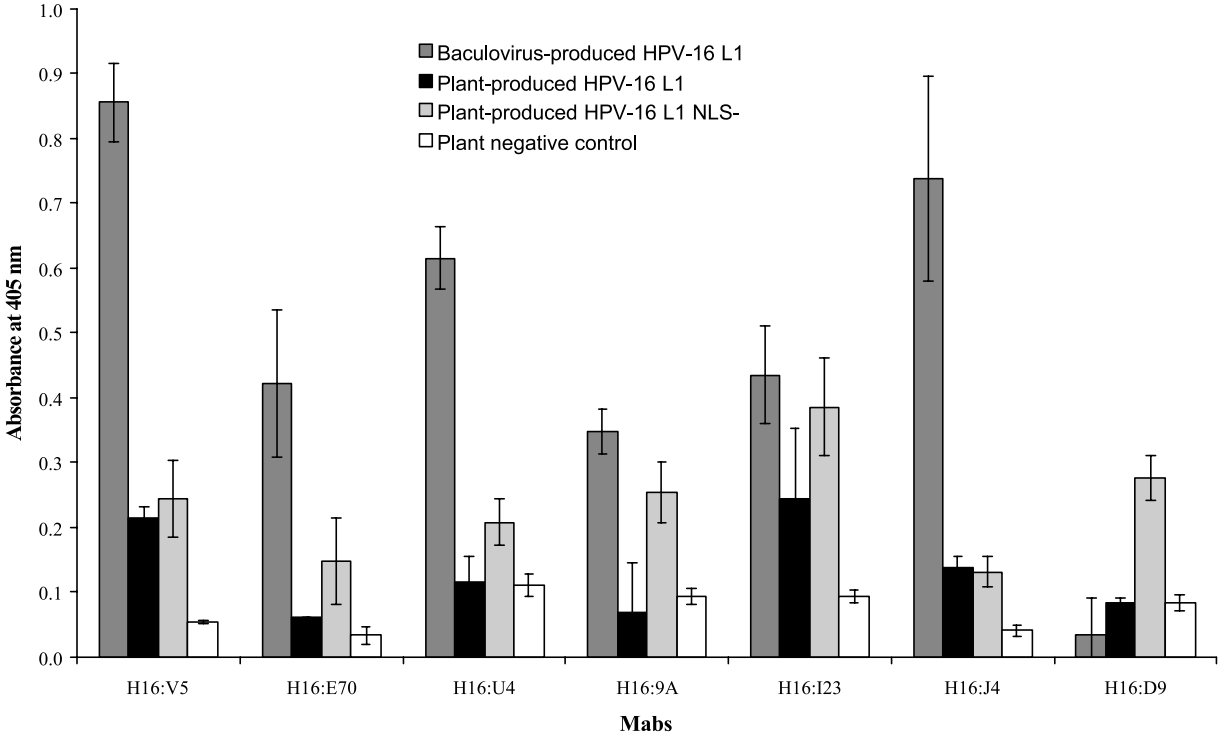


Fig. 2. Monoclonal antibody characterisation of the HPV-16 $L1_{Tr}$ and $L1DC483_{Tr}$, expressed in *N. benthamiana*, and $L1_{Bac}$ control, expressed in Sf21 cells, by capture ELISA. H16:V5 and H16:E70 (binds aa 50, 266 & 282) are conformation-specific and neutralising Mabs. H16:U4 and H16:9A are conformation specific Mabs. H16:I23 (binds between aa 111–130), H16:J4 (binds between aa 261–280) and H16:D9 are Mabs that recognise linear epitopes. Concentration of $L1_{Bac}$ used for ELISA was 0.13 ng/μl. Substrate was incubated for 30 min prior to OD reading

The approximate yield of protein/capsomers from transgenic plants for both $L1_{Tr}$ and $L1\Delta C483_{Tr}$ was determined from the capture ELISA results as follows: a dilution series of $L1_{Bac}$ was assayed for binding in parallel with the plant-produced proteins, and the interpolated concentration of $L1_{Bac}$ was determined in duplicate for each plant sample absorbance value with the "neutralising" conformation-specific Mabs H16:V5, H16:E70 and H16:U4, and the linear epitope-recognising Mab H16:J4. Table 1 summarises the estimated yields. The yield based on H16:V5 and H16:J4 was similar for both $L1_{Tr}$ and $L1\Delta C483_{Tr}$, and there was little variation in the yields determined by the different Mabs. Therefore, based on the ELISA data we estimate the HPV-16 $L1_{Tr}$ yield to be 2–4 μg per kilogram of fresh leaf material, or ∼0.0003% w/w.

Electron microscopy of the plant extracts

Distinct particles were seen in experimental samples, compared to none in non-transgenic plant extract controls. Most of the particles observed by immunotrapping were apparently pentameric capsomers (10 nm) with a few exceptions of higher ordered structures (Figure 3A and B). The full size $L1_{Tr}$ particles observed (Fig. 4) were very similar in size and structure to those produced in insect cell by recombinant baculovirus (Fig. 5); however, similar full size particles were not observed for $L1\Delta C483_{Tr}$. The particles shown in Fig. 4 (except particle IV) seem to have all encapsidated nucleic acid, since the centres of the particles are not heavily stained like those seen for $L1_{Bac}$ VLPs in Fig. 5A. However, baculovirus-expressed VLPs also apparently encapsidate nucleic acid (Fig. 5, particles B and D). There is no marked difference observed in the smaller capsomer-like particles resulting from the transgenic plant expression of L1ΔC483 compared to whole L1 (Fig. 3).

To confirm that the particles produced in transgenic plants were due to $L1_{Tr}$ and $L1\Delta C483_{Tr}$, we immunotrapped the plant extracts using anti-HPV-16 L1 guinea pig serum and probed with H16:V5 and gold-labelled secondary anti-mouse antibodies (10 nm gold particles). Gold particles were observed attached to plant-produced particles, binding to the putative capsomers (Fig. 6). None of these kinds of particles or binding of gold-labelled Mabs were observed for non-transgenic control extracts.

Table 1. Calculated concentrations of $L1_{Tr}$ and $L1\Delta C483_{Tr}$ capsomers based on the binding of the various Mabs to $L1_{Bac}$ dilutions of known concentration

	Concentration of capsomers per kilogram of fresh transgenic leaf material determined using Mabs			
Antigen	H16:V5	H16:E70	H16:U4	H16:J4
HPV-16 $L1_{Tr}$	3.2 μg	1.8 μg	2.4 μg	2.4 μg
HPV-16 $L1\Delta C483_{Tr}$	3.6 μg	4.4 μg	4.3 μg	2.3 μg

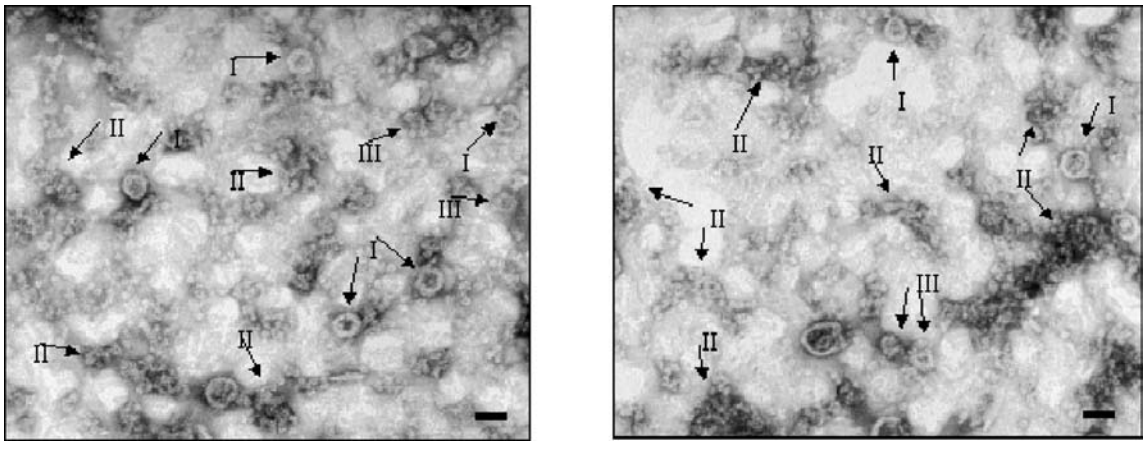


Fig. 3. Electron micrographs of $L1_{Tr}$ (**A**) and $L1\Delta C483_{Tr}$ (**B**) particles immunotrapped using Mab H16:J4 from concentrated transgenic plant extracts (Bar = 50 nm). Mixed population of full size VLPs (*I*), capsomers (*II*) and disassembled/broken down particles (*III*) are observed in both extracts

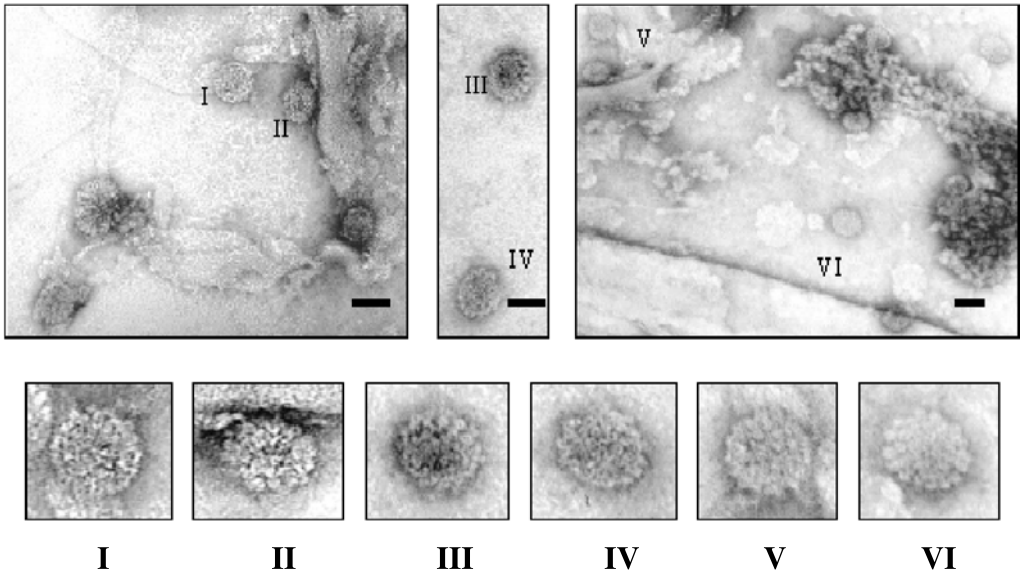


Fig. 4. Electron micrographs of HPV-16 $L1_{Tr}$ VLPs particles immunotrapped with Mab H16:J4 from concentrated plant extracts (Bar = 50 nm). Particles *I*, *II*, *IV*, *V* and *VI* probably contain nucleic acid whereas particle *III* is probably an empty VLP

Analysis of the immune response to transgenic plant derived HPV-16 L1

No adverse effects were observed in the immunised rabbits as a result of immunisations with concentrated transgenic HPV-16 $L1_{Tr}$ plant extract. The rabbit sera were analysed for HPV VLP-specific antibodies by indirect ELISA using

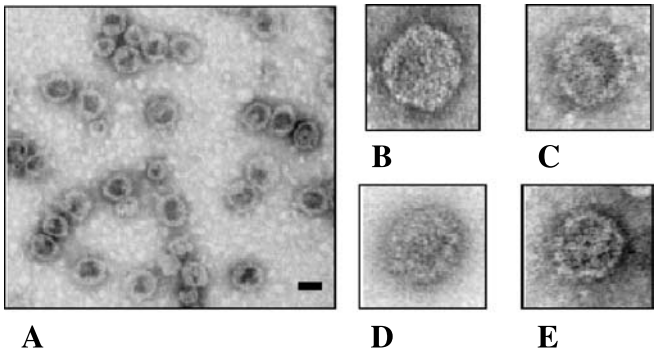


Fig. 5. Electron micrographs of baculovirus-produced HPV-16 $L1_{Bac}$ VLPs (Bar = 50 nm). Empty VLPs shown in micrograph **A** and magnified particles in **C** and **E**. Particles **B** and **D** probably contain nucleic acid since there is no heavy central staining with the uranyl acetate as there would be in the case of empty VLPs (**C** and **E**)

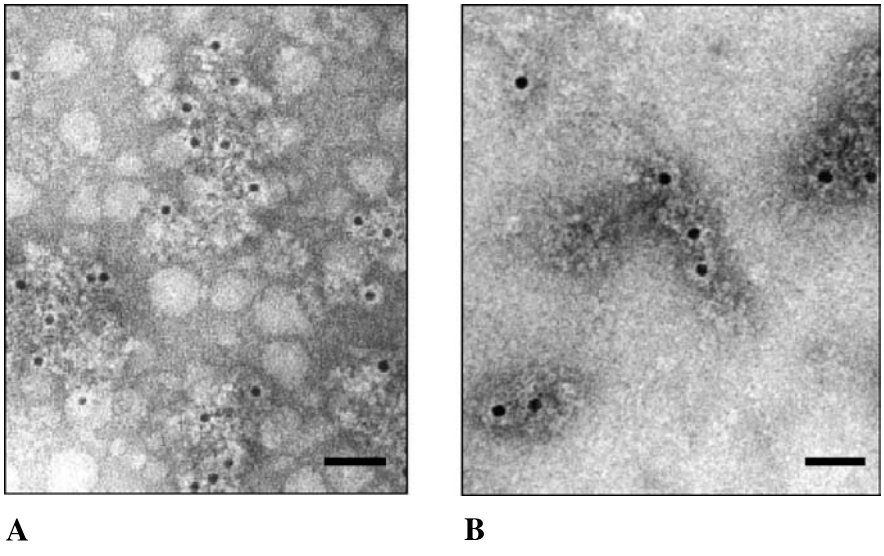
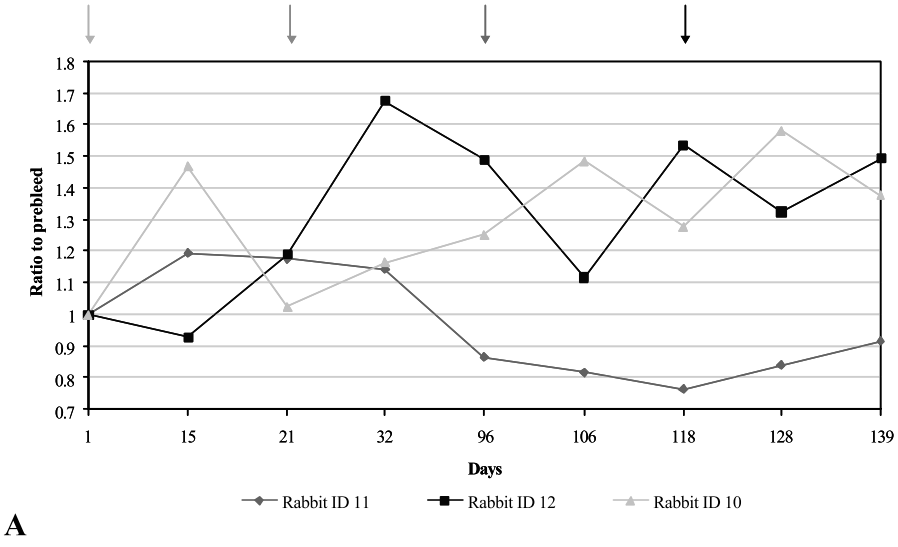


Fig. 6. Electron micrographs of immunogold-labelled capsomers of HPV-16 L_{Tr} (**A**) and $L1\Delta C483_{Tr}$ (**B**) that were trapped onto the copper grids with anti-HPV-16 L1 guinea pig serum and then probed with gold-labelled Mab H16:V5 (Bar = 50 nm). The gold particles are approximately 10 nm in diameter

baculovirus-produced HPV-16 $L1_{Bac}$ VLPs. ELISA plates were coated overnight at 4 °C with 100 µl of HPV-16 $L1_{Bac}$ VLPs at a concentration of 1 µg/ml, and probed with rabbit sera diluted 1:100 in PBS with 1% non-fat milk. An unexpected complication was that the pre-immunised (day 0) sera were found to be highly reactive to HPV-16 $L1_{Bac}$, almost certainly as a result of baculovirus and/or insect cell contamination of HPV-16 $L1_{Bac}$ preparations (see western blots below). This rendered interpretation of the ELISA results difficult; however, a weak but generally increasing immune response was observed for two of the three rabbit



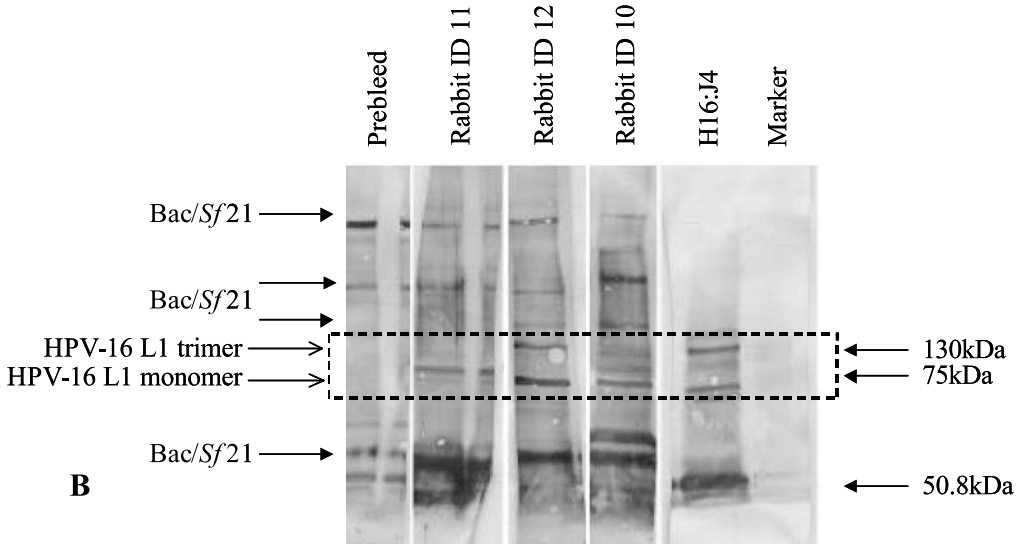


Fig. 7. Analysis of sera from New Zealand White rabbits immunised with plant-produced HPV-16 L1 antigen. **A**: Sera tested against recombinant baculovirus-produced HPV-16 $L1_{Bac}$ VLPs, 100 µl of 1 µg/ml suspension coated per well onto ELISA plates. The rabbits were immunised on days 1, 21, 96 and 118. **B**: Western blot analysis of the rabbit sera with denatured HPV-16 $L1_{Bac}$. Binding of rabbit antibodies from the prebleed to certain baculovirus/*Sf*21 proteins is evident, including at the probable position of the L1 monomer (~50 kDa). The dotted box indicates probable HPV L1-specific antibody binding, and includes possible L1 dimer and trimer bands

sera analysed (Fig. 7A). The weak immune response could be attributed to the low concentration of antigen administered in each inoculation (approximately 50 ng).

Day 15 sera from all three rabbits were checked by western blotting against denatured baculovirus-produced HPV-16 $L1_{Bac}$ VLPs (Fig. 7B). The prebleed

serum and the putative L1-immune sera and the H16:J4 Mab positive control bound a number of protein bands in common in the denatured HPV-16 $L1_{Bac}$ preparations, including in the expected region for L1 monomers (50–60 kDa); however, the three L1 sera also bound to at least one additional higher M_r band in common with the H16:J4 Mab positive control, while the rabbit ID12 serum binds very similarly to the H16:J4 antibodies. This suggests that the preimmune and immune rabbit sera bind common insect cell or baculovirus proteins, while the immune sera and the control Mab also bind L1 protein bands. We note that the western blots were performed on proteins that had been denatured with detergent, but not reduced: this allows retention of intermolecular S–S bridges, which results in dimer and trimer bands for L1 [4]. Thus, the H16:J4 antibodies and probably also at least the ID12 serum bound to putative L1 dimers and trimers in addition to monomers, with binding to the latter being masked by interfering anti-baculovirus and/or Sf21 cell contaminating proteins which also bound antibodies from preimmune sera. Thus, an HPV-16 L1-specific response was elicited in rabbits immunised with concentrated extracts from L1 transgenic plants.

Discussion

A variety of expression systems have been investigated for the expression of HPV capsid protein as potential systems for producing vaccines: these include prokaryotic, baculovirus, yeast and mammalian expression systems. All these recombinant cell expression systems require stringent purification and sterile protocols to obtain sufficient PV L1 antigen. Even with technological improvements, most of these technologies are expensive for developing countries that urgently require these vaccines. Reports have suggested that transgenic plants that express antigens could be used for inexpensive vaccine production systems [1, 22]. This paper reports our successful initial investigation into the use of a transgenic plant system to express the HPV-16 capsid protein.

We reported the use of pooled lines of plants for extraction and concentration for each protein construct, rather than characterising the production levels of protein in individual plant lines. This was done in order to establish at an early stage whether or not there was in fact any VLP or capsomer production, given the difficulties experienced in detection of L1 protein in top mRNA-expressing candidate lines. We therefore maximised the amount of transgenic plant material available in order to isolate putative HPV-16 $L1_{Tr}$ and $L1\Delta C483_{Tr}$ VLPs, as it was felt to be important to prove that VLPs could be produced at all as a proof of concept, rather than to look at their relative expression levels in different lines. As it turned out, expression levels were low enough that we will not be using any of the lines we established for further L1 production. Moreover, the use of ultracentrifugation as described is almost certainly not suitable for bulk purification of a vaccine candidate from plants, and alternate strategies would have to be devised.

Mason et al. [23, 24] have demonstrated that *Hepatitis B virus* surface antigen and *Norwalk virus* capsids can assemble into VLPs when expressed in transgenic plants. Our results in this study suggest that the HPV major capsid protein L1 can

similarly be expressed in transgenic *N. tabacum*: this protein folds into capsomers as shown by electron microscopy, and the unmodified L1 at least forms VLPs that are structurally and antigenically identical to those produced by recombinant baculovirus in insect cells. Moreover, concentrated extracts from transgenic plants could be used to elicit a specific anti-HPV-16 L1 response in rabbits.

We note that a high proportion of capsomers were observed by electron microscopy of plant extracts relative to baculovirus-produced VLPs (Fig. 3A); moreover, very few VLPs were seen for the L1ΔC483 preparations. ELISA and other results suggest that the concentration of $L1_{Tr}$ protein is low in plants, probably no higher than 2–4 μg/kg fresh leaf weight. We note that a high concentration of L1 protein in the plants would presumably drive the free protein → capsomer → VLP equilibrium towards VLP formation; conversely, a low concentration would favour capsomer formation. It is interesting to note that the binding affinity of Mab H16:D9 to $L1\Delta C483_{Tr}$ is almost twice that of full length $L1_{Tr}$ (Fig. 2), yet the binding of both of these proteins to Mabs H16:V5 and H16:J4 is similar. It can be deduced from the latter result that there is a similar quantity of capsomers or higher order L1 structures that result from the transgenic expression, but that there is probably more unassembled protein in the case of $L1\Delta C483_{Tr}$, since Mab H16:D9 has a high affinity for denatured L1 and a very low affinity for intact VLPs [7]. PV virion assembly takes place in the nucleus of infected cells [29]; the nuclear localisation signal (NLS) of HPV-16 L1 is bipartite and located near the C-terminus (aa 484–504; KRK_1atpttsststtaKRKKRKL_2; [37]). There is a strong body of evidence to suggest that C- terminal deletion up to the h5 helix allows the L1 protein to fold into VLPs [4, 5]; therefore, the modification of L1ΔC483 most probably does not affect assembly. However, while relative yields of protein from transgenic plants were similar for the two proteins, the fact that the intact L1 almost certainly localises to plant cell nuclei while the L1ΔC483 does not (A Varsani, EP Rybicki, unpublished results), means that the former probably reaches higher concentrations in nuclei than the latter does in the cytoplasm. This would presumably allow more higher-order assembly to occur for unmodified L1 than for the truncated version.

Capsomers have been shown to be as immunogenic as VLPs, and can be used to elicit neutralising antibodies [10, 13, 33]. Our electron microscopy data also indicated that there is no marked difference in the capsomer-like particles formed from the expression of the HPV-16 $L1_{Tr}$ gene with or without the nuclear localisation signal ($L1\Delta C483_{Tr}$); thus, preparations from both types of transgenic plant are probably equally viable as potential vaccines.

Our estimates of the HPV-16 $L1_{Tr}$ yield (2–4 μg per kilogram of fresh leaf material; ~0.0003% w/w) are similar to yields reported for expression of hepatitis B surface antigen in lettuce [18]. In general, the expression levels of foreign proteins in transgenic plants have been variable, ranging from 0.001% of total soluble protein (as opposed to leaf mass, certainly a lower value than ours) for rabies virus glycoprotein [25], to 0.37% for *Norwalk virus* capsid protein [23].

Gerber et al. [12] have shown that intramuscular immunisation of mice with 0.3 μg of HPV-16 VLPs produced in insect cells elicited a strong systemic immune response to L1. However, the immune response we observed in rabbit experiments

with ~0.1 μg per dose was very low: we believe that this was due to a much lower dose/body weight ratio for rabbits compared to mice, and that the response can be improved markedly by using a plant extract that has a higher concentration of $L1_{Tr}$ antigen. This can be done by developing a bulk extraction protocol from a larger source of plant material in addition to increasing expression levels in the transgenic plants. Various approaches have been suggested to increase the expression levels in transgenic plants. These include codon optimisation, modification of plant promoters for transcription of the genes, insertion of 5′- or 3′-untranslated regions to increase mRNA stability [27, 30], introduction of a 5′-untranslated *Tobacco mosaic virus* sequence or other translational enhancer ([6], and the use of a CaMV 35S-dual enhancer promoter [24]. Another very important approach, which is not suitable for all proteins given the kinds of modifications required, is the enhancement of protein stability and accumulation. This usually involves targetting the protein to different subcellular compartments such as the chloroplasts or the endoplasmic reticulum, or potentially membrane anchoring, where there is better accumulation and usually less proteolytic activity [2]. Nonetheless our data on the expression of the HPV-16 L1 in transgenic plants suggests that this system is potentially viable for vaccine production.

In summary, the HPV-16 L1 and L1ΔC483 genes were introduced into the *N. tabacum* plant genomes by *Agrobacterium*-mediated gene transfer. Our data confirm the successful and stable *in planta* production, with both of the constructs, of HPV-16 L1 particles that are capable of binding conformation-specific neutralising monoclonal antibodies (Mabs). Electron microscopy showed that the particles that result from the transgenic expression of HPV L1 in *N. tabacum* plants were very similar to those produced in insect cells by using recombinant baculovirus, and antigenic analysis showed them to be effectively identical. It therefore appears quite feasible that transgenic plants could be used for the production of HPV vaccines, provided expression levels and assembly of the HPV-16 L1 can be improved significantly.

Acknowledgements

This study was supported by a Innovation Fund grant (grant no. 41224) from the Department of Arts, Culture, Science and Technology (DACST). We thank Dr. Neil Christensen for providing the panel of monoclonal antibodies used in this study, Mr. Rodney Lucas, Ms. Debbie de Villiers and Ms. Marleze Rheeder for help with the animal work, Ms. Tracey Seamen for the help with some of the plant tissue culture and Mr. Donald Solomons and Ms. Jackie McCann for maintaining the plant growth rooms.

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