

Transient expression of Human papillomavirus type 16 L1 protein in *Nicotiana benthamiana* using an infectious tobamovirus vector

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Abstract

A Tobacco mosaic virus (TMV)-derived vector was used to express a native Human papillomavirus type 16 (HPV-16) L1 gene in *Nicotiana benthamiana* by means of infectious in vitro RNA transcripts inoculated onto *N. benthamiana* plants. HPV-16 L1 protein expression was quantitated by enzyme-linked immunosorbent assays (ELISA) after concentration of the plant extract. We estimated that the L1 product yield was 20–37 µg/kg of fresh leaf material. The L1 protein in the concentrated extract was antigenically characterised using the neutralising and conformation-specific Mabs H16:V5 and H16:E70, which bound to the plant-produced protein. Particles observed by transmission electron microscopy were mainly capsomers but virus-like particles (VLPs) similar to those produced in other systems were also present. Immunisation of rabbits with the concentrated plant extract induced a weak immune response. This is the first report of the successful expression of an HPV L1 gene in plants using a plant virus vector.

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1. Introduction

Human papillomaviruses (HPV; family *Papillomaviridae*, genus *Papillomavirus*) cause epithelial lesions in humans. Certain sexually transmitted HPV types are the principal cause of cervical cancer (Walboomers et al., 1999): HPV-16 is present in more than 50% of cervical cancers and types 18, 31 and 45 are present in approximately an additional 30% (WHO, 1999). Cervical cancer is the most common malignancy of women in developing countries, with about 500,000 new cases worldwide each year (Clifford et al., 2003).

Papillomavirus virus-like particles (VLPs) made from the main capsid protein L1 alone, or by co-expression of L1 and L2, have been proven to induce protective immunity in animal

models (Christensen et al., 1996; Stanley et al., 2001; Suzich et al., 1995). Successful phase II trials have shown protection from HPV infection in volunteers immunised with HPV L1 VLP-based vaccines made in *S. cerevisiae* (Koutsky et al., 2002) or in insect cells via recombinant baculovirus (Harper et al., 2004). These vaccines are expensive to produce, however, meaning developing countries may not be able to afford them (Sanders and Taira, 2003; Taira et al., 2004).

The use of plants as expression systems for foreign antigens has been proposed as a cheaper alternative for human vaccine production (Awram et al., 2002; Mason and Arntzen, 1995), and a number of human viral antigens have now successfully been expressed in transgenic plants (Huang et al., 2005; Mason et al., 1996; McGarvey et al., 1995; Richter et al., 2000). An important limitation of using transgenic plants as a system for expression of foreign proteins is the low yield in many cases: this is particularly true in the case of HPV-11 and HPV-16 L1 proteins, as recently reported by our group and others (Biemelt et al., 2003; Liu et al., 2005; Varsani et al., 2003; Warzecha et al., 2003). In separate trials with transgenic tobacco and potatoes expressing HPV-16 L1, and potatoes expressing HPV-11 L1, none of the groups

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achieved high yields or more than weakly orally or parenterally immunogenic extracts despite using codon-optimised genes in two instances (Biemelt et al., 2003; Warzecha et al., 2003).

Plant viruses are becoming increasingly feasible as antigen expression systems (Canizares et al., 2005; Mechtcheriakova et al., 2006). Major potential advantages over transgenic plant expression are the relatively rapid transient expression possible in plants, and potentially much higher product yields (Shivprasad et al., 1999). However, while codon optimisation can increase product yield in transgenic plants, yield of plant codon-optimised HPV-11 was very low (Warzecha et al., 2003), and for HPV-16 L1 expression a human codon-optimised gene was found to be significantly better than a plant-optimised version (Biemelt et al., 2003). Given that codon optimisation is not guaranteed to work, therefore, optimising gene expression by altering codon usage could be a risky and very expensive procedure. Accordingly, we investigated the prospects of transiently expressing the same native HPV-16 L1 gene as we previously used for transgenic expression in tobacco (Varsani et al., 2003), in *Nicotiana benthamiana* using a Tobacco mosaic virus (TMV)-based vector. Additionally, we tested parenteral immunisation of rabbits with concentrated plant extracts rather than oral immunisation with plant material, given the lack of success with the latter in other work.

2. Materials and methods

The TMV-based GenewareTM vector pBSG1057, derived from the 30B TMV-based vector of Shivprasad et al. (1999), was obtained from Large Scale Biology Corporation (Vacaville, USA). This contains sequences encoding the coat protein subgenomic mRNA promoter, coat protein ORF, and 3' nontranslated region from Tobacco mild green mosaic virus (TMGMV) variant U5. The GFPC3 gene was replaced with our previously described native HPV-16 L1 gene (Varsani et al., 2003) to obtain the clone pTMV-L1. In vitro transcripts were made using the T7 RNA polymerase (RiboMAXTM Large Scale RNA production System-T7, Promega) and capped using RNA cap-structure analogue m7G(5)ppp(5)G (New England Biolabs). RNA transcripts made in 100 μ l reactions were diluted to 1 ml with DEPC-treated water, and mechanically inoculated with sterile cotton buds and Celite onto the bottom leaves of 3-week-old *N. benthamiana* plants. Plants were grown at 22 °C with 16-h light and 8-h darkness.

Total RNA was extracted from fresh or stored (–70 °C) leaves using the TRIzolTM reagent (Life Technologies). The L1 and TMGMV U5 coat protein (U5 CP) mRNAs were detected by RT-PCR amplification using the Access RT-PCR system (Promega) using the primers forward 5'-GGTCCATTAGGTGTGGG-3'/reverse 5'-GCTGTGCGCCATATGGTTCTG-3' for the HPV-16 L1 gene and forward 5'-GTTCCGCTTATGCAGATCCTG-3'/reverse 5'-AAGTAGCCGGAGTTGTGGTCC-3' for the U5 CP gene, both amplifying an internal 500 bp fragment.

Twenty-five *N. benthamiana* plants were inoculated with the freshly prepared RNA transcripts of TMV-L1, and harvested 14 dpi (~80 g) and homogenised in 1:2 (w/v) ice cold PBS/0.5 M NaCl pH 7.4. The homogenate was strained through cheesecloth

and centrifuged at 6000 \times g for 10 min, and the vector TMV particles largely removed by addition of polyethylene glycol (PEG) MW 8000 to 4% (w/v) final concentration, incubating the mixture at 4 °C for several hours, and pelleting the precipitated particles by centrifugation. Putative HPV L1 VLPs in the supernatant were pelleted by ultracentrifugation at ~77,000 \times g for 6 h and were resuspended in cold PBS. For antibody characterisation putative L1 protein was further concentrated using Centricon YM-30 (30,000 kDa cut-off) centrifugal filters (Amicon Separation).

Concentrated extracts from plants infected with TMV-L1 and uninfected negative controls were tested with a panel of monoclonal antibodies (Mabs) by capture ELISA, and subjected to electron microscopy, as described (Varsani et al., 2003). The Mabs and their target epitopes were H16:V5, H16:E70, H16:U4, H16:9A (conformational/neutralising epitope-specific); H16:J4, H16:I23 (linear/neutralising epitope); H16:D9 (linear epitope, predominantly binds denatured L1 (Christensen et al., 1996, 2001; Combata et al., 2002; Roden et al., 1997; White et al., 1999)). This panel has previously been used to compare antigenicity of baculovirus-produced and transgenic tobacco-produced HPV-16 L1 (Varsani et al., 2003). Control VLPs were made via baculovirus expression in Sf21 insect cells as described elsewhere (Varsani et al., 2003).

Three white New Zealand rabbits were injected with concentrated sap extract from *N. benthamiana* infected with TMV-L1 (~65 fold concentrated): aliquots containing approximately 0.4 μ g of L1 antigen were diluted to 500 μ l in PBS and injected 1:1 with Freund's incomplete adjuvant, in two subcutaneous sites and an intramuscular site at days 1, 21, 67 and 87. Serum diluted 1:50 was analysed for VLP-specific antibodies by ELISA using the "gold standard" baculovirus-produced HPV-16 L1 (Bac-L1) VLPs, purified as described (Varsani et al., 2003), and by Western blot. Bac-L1 protein was partially purified, resolved on a 10% SDS PAGE gel, and blotted as described previously (Varsani et al., 2003), then probed with sera derived by immunisation with plant extracts in order to prove that these were reacting with the L1 polypeptide.

3. Results

The TMV vector only infection produced by inoculation of RNA derived from pBSG1057/30BGFP3 (which expresses jellyfish green fluorescent protein, GFP) was monitored using a hand-held UV lamp. GFP fluorescent spots were seen on the infected leaves 3 dpi, and systemic infection was generally observed after 8 dpi. TMV vector infection symptoms—mosaic, and curling of the top infected leaves, were evident 10–14 days post inoculation (dpi) for both 30BGFP3 and TMV-L1. These were confirmed by RT-PCR of total RNA extracted from the top leaves of plants infected with TMV-L1, with a strong 500 bp RT-PCR product observed for both the TMGMV U5 coat protein (U5 CP) and the L1 genes (not shown). However, only U5 CP could be detected by chromogenic or even chemiluminescent Western blot using antibodies specific for either TMV CP or L1 protein in unconcentrated extracts from these plants (not shown): this indicated that the expression of L1 protein by TMV-

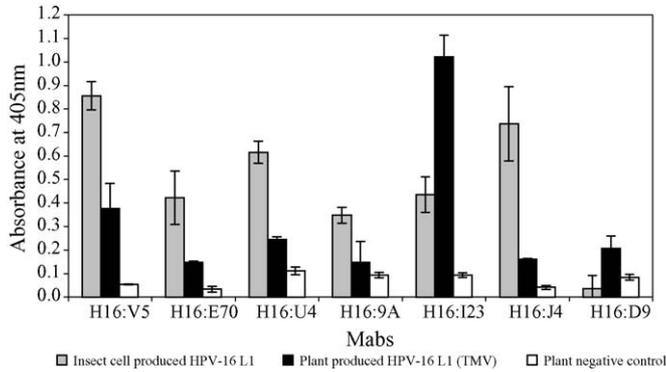


Fig. 1. Characterisation by capture ELISA using monoclonal antibodies of the HPV-16 L1 transiently expressed in *N. benthamiana*. Mabs H16:V5, E70, U4 and 9A bind conformational epitopes; Mabs I23, J4 and D9 bind linear epitopes. HPV-16 Bac-L1 was expressed in insect cells by recombinant baculovirus: Bac-L1 coating concentration was 0.13 $\mu\text{g}/\text{ml}$, and was adjusted to give a roughly comparable OD_{405} to plant extract using Mab V5.

L1 was significantly lower than the ~ 0.1 ng/sample or ~ 5 $\mu\text{g}/\text{g}$ leaf level, and all further work was accordingly done on concentrated extracts.

The binding of the conformation-specific Mabs H16:V5, H16:U4 and H16:E70 to the TMV-L1 plant concentrate (Fig. 1) indicates not only the presence of HPV-16 L1, but also the formation of higher order L1 structures, such as capsomers or VLPs. However, the strong binding to Mab H16:I23 and H16:D9

to the plant concentrate compared to their binding to insect cell-produced L1 protein suggests a higher proportion of denatured/unassembled particles in the plant extracts, since H16:I23 has almost half the binding affinity for intact VLPs as for free protein (Christensen et al., 1996).

Electron microscopy of leaf sap from top leaves of plants infected with TMV-L1 at 12 dpi (Fig. 2) appeared to show TMV-like rods of two clearly different size classes, 300 and 370 nm, compared to the apparently single size class of 300 nm virions from plants infected with 30BGFPC3 (not shown). The apparent presence of wild type TMV rods in extracts from TMV-L1 infected plants could indicate deletion of the L1 gene somewhere in the cycle of replication and movement of the virus within the plants (KE Palmer, LSBC Corp., personal communication): however, conclusive proof would require a detailed measurement study, and this was not done. Particles, which could be HPV-16 L1 VLPs and aggregates of pentamers could also be seen (Fig. 2).

Concentrates from 30BGFPC3- and TMV-L1-infected plants were also immunotrapped with Mabs H16:J4 and H16:V5: again, mostly pentameric capsomers (10 nm) with a few larger particles were seen for the L1-containing extracts (Fig. 2), with nothing visible in 30BGFPC3 extracts (not shown). The VLPs were qualitatively identical in size and structure both to those produced in insect cell by recombinant baculovirus (Fig. 2), and those previously produced in transgenic plants (Varsani et

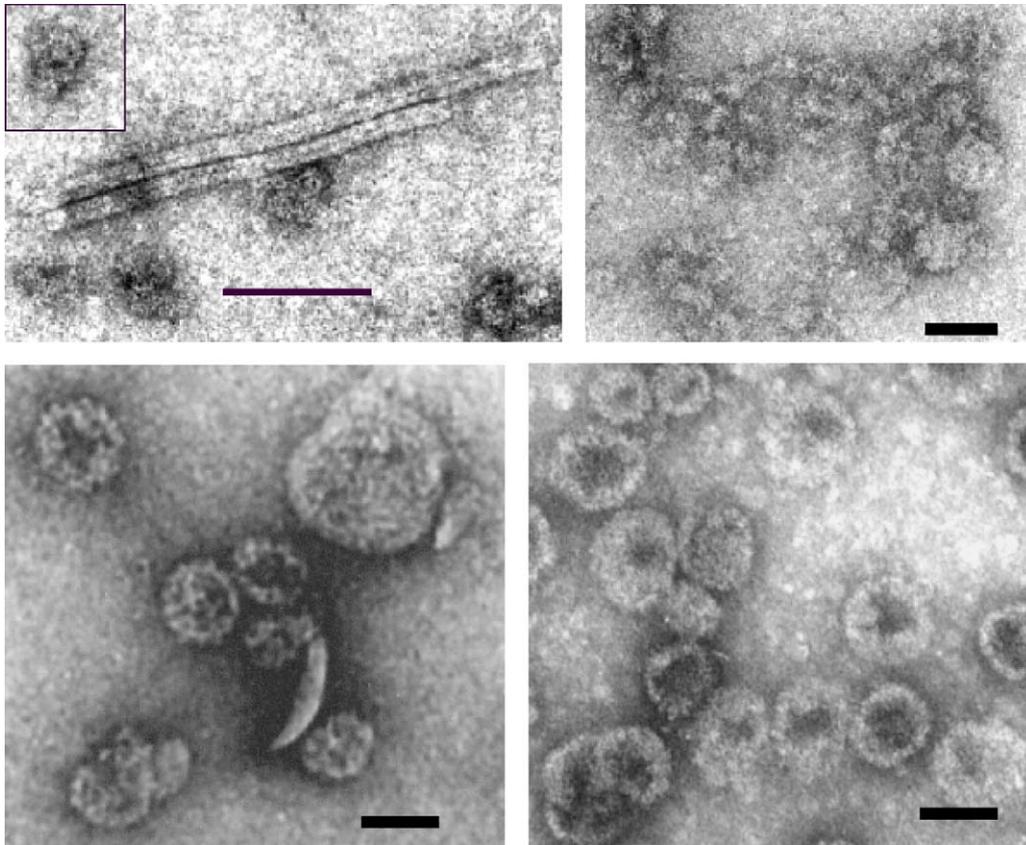


Fig. 2. Electron micrographs of uranyl acetate-stained preparations. (A) Crude leaf extracts of top leaves (Bar = 100 nm): TMV rods of two distinct lengths, 300 and 370 nm length are shown, with (inset) a possible VLP. (B) Apparent capsomer aggregates trapped with Mab H16:J4 (Bar = 50 nm); (C) plant-produced L1 protein trapped with Mab H16:V5 (Bar = 50 nm); (D) CsCl-purified VLPs made via baculovirus (Bar = 50 nm).

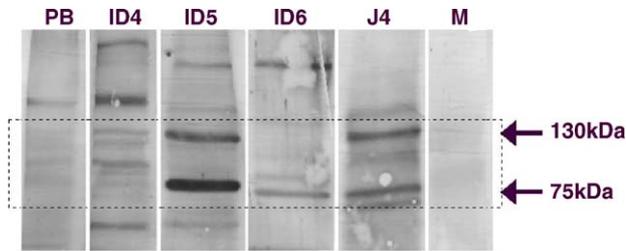


Fig. 3. Western blot analysis of the rabbit sera reacting against HPV-16 Bac-L1 run in a denaturing gel system with no disulphide bond cleavage. PB: prebleed serum; ID4, 5, 6=rabbit sera from animals injected once with concentrated putative L1 extract, 21 dpi; J4=Mab 16:J4 (reacts with linear epitope in HPV-16 L1); M: molecular weight marker lane. 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 phosphatase-labelled secondary goat anti-rabbit antibody diluted 1:2000, for 1 h at room temperature. Reaction was detected colorimetrically using BCIP and NBT substrates. Bands shown at 75 and 130 kDa probably represent dimerised and trimerised L1 protein, respectively.

al., 2003). Immunotrapping of TMV-L1 extracts using the anti-HPV-16 guinea pig serum and probing with H16:V5 and gold-labelled secondary anti-mouse antibodies (10 nm gold particles) resulted in gold labelling of capsomers/protein from TMV-L1-infected plants only; no binding was observed in the negative control (results not shown).

The approximate yield of L1 protein/capsomers from TMV-L1-infected plants was determined by capture ELISA as described previously (Varsani et al., 2003). The yield/kg wet leaf weight based on the four Mabs was H16:V5: 28 µg, H16:E70: 22 µg, H16:U4:36 µg, H16:J4:24 µg. Thus, the HPV-16 L1 yield was between 20 and 37 µg/kg of fresh leaf material, or ~0.003% (w/w). This is ~10× the yield we previously reported from transgenic *N. tabacum* plants for the same gene (Varsani et al., 2003).

A weak serum immune response was observed in indirect ELISA using baculovirus-produced VLPs for two out of the three rabbits immunised with putatively L1-containing sap concentrates. One rabbit (ID 5) responded after only one injection, and the other (ID6) apparently only after 2 (results not shown).

Sera from day 21 after first injection (=after one injection) was checked by Western blotting against denatured HPV-16 Bac-L1 VLPs produced in insect cells (Fig. 3): sera collected at later times were not noticeably more reactive (not shown). Gels were run without reducing agent to allow for multimerisation of the L1 protein: this allows easier identification of reactions with weakly-reacting sera (Varsani et al., 2003). Both sera that reacted in ELISA tests (ID 5 and ID 6) reacted specifically with L1 bands when compared to the positive control Mab 16:J4 and a prebleed serum, with ID 5 reacting more strongly than ID6. The ELISA non-responding ID4 serum did not react specifically. All sera, including the prebleed, bound strongly to baculovirus/Sf21 cell antigens.

4. Discussion

This study has shown that the native HPV-16 L1 gene could be successfully expressed in *N. benthamiana* plants via a recom-

binant TMV-based viral vector, to produce L1 protein at levels about 10× higher than we previously obtained by transgenic expression of the same gene in tobacco. Detailed antigenic characterisation showed the plant-produced L1 was very similar if not identical to that produced in insect cells by recombinant baculovirus (Fig. 2); moreover, rabbits injected with L1-containing plant sap concentrates produced antibodies which reacted with L1 protein (Fig. 3). This is a significant result, in that previous attempts at expression of the native HPV-16 L1 gene in plants resulted either in no apparent expression at all (Biemelt et al., 2003), or very low levels of expression (Liu et al., 2005; Varsani et al., 2003).

As also occurred with transgenic plants, ELISA results showed there was apparently a significant amount of unassembled L1: this is probably due to the low concentration of monomeric protein in plant cells not driving assembly to pentamers and higher (Biemelt et al., 2003; Varsani et al., 2003). The EM study also showed that there were an appreciable number of capsomers compared to the numbers of VLPs (Fig. 2). However, capsomers have been shown capable of eliciting neutralising antibodies (Fligge et al., 2001; Giroglou et al., 2001), so this should not be seen as a drawback for a plant-produced HPV vaccine.

The weak immune response seen in this work echoes our previous work with transgenic plants expressing HPV-16 L1, and in fact all other work using plant-produced HPV L1 antigen (Biemelt et al., 2003; Varsani et al., 2003; Warzecha et al., 2003). In the case of other investigators, this was probably due to orally immunising with too little unconcentrated antigen in plant tissue given that more than an order of magnitude greater amount is required for a mucosal as opposed to a humoral response with the same antigen (Rose et al., 1999). In our case, however, the antigen was injected as a concentrate that should have contained at least 1 µg of L1 protein: we produced 20–37 µg of L1/kg of fresh leaf material, which was concentrated ~60-fold for injection. While purified baculovirus-produced HPV or other PV VLPs are very highly immunogenic, with good responses down to doses as low as ~100 pg in dogs (Suzich et al., 1995), we note that in none of the reports on plant-produced HPV L1 were VLPs purified from extracts, and that in two cases (Biemelt et al., 2003; Varsani et al., 2003) the majority of the L1 protein was assumed to be only partially assembled. Thus, the weak immunogenicity of our inoculum was probably due to the preponderant presence of subunit protein rather than of assembled capsomers and higher. This should easily be obviated by further concentration and purification of the protein from larger batches of infected plants.

We note that our results were achieved with a native L1 gene. While use of a suitably codon-optimised version could potentially very considerably enhance the expression level, the expression level of a “plantised” HPV-11 L1 gene in potato was very low (Warzecha et al., 2003), and our group has found that native HPV-11 and Cottontail rabbit papillomavirus (CRPV) L1 genes express very well in transgenic tobacco (TO Kohl, I Becker-Hitzeroth and EP Rybicki, unpublished data). This indicates to us that improvement of PV L1 gene expression in plants

is a strictly empirical process, and one that is not subject to prediction.

Our electron microscopy results indicated that the L1 gene could be deleting from the TMV 30B vector in top leaves of plants 12–14 dpi: while this was not conclusive, we also found that use of the sap from these leaves as infective inoculum for fresh plants resulted in rapid appearance of necrotic symptoms typical of TMV-only controls, and significantly lower yield of L1 protein (results not shown). Dawson and co-worker (Rabindran and Dawson, 2001) reported recombinational deletion occurring when they used TMV to express GFP (30 kDa) in *N. benthamiana*. However, the fact that the native L1 gene was only deleted, if at all, from a subset of the replicating TMV at the top of plants, indicates the system is stable enough for L1 expression if freshly-generated RNA inoculum is used.

In summary, this paper describes the successful transient expression via a TMV-based vector of a HPV L1 protein in plants. While at least one larger protein, the Foot-and-mouth disease virus P1 protein (Wigdorovitz et al., 1999), has been expressed using TMV vectors, we report one of the largest proteins ever successfully expressed in this system. The L1 protein is capable of assembling into vaccine-appropriate structures such as pentameric capsomers and VLPs, and appears to be suitably immunogenic. While other studies of HPV L1 expression in plants have focussed on oral immunisation of experimental animals, we have tested injection of concentrated extracts, and shown that this elicits L1-reactive antibodies. Further development of this expression system could provide a considerably cheaper means of producing HPV vaccines or even L1-based reagents compared to the presently-used yeast and baculovirus systems.

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